

**A MORPHOLOGICAL STUDY OF HUMAN ENDOMETRIAL STROMA
IN VIVO AND *IN VITRO***

**BY
HÜSEYİN ERAY BULUT**

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TO MY MOTHER

DECLARATION

I declare that this work was performed by the author and it has not been submitted for acceptance in any previous application for a degree. This thesis is a record of the work that was undertaken by the candidate. All verbatim extracts have been distinguished by quotation marks and the sources of the candidates information have been specifically acknowledged.

H. Eray Bulut

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SUMMARY

A Morphological Study of Human Endometrial Stroma *In Vivo* and *In Vitro*

by Hüseyin Eray Bulut

Despite its crucial role in fertility, relatively little has been published on the human endometrial stroma. The first experiment reported in this thesis was designed to provide quantitative baseline data on the human stroma during the mid to late luteal phase, when it plays a major part in normal pregnancy. Subsequent chapters investigate the effects of an anti-oestrogen (clomiphene citrate-CC); growth hormone (GH) supplementation of patients on hormone replacement therapy (HRT) due to lack of endogenous ovarian steroids; and patients with unexplained recurrent miscarriage. Finally a novel 3-dimensional *in vitro* model of human stroma is described along with the effects of steroid supplementation on stromal cells grown in the model.

In all cases of *in vivo* studies, conventional stereological methods were used to obtain quantitative morphological data from at least 6 subjects per group, from both control (fertile) and experimental (infertile) subjects using light and electron microscopy. In addition, several staining techniques were also used to demonstrate qualitative changes that occur in human endometrial stroma.

During the mid to late luteal phase, endometrial stroma and blood vessels underwent substantial changes, and thus quantitative and qualitative baseline data have been established to fill a gap in this important area of reproductive biology. CC caused no substantial changes in stromal structure in women of proven fertility and therefore its advantageous effects on ovulation are not negated at the level of the endometrium. GH supplementation had no effects on infertile human endometrial stroma from subjects on HRT, (in either premature ovarian failure or Turner's syndrome groups). However, both infertile groups had endometrial stroma which significantly differed from matched fertile endometrium, suggesting an impaired endometrial development in infertile subjects which was not reversed by HRT, either with or without GH treatment. Stromal morphology did not differ between the recurrent miscarriage groups, however data suggested the existence of several subgroups which made firm conclusions difficult. The tissue culture model provided preliminary data suggesting it to be a potentially very useful technique for the study of stromal cell biology.

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

The human endometrium consists of 2 compartments; epithelium and stroma, which also includes the blood vessels. All of the endometrial compartments undergo dramatic sequential changes every 28 days (the menstrual cycle) under the influence of ovarian sex steroid hormones. These changes require a precisely timed sequence of hormonal action and are designed to co-ordinate endometrial events for implantation of the blastocyst and the maintenance of pregnancy. Although there is a large number of reports dealing with the glandular epithelial morphology throughout the menstrual cycle, very little is published about the morphological changes that occur in human endometrial stromal and vascular compartments during the menstrual cycle. It is the aim of the present study to evaluate the human endometrial stromal and vascular structures quantitatively and qualitatively during the mid to late secretory phase of the menstrual cycle (when stromal changes are most prominent) for a better understanding of the endometrium in fertility and infertility. In addition to examining stroma and blood vessels, some glandular epithelial features will also be examined for a complete evaluation of the endometrial structure at this time.

There are many reproductive tract disturbances causing fertility problems in women, such as premature ovarian failure (POF) (Aiman and Smentek 1985; Coulam *et al.* 1986), unexplained infertility (Fisch *et al.* 1989; Graham *et al.* 1990), polycystic ovary syndrome (PCOS) (Kettel *et al.* 1993), unexplained recurrent miscarriage (Crosignani and Rubin 1991) and so on. Many of these conditions occur in association with dysfunction of the ovary which may also adversely effect endometrial development and thus affect fertility. To overcome these infertility problems, several drug treatments are used such as clomiphene citrate (CC) (Glasier 1990; Guzick and Zeleznik 1990) and growth hormone (GH) (Katz *et al.* 1993) treatments for ovulation induction along with hormone replacement therapy (HRT) (Navot *et al.* 1986) to control endometrial development when the ovary is dysfunctional. However, there is little published information on the effects of these treatments on endometrial structure, especially on the stromal and the vascular elements. In the present study to examine the effects of these ovulation induction and ovulation enhancement protocols on endometrial morphology, a large number of stromal and vascular features will be evaluated quantitatively after

treatment with CC and after treatment with GH with or without HRT compared to normal fertile controls.

Recurrent miscarriage is another fertility problem, but one that occurs after the implantation of the fetus. Many factors may be involved in recurrent miscarriage, such as abnormal genetic content of the embryo, infection and so on (see later). However, when these factors can be excluded it remains likely that the endometrium may play a role in recurrent miscarriage. Disturbances in stromal and vascular compartments of the human endometrium are likely to be involved in otherwise unexplained repeated pregnancy losses. It is intended to evaluate the morphological differences in the stromal and blood vessel components of the endometrium between persistently miscarrying women and women with a similar previous history of recurrent miscarriage but who had then had a successful pregnancy. Comparisons will also be made between fertile women and these recurrent miscarriage groups where possible.

Stromal cells play an important role in preparation of the endometrium for a successful implantation and maintenance of pregnancy. However, for ethical and technical reasons, it is difficult to conduct *in vivo* experiments in humans. Using *in vitro* tissue culture techniques would be a more convenient way to determine the cellular events that take place in human endometrial stromal cells during pregnancy, but despite its potential importance in understanding reproduction, the function and structure of the stromal cell *in vitro* is apparently a poorly studied subject. It is an aim of the present study to determine the endometrial stromal cell morphology in a 3-dimensional culture system and establish a novel *in vitro* culture model that could be used subsequently as a reliable and accurate model for *in vivo* conditions. Preliminary evaluations of the effects of sex steroid hormones (oestrogen and progesterone) on stromal cell structure *in vitro* at different stages of the menstrual cycle are also presented. Throughout, *in vitro* results will be compared where possible to those *in vivo* results.

In order to establish a complete understanding of the subject, related topics such as hormonal control of the endometrium, implantation, endometrial morphology throughout the menstrual cycle, angiogenesis (blood vessel formation) and decidualization will be either reviewed in this section or in later sections where relevant.

1.2. AIMS

- ⇒ To establish baseline data for the quantitative and qualitative morphology of fertile human endometrial stromal and vascular compartments during mid to late luteal phase of the menstrual cycle.
- ⇒ To examine quantitatively and qualitatively the effects of ovulation induction with CC on the stromal and vascular structures of normally ovulating fertile women.
- ⇒ To evaluate the effects of GH treatment either with or without HRT on the morphology of stroma and blood vessels in 2 groups of patients with non-functioning ovaries; those with premature ovarian failure and those with Turner's syndrome.
- ⇒ To compare the morphological differences of stromal and vascular structures between patients with unexplained persistent recurrent miscarriage and patients with the history of unexplained recurrent miscarriages but at least one successful pregnancy.
- ⇒ To establish a novel 3-D culture model for human endometrial stromal cells *in vitro* which could be used as a model for *in vivo* conditions, and also to determine the effects of steroid hormones on the structure of stromal cells using this culture system compared with stromal cell changes seen *in vivo*.

1.3. ENDOMETRIAL STROMAL CELL DIFFERENTIATION (DECIDUALIZATION)

One of the many dramatic events occurring in the human endometrium is decidualization of the stroma. During this process fibroblast-like mesenchymal cells undergo dramatic morphological changes and extensive biochemical differentiation is reflected by the expression of a multiplicity of new products (Tabanelli *et al.* 1992; Huang *et al.* 1987; Clemmons *et al.* 1990; Thrailkill *et al.* 1990; Sakbun *et al.* 1990; Petraglia *et al.* 1990; Hoffman *et al.* 1991) which are assumed to play a still undetermined role in embryo implantation and maintenance of pregnancy. In humans, this process takes place during the normal luteal phase under the influence of progesterone, probably in concert with relaxin which is also secreted by the corpus luteum (Bryant-Greenwood 1991). It has been suggested that growth factors (such as epidermal growth factor, basic fibroblast growth factor and platelet-derived growth

factor) might be required for growth and differentiation of human endometrial stromal cells as well as progesterone and relaxin (Irwin *et al.* 1991).

Decidualization occurs even in the absence of an implanting blastocyst in human (Nilsson *et al.* 1978). Decidualization is evident in non-pregnant women treated with progestins and its hormonal dependence can be further inferred from the extensive decidual changes observed in the endometrium during ectopic pregnancies in the absence of intrauterine fetal tissue (Meuris *et al.* 1980; Albert and Teiltman 1986). The stromal cells display a similar type of modification during the menstrual cycle at around day LH+7, which is called predecidualization or more accurately pseudo-decidualization (Flamigni *et al.* 1991). Decidual cells are believed to play a role in implantation and in the maintenance of pregnancy through control of trophoblast invasion (Pijnenborg *et al.* 1980), nutrition of the blastocyst (Kearns and Lala 1983), endocrine secretion (Maslar *et al.* 1980), and protection of the embryo from maternal immune rejection (Golander *et al.* 1981).

Biochemically, stromal cells at this stage of differentiation produce prolactin (PRL) and laminin, secrete prostaglandins and express oestrogen and progesterone receptors (ER and PR) which provide the mechanism for these stromal cell functions (Leavitt and Takeda 1986). ER and PR formation in decidual cells are also under hormonal control and there is a significant loss of ER during the first four days of decidualization (Leavitt and Takeda 1986). It has been suggested that prostaglandin E₂ (PGE₂) enhances human endometrial cell differentiation and PRL production in the presence of oestradiol and medroxyprogesterone acetate (MPA). This indicates that there are synergistic effects among PGE₂, oestradiol and MPA, resulting in acceleration of endometrial stromal cell differentiation and enhanced PRL production (Frank *et al.* 1994). On the other hand, interleukin-1 (IL-1), a critical cytokine for the initiation of the immune response to infection or antigenic challenge, blocks *in vitro* decidualization (Kariya *et al.* 1991). In addition, human chorionic gonadotrophin (hCG) and luteinizing hormone (LH) receptors have been demonstrated in the non-pregnant human uterus, human placenta, fetal membranes and decidua indicating that hCG and LH may play a direct role in regulating functions of these tissues by endocrine, autocrine or paracrine mechanisms (Reshef *et al.* 1990).

Progress in understanding the basic mechanisms that regulate uterine function has been limited by the complexity of the hormonal and cellular interactions that regulate this tissue *in vivo*. To overcome some of these limitations, an alternative approach is to use *in vitro* model systems. Cultured human endometrial stromal cells respond to ovarian steroids with increased proliferation and the production of markers characteristic of decidualization such as extracellular matrix (ECM) proteins and PRL (Irwin *et al.* 1989, 1991; Faber *et al.* 1986; Maslar and Riddick 1979; Benedetto *et al.* 1990). In addition, an increase in intermediate filaments has been reported in rat uterine stromal cells undergoing decidualization; vimentin and desmin have been identified as intermediate filament proteins in rat endometrium and these proteins have been quantified during the process of decidualization (Glasser and Julian 1986). Another marker for the decidual reaction is alkaline phosphatase activity. It has been demonstrated that there is no alkaline phosphatase activity before day 23 of the menstrual cycle but between days 23 and 27 alkaline phosphatase activity is present in the stromal cells surrounding spiral arterioles (Wilson 1969).

Short term cultures of human decidua from early pregnancy revealed active synthesis of all major basement membrane (BM) components such as collagen type IV, laminin, heparan sulphate proteoglycan, entactin and fibronectin (Wewer *et al.* 1985; Kisalus *et al.* 1987; Welsh and Enders 1985). In contrast, in the non-pregnant uterine endometrium, there is no immunohistochemically demonstrable collagen type IV or laminin, except in the perivascular spaces and at the interface between stroma and the glandular epithelium (Damjanov and Wewer 1991). The formation of pericellular membranes around decidual cells appears to be coincidental with changes in stromal cell morphology.

When compared to other adult tissues, endometrial stromal cells have a much higher proliferative capacity and doubling potential *in vitro* and distinct changes in morphology are observed when primary stromal cell cultures are exposed to progesterone, indicating that progestins may affect cytoskeletal proteins (Holinka and Gurpide 1987).

1.3.1. DECIDUAL CELL MORPHOLOGY

As the pregnancy advances, the stromal cells gradually enlarge and shortly after implantation three groups of decidual cells can be distinguished: large mature polygonal cells measuring more than 25µm in diameter, medium sized polygonal or round decidual cells measuring 15-25µm in diameter preferentially located close by the dilated endometrial glands, and small elongated cells measuring less than 15µm in diameter resembling the fibroblasts or stromal cells from non-pregnant endometrium (Damjanov and Wewer 1991). It has been hypothesised that the medium-sized and large decidual cells evolve from the small elongated cells (Welsh and Enders 1985). After the sixth week to the end of the pregnancy, the decidua consists of mostly large mature cells, the ratio for the large, intermediate and small cells is 10:3:0.1 (Damjanov and Wewer 1991).

Where decidual cells are largest and most differentiated, the amount of matrix is less, the surface processes in decidual cells are flattened and the cells contain few of the membrane-bound inclusions, however specialised gap junctions are present between decidual cells (Lawn *et al.* 1971). Specialized gap junctions are also found between predecidual cells around small arterioles in non-pregnant endometrium at the late luteal phase of the menstrual cycle (Lawn *et al.* 1971). There are several hypothesis concerning the function of the junctional regions between decidual cells. Either it works as a mechanical barrier, protecting the blastocyst and preparing for deeper invasion of the uterus or the junctions contain regions of high permeability where ions and large molecules are transferred from one cell to another (Finn and Lawn 1967). Yet another alternative is that signals initiating the differentiation of fibroblasts into decidual cells might be transmitted through this specialised junctions (Lawn *et al.* 1971). It is of course possible that all these hypothesised functions might work together in a synchrony.

Between the 20th and 40th days of pregnancy, human decidual cells in the endometrial stroma come into direct contact with the trophoblastic cells eroding the stromal tissue (Tekelioglu-Uysal *et al.* 1975). The aggregates of decidual and trophoblastic cells (deciduo-trophoblastic complex) in the human during early pregnancy may function as a local humoral regulator releasing chemical factors and taking part in the establishment of immunological privilege at the site of implantation (Tekelioglu-Uysal *et al.* 1975). During this process, glycoprotein biosynthesis and glycosyl

transferase activity are altered and these changes in patterns of glycosylation may give rise to altered decidual cell-matrix and cell-cell interactions during differentiation and play a role in the modulation of decidual cell interactions with trophoblast during early placentation (Jones *et al.* 1993). It has been suggested that the development of this type of connection between decidual cells is associated with a change from independent stromal cells into a co-ordinated assembly of interconnected cells forming a decidual organ (Finn and Lawn 1967).

The Golgi apparatus of the endometrial stromal cells of pregnant mice increases in size with the differentiation of stromal cells into decidual cells and the activity of acid phosphatase in this organelle increases at this time (Bijovsky and Abrahamson 1992). However, the most internal decidual cells (antimesometrial decidual cells), which are closest to the embryo, degenerate and morphological aspects of this decidual regression and involution have been described in rats (Welsh and Enders 1985) and in mice (Katz and Abrahamson 1987; Bijovsky and Abrahamson 1992). Active growth and differentiation of endometrial stromal cells during the decidualization of early pregnancy are followed by programmed cell death (apoptosis) in the antimesometrial region of the decidua. The location and temporal specificity of decidual cell apoptosis and the identification of transforming growth factor- β 1 (TGF β 1) and TGF β 2 protein and mRNA in the decidua suggests that members of the TGF β family control stromal apoptosis by autocrine or paracrine mechanisms (Moulton 1994). The involuting decidual cells show morphological and cytochemical signs of Golgi regression, such as dilated cisternae and lack of enzymatic activity, together with the numerous pleomorphic lysosomes suggesting that decidual cell death occurs by autophagic degeneration in mouse and rat decidua (Bijovsky and Abrahamson 1992; Jollie and Bencosme 1965). However, homologies between human decidual cells and primary decidual cells of the rodents are uncertain. In ovariectomized, oestrogen and progesterone treated rhesus monkeys, the decidual cells have rounded nuclei and elaborate arrangements of interconnected cisternae of rough endoplasmic reticulum (RER) (Sengupta *et al.* 1990). Granular cells are also present in rhesus monkey endometrium with eccentrically located nuclei and numerous membrane-bound, electron-dense granules in their cytoplasm and they are

found in increasing numbers around decidual cells, blood vessels and glandular epithelia suggesting that these cells have secretory functions (Sengupta *et al.* 1990).

1.3.2. DECIDUAL CELL TYPES

Decidual tissue contains number of cell types. It has been clearly demonstrated that the human decidua contains stromal cells of endometrial origin, together with large granular lymphocytes (LGLs), macrophages, T-lymphocytes, fibroblasts and polymorphonuclear leukocytes (Bulmer *et al.* 1988b; Starkey *et al.* 1988; Casey and MacDonald 1988; Haller *et al.* 1993). The lymphoid tissue in human decidua from early normal pregnancy has a unique cellular composition and functional properties different from other mucosal tissues. The role of the individual cell components is not yet known, but there are indications that their role is to adapt the immune system of the mother to the presence of the fetus, thus preventing an immunological rejection.

Khan *et al.* (1991) has observed two populations of cells from term human decidua by immunocytochemistry. The first group of cells were a mixed cell population, predominantly macrophages (80%) but small numbers of T- and B-lymphocytes were also present. The second group were a less-dense cell population which did not contain significant numbers of the above cell types but did release PRL, suggesting that they are decidual stromal cells (Khan *et al.* 1991). The proportions of these different cell types vary throughout pregnancy. In the first trimester the LGLs are the dominant cell type, although by the third trimester a majority of the cells are stromal in origin, the percentage of LGLs is decreased and significant numbers of polymorphonuclear leukocytes are now present (Bulmer *et al.* 1988b; Finn and Pope 1991). Leukocytes classified as CD56+, CD3-, CD16- have been detected predominantly in first trimester decidua. While they are intensely positive for CD56, they do not express other natural killer (NK) lineage markers such as CD16, CD57 and CD11b (Bulmer *et al.* 1991). These phenotypically unusual cells were described in early pregnancy decidua (Ritson and Bulmer 1987) and a comparable population was also described in non-pregnant endometrial stroma in the late secretory phase of the menstrual cycle (Marshall and Jones 1988; Bulmer *et al.* 1988a).

1.3.3. HORMONES SECRETED BY DECIDUAL CELLS

Postovulatory endometrial cells produce PRL (Maslar and Riddick 1979; Healy 1991) which has been found to be one of the major secretory proteins of endometrial stromal cells (Zhu *et al.* 1990). PRL secretion by the human endometrium extends from the luteal phase of the menstrual cycle over the entire gestation period. The production of PRL positively correlates with the degree of decidualization (Irwin *et al.* 1989). Much of the PRL produced by secretory endometrium is glycosylated (G-PRL) and as pregnancy advances, the decidua secretes less G-PRL and more non-glycosylated PRL (Markoff *et al.* 1988). PRL synthesis in the endometrium is regulated directly by progesterone and indirectly by oestrogen (Casslen *et al.* 1990). Relaxin, insulin-like growth factor-1 (IGF-1) and some progestogens enhance endometrial PRL secretion and the progesterone antagonist RU 486 inhibits it (Huang *et al.* 1987; Thrailkill *et al.* 1988; Bell *et al.* 1991). The highest production rate of PRL has been shown in stromal cells treated first with medroxyprogesterone then with relaxin (Zhu *et al.* 1990). On the other hand, it has been recently demonstrated that the tumour necrosis factor- α (TNF- α) and endothelin (ET), which are synthesized and released from macrophages and decidual cells, inhibit the synthesis and release of human decidual PRL (Chao *et al.* 1993; Jikihara and Handwerger 1994), indicating that the inhibitory action of TNF- α and ET on basal and stimulated PRL release may result from an autocrine and/or paracrine effect.

Recent evidence indicates that, in addition to the corpus luteum, relaxin is also produced in decidualized endometrium and the trophoblast (Seppala *et al.* 1992). In the human genome, there are two relaxin genes (H1 and H2) encoding different amino acid sequences. Human corpus luteum transcribes the H2 relaxin gene, whereas the H1 gene is expressed in the decidua (Hansell *et al.* 1991; Bryant-Greenwood 1991). Relaxin stimulates endometrial and decidual secretion of PRL (Huang *et al.* 1987), prorenin (Poisner *et al.* 1990), insulin-like growth factor binding protein-1 (IGFBP-1) (Thrailkill *et al.* 1990) and pregnancy-associated plasma protein-A (PAPP-A) (Bischoff and Tseng 1986). In the monkey and the human, relaxin promotes endometrial growth and induces dilatation of superficial vessels (Dallenbach-Helweg *et al.* 1967). In addition, relaxin inhibits uterine contractions and oxytocin release (Jones and Summerlee 1986; Downing

and Hollingsworth 1993) suggesting that relaxin and oxytocin work in an antagonistic manner.

Human decidua also synthesises and secretes placental protein 14 (PP14). The PP14 content in decidua is higher than in chorion, amnion and placenta (Julkunen 1986). During pregnancy, serum PP14 concentrations are highest between 6 and 12 weeks, after 16 weeks the levels decrease then plateau at the end of the second trimester and remain low during the third trimester (Julkunen 1986).

Human decidua also actively produces inhibin subunits suggesting that the decidua may be a further source of inhibin-related proteins during pregnancy (Petraglia *et al.* 1990). In addition, human decidua produces significant amounts of somatostatin (Kumasaka *et al.* 1979) and corticotrophin releasing hormone (CRH) (Jones *et al.* 1989). It may be suggested that the decidual tissue, as well as being a hormonal target, produces polypeptide hormones as well as prostaglandins, indicating an important endocrine capacity.

1.3.4. EXTRACELLULAR MATRIX (ECM) PROTEINS SECRETED FROM DECIDUAL CELLS

It has been shown that the first trimester human decidua synthesises and releases ECM proteins and several collagen types. An extensive network of collagen types I and III are present in the first trimester human decidua and intracytoplasmic staining by immunocytochemistry has demonstrated the presence of these collagens in decidual cells (Kisalus *et al.* 1987). Collagen type IV and laminin localise in the external lamina which surrounds the differentiated decidual cells. Immunoreactive collagen type V is observed in close association with the external lamina and in the peridecidual matrix. Fibronectin localises throughout the decidual ECM in fibrillar and punctuate patterns in the decidual cell cytoplasm (Kisalus *et al.* 1987). However, it has been shown that a significant loss of collagen type VI occurs in the rat endometrium during decidualization (Mullholland *et al.* 1992). Differentiated decidual cells also have a mesenchymal intermediate filament cytoskeleton and a very few keratin filaments (Kisalus *et al.* 1987). Glasser and Julian (1986) have demonstrated intermediate filaments desmin and vimentin in the rat endometrium.

1.4. BLOOD VESSEL FORMATION (ANGIOGENESIS)

The term angiogenesis refers to formation of new blood vessels, or neovascularization, and is an essential component of tissue growth and development (Hudlicka 1984). The angiogenic process begins with capillary proliferation, and results in formation of a new microcirculatory bed composed of arterioles, capillaries and venules (Folkman and Klagsbrun 1987). The initial component of angiogenesis (capillary formation) has been shown to consist of 3 processes: 1) fragmentation of the basal lamina of the existing vessel, 2) migration of endothelial cells from the existing vessel toward the angiogenic stimulus, and 3) proliferation of endothelial cells (Shepro and D'Amore 1984; Folkman and Klagsbrun 1987). Neovascularization is completed by formation of a capillary lumen and differentiation of some of the newly formed capillaries into arterioles and venules (Hudlicka 1984).

In most adult tissues, capillary growth occurs rarely, and vascular endothelium represents an extremely stable population of cells with a low mitotic rate (Hudlicka 1984). The female reproductive organs (ovary, uterus, placenta) contain some of the few tissues in the adult that exhibit periodic growth and regression. In addition, growth and regression of these tissues are extremely rapid. Therefore, female reproductive tissues are some of the few tissues in which regular angiogenesis occurs as a normal process (Hudlicka 1984; Klagsbrun and D'Amore 1991). Rapid growth and regression of female reproductive tissues are accompanied by equally rapid changes in rates of blood flow (Rosenfeld *et al.* 1974; Ferrell 1989). In addition, vascular endothelial cells of these tissues exhibit mitotic rates equal to or greater than those observed for tumour endothelial cells (Gaede *et al.* 1985). Since the tissues of the female reproductive system are so dynamic, they provide a unique model for studying regulation of angiogenesis during the process of growth, differentiation, and regression in normal adult tissues.

1.4.1. OVARIAN VASCULAR GROWTH

After ovulation, the remaining cells of the ruptured follicle form a transient endocrine gland known as the corpus luteum (Niswender and Nett 1988; Adashi 1994). Following rupture of the follicle, capillaries and fibroblasts from the surrounding stroma proliferate and penetrate the basal lamina. This rapid vascularization of the corpus luteum may be

guided by angiogenic factors which have been detected in the follicular fluid (Frederick *et al.* 1984). Approximately 50% of the cells of a mature corpus luteum are endothelial cells, and the majority of parenchymal (steroidogenic) cells are adjacent to one or more capillaries (Dharmarajan *et al.* 1985; Niswender and Nett 1988). The mature corpus luteum receives most of the ovarian blood supply, and ovarian blood flow is highly correlated with progesterone secretion (Reynolds 1986). In addition, inadequate luteal function has been associated with decreased luteal vascularization (Jones *et al.* 1970), and several investigators have suggested that reduced ovarian blood flow may play a role in luteal regression (Reynolds 1986; Niswender and Nett 1988).

1.4.2. UTERINE AND PLACENTAL VASCULAR GROWTH

In primates, growth of endometrial vasculature begins during the proliferative phase and continues throughout the secretory phase of the menstrual cycle (Meschia 1983). Associated with endometrial capillary proliferation is increased DNA synthesis of vascular endothelial cells (Ferenczy *et al.* 1979). Endometrial growth and development in response to systemic concentrations of ovarian steroids was demonstrated in the classic experiments of Markee (1932, 1940). In these studies, endometrial explants of rabbits or monkeys were transplanted to the anterior chamber of the eye. Not only did these explants quickly recruit a vascular supply, but they also underwent cyclic periods of growth and regression, which were associated with changes in systemic concentrations of steroids.

Rate of blood flow to uterine tissues varies regularly throughout the non-pregnant cycle, being greatest around ovulation time, when systemic levels of oestrogen are greatest, and least during the luteal phase of the cycle when systemic levels of progesterone are greatest (Meschia 1983; Reynolds 1986; Reynolds *et al.* 1992). This varying uterine blood flow rate is temporally associated with the ratio of oestrogen to progesterone in systemic blood (Meschia 1983; Reynolds 1986). On the other hand, progesterone has the effect to modulate responsiveness of the uterine vascular bed to vasodilatory agents (Meschia 1983).

It has been shown that the uterine endothelial cell proliferation is dramatically increased after embryo implantation in rats (Goodger and Rogers 1993). In their study,

the endothelial cell proliferative index in the rat endometrium rose significantly from approximately 1% on the first 2 days of pregnancy to 13% on day 3; and continued to rise to 28% on day 5, and it further rose to 71% on day 7 of the pregnancy at embryo sites only. These authors suggested two separate mechanisms controlling rat uterine endothelial cell proliferation during early pregnancy. The first mechanism is maternally controlled and is apparent throughout the entire endometrium from day 3; and the second mechanism is apparent in the entire embryo (Goodger and Rogers 1993).

In mammals, the placenta consists of maternal (endometrial) and fetal (chorio-allantoic) tissues and is the site of physiological exchange between the maternal and fetal vascular systems. Placental vascular growth begins early in pregnancy and continues throughout gestation in association with a continual and dramatic increase in rates of uterine and umbilical blood flows (Rosenfeld *et al.* 1974; Ferrell 1989). The importance of placental vascular development in supporting fetal growth and development has long been recognized (Meschia 1983; Ferrell 1989). Inadequate placental vascular development may be a major contributor to embryonic wastage and reduced birth weights (Meegdes *et al.* 1988). Although quantitative data on the effects of ovarian steroids on uterine vascularity are lacking, these hormones may have substantial effects on the regulation of vascular growth and development of uterine tissues.

1.4.3. ANGIOGENIC FACTORS

In vivo and *in vitro* methods for the isolation and the characterization of angiogenic and anti-angiogenic factors have been shown by several investigators (Folkman and Klagsbrun 1987; Klagsbrun and D'Amore 1991; Reynolds *et al.* 1992). The two most commonly used *in vivo* assays for the detection of angiogenic factors are the "corneal pocket" assay, which permits linear measurement of capillary growth induced by a substance implanted in the cornea of the rabbit, mouse or rat, and the "chorio-allantoic membrane" (CAM) assay, which evaluates the ability of a substance to stimulate or inhibit neovascularization when placed onto the CAM of the chicken embryo (Folkman and Klagsbrun 1987; Klagsbrun and D'Amore 1991; Rizzo *et al.* 1995). In contrast with *in vivo* techniques, *in vitro* assays can be used to evaluate the ability of a factor to influence one of the angiogenic processes such as: 1) production of proteases by

endothelial cells, 2) migration of endothelial cells, and 3) proliferation of endothelial cells (Folkman and Klagsbrun 1987; Reynolds *et al.* 1992).

Media conditioned by thecal tissues stimulates proliferation of endothelial cells regardless of the stage of follicular development, however granulosa cells produce an endothelial cell mitogen only when they are obtained from non-atretic follicles just before ovulation (Taraska *et al.* 1989). Based on these observations, it is possible to conclude that production of angiogenic factors by granulosa cells may help maintain the vasculature, and thereby the health of the preovulatory follicle. However, thecal production of angiogenic factors seems to be independent of the stage of preovulatory development or follicle status.

The endothelial mitogen produced by preovulatory bovine follicles appears to be heparin-binding (Reynolds *et al.* 1992). Heparin-binding growth factors (HBGF) are a family of closely related proteins (Folkman and Klagsbrun 1987; Burgess and Maciag 1989). The prototypes of this family, HBGF-1 (also known as acidic fibroblast growth factor) and HBGF-2 (basic fibroblast growth factor), are potent angiogenic factors (Folkman and Klagsbrun 1987; Burgess and Maciag 1989). It has been suggested that the corpus luteum could be the major source for angiogenic factors, and thus angiogenic factors other than HBGFs could be produced by the corpus luteum and may play an important role in luteal angiogenesis (Redmer *et al.* 1988).

Media conditioned by uterine tissues from non-pregnant cows and ewes stimulate migration and proliferation of endothelial cells (Millaway *et al.* 1989). Production of angiogenic factors by endometrial tissues of ovariectomized ewes can be modulated by *in vivo* treatment with progesterone and oestradiol (Reynolds and Redmer 1988a). Oestradiol has been shown to stimulate HBGF-2 production by human endometrial adenocarcinoma cells *in vitro* (Presta 1988).

Angiogenic activity of placental tissues from human, bovine, and ovine sources has been evaluated using *in vivo* (CAM) and *in vitro* (endothelial protease production, migration and proliferation) assays (Reynolds *et al.* 1987; Millaway *et al.* 1989). In cows and ewes, these angiogenic factors are primarily produced by maternal placental (endometrial) tissues but not by fetal placental tissues (Millaway *et al.* 1989). It has been suggested that maternal placental (endometrial) tissues may regulate placental

vascularization (Reynolds *et al.* 1992). If this hypothesis is correct, maternal placental production of angiogenic factors could have a significant effect on placental size, transport and blood flow, and thereby on fetal growth and development.

Throughout most gestation, fetal placental tissues of ewes and cows produce a factor or factors that inhibit endothelial cell migration and proliferation (Reynolds and Redmer 1988b). The target of these fetal placental anti-angiogenic factors is likely to be the maternal placental vasculature, where they may function to limit vascular development and thereby prevent uncontrolled growth of capillaries and invasion of fetal tissues. Compounds such as transforming growth factor- β , tumour necrosis factor- α , interferons, or certain steroid metabolites that have been termed angiostatic steroids, may be physiological inhibitors of angiogenesis in female reproductive tissues (Folkman and Klagsbrun 1987; Reynolds and Redmer 1988b).

Other growth factors found in female reproductive tissues, such as vascular endothelial growth factor, insulin-like growth factors, epidermal growth factor and transforming growth factors, may also have a role in regulating the angiogenic process (Folkman and Klagsbrun 1987; Koos 1989; Klagsbrun and D'Amore 1991; Li *et al.* 1994). In the human endometrium, the immunohistochemical localization of vascular endothelial growth factor (VEGF), which has a mitogenic activity specific for endothelial cells, was demonstrated in different concentrations at different times of the menstrual cycle in different cell types (Li *et al.* 1994). These authors concluded that VEGF might serve as a paracrine mediator of the effects of ovarian steroids on endometrial vascular development. In addition to growth factors, other low molecular weight (e.g., nucleotides, eicosanoids) or high molecular weight (e.g., extracellular matrix components such as glycosaminoglycans or fibronectin) molecules are probably involved in regulating the angiogenic process in reproductive as well as other tissues (Eisenstein 1991; Odedra and Weiss 1991).

Although the production of angiogenic factors by female reproductive tissues varies with the stage of development, and is responsive to tropic hormones, much remains to be discovered about the physiological role of angiogenic factors in reproductive tissue vascularization. This is an especially important field in reproductive research because of

its implications for the control of fertility and for vascular growth in other normal and pathological processes, the regulation of which is poorly understood.

Previous studies were involved the differentiation of stromal cells into decidual cells, types of decidual cells, hormones and ECM proteins secreted from decidual cells, blood vessel formation of endometrium and placenta in Man. However, there is little published data on the quantitative morphology of normal fertile human endometrial stroma and blood vessels during mid to late luteal phase of the menstrual cycle which is likely to be crucial for further development of decidual and placental tissues. Therefore the next chapter of the present study is designed to establish baseline quantitative data on the morphology of the endometrial stroma and blood vessels during mid to late secretory phase of the menstrual cycle.

CHAPTER 2

A QUANTITATIVE DESCRIPTION OF NORMAL FERTILE

HUMAN ENDOMETRIAL STROMA

2.1. INTRODUCTION

Although the main aim of this study is to investigate the post-implantation human endometrial stromal and vascular morphology, it is also necessary to consider the morphological changes that lead-up to this stage, such as the proliferative and pre-implantation periods. It is also important to consider the structural changes that occur in the endometrial glandular and luminal epithelial cells, as well as the stromal cells, since these different compartments function as a single unit during the menstrual cycle.

The morphological changes that occur in the human endometrium in response to ovarian steroids are well documented (Wynn 1984; Cornillie *et al.* 1985; Dockery and Rogers 1989; Ferenczy and Bergeron 1991; Li *et al.* 1991a), and a number of morphometric measurements on endometrial biopsies has been published by several investigators (Johannisson *et al.* 1982; Dockery *et al.* 1988a; Dockery *et al.* 1990).

During the proliferative phase of the menstrual cycle the endometrium is mainly under the influence of oestrogen. The histologic changes that occur during this phase of the cycle are not very specific and thus are not useful for precise dating of the endometrium. All of the tissue components, including the glands, stromal cells and endothelial cells demonstrate proliferation which peaks around cycle days 8 to 10 (Ferenczy *et al.* 1979; Ferenczy and Guralnick 1983). These alterations are manifest as increased mitotic activity, nuclear DNA and cytoplasmic RNA synthesis (Ferenczy 1980; Ferenczy and Bergeron 1991). Ultrastructural changes seen during this tissue proliferation include increased free and bound ribosomes, mitochondria, Golgi apparatus, and primary lysosomes in gland cells (Ferenczy *et al.* 1979) and stromal fibroblasts (Holinka and Gupide 1987). Biochemically, these organelles provide for protein matrix, energy and synthesis of enzymes, all of which are required during cell proliferation.

2.1.1. PROLIFERATIVE PHASE GLANDULAR EPITHELIUM

During the proliferative phase of the menstrual cycle, endometrial cells undergo an extensive proliferation. Early in the phase the glandular epithelial cells are tall, columnar and relatively unspecialized (Cornillie *et al.* 1985). Increased ciliogenesis and microvillogenesis proceed at an increased rate in proliferative phase gland cells (Ferenczy and Bergeron 1991). These events are considerably decreased in endometrial cells that

are exposed to endogenous progestogens and are increased in the case of hyperoestrogenism (Ferenczy and Guralnick 1983) indicating that the glandular ciliogenesis and microvillogenesis are oestrogen dependent. Intercellular lateral membranes are unfolded and desmosomes (Cavazos *et al.* 1967), tight junctions and gap junctions may be detected (Davie *et al.* 1977).

The nucleus is large and ovoid having an appearance of a cell with a high level of synthetic activity during this time of the cycle (Verma 1983; Cornillie *et al.* 1985). Polysomes, annulate lamella and a few isolated cisternae of rough endoplasmic reticulum (RER) are seen in the cytoplasm (Cornillie *et al.* 1985). Mitochondria are of normal dimensions and poorly developed Golgi profiles are found in the apical cytoplasm (Wynn 1984). The Golgi complex in those glandular cells elaborates during the mid to late proliferative phase of the cycle along with increased interdigitation of the lateral walls. Immediately prior to ovulation small amounts of glycogen accumulation is evident in the cytoplasm (Cornillie *et al.* 1985).

2.1.2. PROLIFERATIVE PHASE ENDOMETRIAL STROMA

In contrast to the glands, the stromal compartment of the human endometrium appears to have received little attention. However, its contribution to successful implantation and to the maintenance of pregnancy may be crucial. During the proliferative phase, mitoses of stromal cells reach a maximum (about 10 per 1000 cells) around the time of ovulation (Johannisson 1985). The proliferative potential of human endometrial stromal cells has been shown *in vitro* to be greater than that of most other adult tissues (Holinka and Gurspide 1987). At this time of the cycle, stromal cells resemble those of undifferentiated fibroblasts, and become mature fibroblasts as the proliferative phase advances (More *et al.* 1974a; Dallenbach - Helweg 1981). Around the time of ovulation, stromal cells are involved in the formation and remodelling of the extracellular matrix (Cornillie *et al.* 1985). The size of stromal cells increase and the nuclei contain elevated amounts of euchromatin with prominent nucleoli (More *et al.* 1974a). The amount of RER increases and glycogen is evident in the cytoplasm at this time (Dockery and Rogers 1989). During the proliferative phase stromal cell

mitochondria are small and simple in structure and the smooth endoplasmic reticulum and Golgi complexes are poorly developed (Sengel and Stoebner 1970).

2.1.3. SECRETORY PHASE GLANDULAR EPITHELIUM

During the early secretory phase of the menstrual cycle endometrial glandular cells undergo extensive changes which are highly correlated to the rising levels of progesterone. These cellular features include the nuclear channel system (NCS) (Terzakis 1965; Wynn and Wooley 1967; More *et al.* 1974b; More and McSeveney 1980; Dockery *et al.* 1988b), giant mitochondrial profiles (Nilsson 1962; Wynn and Wooley 1967; Kohorn *et al.* 1972; Armstrong *et al.* 1973; Dockery *et al.* 1988b) and massive deposits of glycogen-rich material in the subnuclear region of the cell (Nilsson 1962; Wynn and Wooley 1967; Cornillie *et al.* 1985; Dockery *et al.* 1988b).

The ultrastructural changes in the human glandular epithelial cells in the early secretory phase are well co-ordinated (Dockery *et al.* 1988b). At day LH+2 glandular epithelial cells have large euchromatic nuclei and small amounts of glycogen are seen in the basal region of the cells. The Golgi apparatus is poorly developed (Dockery and Rogers 1989). By day LH+3, increased amounts of glycogen-rich material are evident in the subnuclear region (Cornillie *et al.* 1985). Giant mitochondria are seen, often surrounded by areas of endoplasmic reticulum. The occasional nuclear channel systems may be seen in glandular secretory cells of the mid luteal phase (Dockery and Rogers 1989). At day LH+4 gland cells contain abundant NCS, giant mitochondria and subnuclear glycogen (Dockery *et al.* 1988b). Prominent Golgi complexes are seen in the supranuclear region of the gland cells. By day LH+5 the glycogen content of the gland cells starts to mobilize through the apical region, which also contains dilated Golgi complexes. NCSs are still present on day LH+6 and the nucleus become more heterochromatic, indicating a reduced transcriptional activity in the gland cells (Dockery and Rogers 1989). Day LH+6 gland cells also contain extensive Golgi apparatus and secretory vesicles which strongly indicates an increased secretory function. Lateral cell membranes show extensive membrane folding and interdigitations. By day LH+8, the secretory activity decreases in gland cells, the nuclei become more heterochromatic and

lateral membrane interdigitations are most prominent (Cornillie *et al.* 1985; Dockery and Rogers 1989).

2.1.4. LUMINAL EPITHELIUM OF THE ENDOMETRIUM

When compared to the glandular epithelium, there are relatively few studies on luminal epithelium. Although the latter have had relatively little consideration, it is the first maternal cell layer encountered by the implanting blastocyst and its functions are likely to be at least as important as the other endometrial compartments. Luminal epithelium shows both ciliated and secretory cells which are similar to those seen in glands. However, the cyclical changes are less dramatic than that of glandular cells (Cornillie *et al.* 1985). Although NCS and glycogen deposits have been reported, giant mitochondrial profiles are absent in the luminal epithelial cells (Dockery and Rogers 1989). In the rat, ultrastructural changes in the luminal epithelium coincide with the 'window' of receptivity (Ljungkvist 1972; Murphy and Rogers 1981; Murphy *et al.* 1982; Murphy and Martin 1985). It is generally agreed that a certain degree of synchrony is required between the arrival of the blastocyst at the site of implantation and the stage of development of the endometrium in order to achieve successful implantation (McLaren 1984; Edwards and Fishel 1986). In rats and mice, there is a narrow window during which implantation can be initiated. The ensuing 36-48 hours is a period in which embryos could survive in the uterus, but implantation would not be possible. Then there is a narrow window of just a few hours during which implantation can occur. After this time, the uterine environment becomes hostile, and any unimplanted embryo degenerates (Li *et al.* 1991a). A similar window is thought to exist in man. However, there is as yet no clear evidence for a comparable window of receptivity and no detailed study has yet been carried out on well-timed endometrial material in human.

2.1.5. SECRETORY PHASE ENDOMETRIAL STROMA

In contrast to the extensive literature on epithelial cells, there is relatively little published on stromal cell changes during the menstrual cycle. In the early secretory phase, stromal cells become steadily larger, with nuclei containing less heterochromatin, and structures referred to as 'nuclear bodies' being seen (More *et al.* 1974a). The rough endoplasmic reticulum increases in size and activity and the cytoplasm contains many

vesicles, possibly concerned with transport of material to the extracellular matrix (Dockery and Rogers 1989). Glycogen is commonly found in the cytoplasm (Weinke *et al.* 1968; More *et al.* 1974a). Junctions between stromal cells are described as simple membrane thickenings (More *et al.* 1974a; Wynn 1984). The later luteal phase begins with stromal oedema on LH+8 to LH+10 and continues to the predecidual reaction of stromal cells. Since these changes occur regardless of whether a blastocyst is present or not (Li *et al.* 1991a) the term “pseudo-decidual” may be more appropriate. Stromal oedema is possibly mediated by prostaglandin E₂ (PGE₂) and prostaglandin F_{2α} (PGF_{2α}) and coincides also with rising oestrogen and progesterone levels (Ferenczy and Bergeron 1991). Oestradiol stimulates the production of PGF_{2α}, where as progesterone stimulates the synthesis of both PGF_{2α} and oestrogen in *in vitro* conditions (Neulen *et al.* 1988). PGE₂ presumably promotes capillary permeability either directly or by increasing histamine release resulting in stromal oedema.

The stroma of the endometrium, especially during the mid to late secretory phase, is composed of highly specialized stromal cells. They are not simply fibroblasts, but respond to hormonal stimuli and have receptors for both oestrogen and progesterone (Press *et al.* 1984; Bergeron *et al.* 1988; Lessey *et al.* 1988). In addition they synthesize prostaglandins (Smith *et al.* 1984), and when transformed into decidual cells actively secrete prolactin, immunosuppressive substances and a variety of serum proteins (Riddick *et al.* 1983). It has been suggested that the endometrial granulocytes are differentiated from stromal cells between days LH+8 and LH+10 (Dallenbach-Helweg 1981). However, recent work suggests that “endometrial granulocytes” are of bone marrow origin and are granulated lymphocytes (Bulmer *et al.* 1988a). The predecidual cells are larger than the stromal cells of the early secretory phase, with rounded nuclei and dilated rough endoplasmic reticulum. Golgi profiles and lysosomes are more obvious (Verma 1983; Cornillie *et al.* 1985). The decidual tissue has been considered to play a role in restraining the invasiveness of the conceptus (Johnson and Everitt 1988). A detailed review of decidual cells and their possible functions has been given in Chapter 1.

2.1.6. ENDOMETRIAL BLOOD VESSELS

The uterine arteries arise from the hypogastric arteries, which derive from the common iliac arteries. The arteries supplying the endometrium are of two types: short branches supplying the basal region of the endometrium called 'basal arteries' or sometimes 'straight arteries'; and longer spiral arteries which vascularize the functionalis layer of the endometrium. The spiral arteries are involved in the process of menstruation whereas the basal arteries are largely unaffected. Stromal capillaries empty into veins which form a plexus in the stroma. It has also been suggested that a close connection exists between the thin capillaries and the venules by arteriovenous anastomoses and this connection may form the so-called "venous lakes" (Schlegel 1945; Dalgaard 1945; Bartelmez 1956). Ramsey (1984) suggested that the function of these structures was to regulate the blood volume and the rate of blood flow in the superficial part of the endometrium. On the other hand, the spiral arteries have also been reported to develop small branches in the functional layer which improves its blood supply directly (Fanger and Barker 1961; Ramsey 1984).

In primates, the growth of endometrial vasculature begins during the proliferative phase and continues throughout the secretory phase of the menstrual cycle (Reynolds *et al.* 1992). In the proliferative phase of the menstrual cycle, growth of the vascular tree accompanies the increase in endometrial thickness (Dockery and Rogers 1989). Using intra-ocular endometrial transplants in Rhesus monkeys, Markee (1940) described a five-fold increase in the length of the spiral arteries during the late pre-ovulatory phase whereas the endometrial stroma did not exhibit the same rate of growth and the endometrial thickness only doubled during the same period of time. This discrepancy between the growth rate of the vessels and the thickness of the endometrium may be explained by coiling of the arteries which takes place in the late pre-ovulatory phase. The enzyme arachidonic acid-prostaglandin related cyclooxygenase has been localized in smooth muscle cells and endothelium of endometrial arterioles in the human by immunohistochemistry (Rao *et al.* 1989). Prostaglandins are likely to be the primary stimulator of vascular mitotic activity, which leads to coiling of the arteriolar-capillary system. During the pre-ovulatory phase of the menstrual cycle, the most distal part of the spiral arteries become connected to the sub-epithelial capillary plexus via arterioles.

Like the distal arteries, the sub-epithelial capillary plexus is likely to undergo changes during the normal menstrual cycle. Receptors for oestrogen and progesterone are localized in the muscular wall of the endometrial vessels (Perrot-Applanat *et al.* 1988), and sex steroids may also play a role in vascular proliferation in the endometrium. The endothelial lining of endometrial vessels is uniformly free of sex steroid receptors (Bergeron *et al.* 1988; Perrot-Applanat *et al.* 1988). It has been suggested that the endometrial microvascular density did not change throughout the menstrual cycle whereas it increased in women using contraceptive agent levonorgestrel (Rogers *et al.* 1993). Goodger *et al.* (1994) also suggested that the endometrial endothelial cell proliferative index of levonorgestrel users was reduced compared to that seen in normally cycling women, whereas total numbers of endothelial cells per mm² in levonorgestrel users were higher than in controls. They postulated that it was either a reduced rate of blood vessel regression associated with contraceptive use or a reduced rate of endothelial cell death or turnover. These findings may indicate a direct involvement of sex steroids in the mechanisms of endometrial bleeding as well as in the menstruation process.

2.1.7. ENDOMETRIUM DURING MENSTRUATION

Withdrawal of sex steroids (especially progesterone) from a previously primed endometrium results in menstrual bleeding in women. However, two mechanisms, related to steroid levels, appear to be involved in menstrual bleeding: 1) bleeding associated with declining levels of peripheral steroids and 2) bleeding associated with steady levels of peripheral steroid levels (Smith 1989). On the other hand, daily measurements of oestradiol and progesterone in women with and without heavy periods have failed to demonstrate any correlation between steroid levels and severity of menstrual bleeding (Haynes *et al.* 1979).

Similar to other tissues, haemostatic mechanisms exist in the uterus and they are involved in limiting excessive blood loss during menstruation. One of the early events in a haemostatic reaction is the attachment of platelets to the damaged vessel wall via the interaction with von Willebrand factor (vWF) secreted by the endothelial cells (Au and

Rogers 1993). The involvement of the activation of both coagulation and fibrinolytic enzyme systems in menstrual bleeding has also been suggested (Sheppard 1989).

There are 2 different proposed mechanisms involved in the shedding of the endometrial tissue during menstruation. In one view, the functionalis layer of the endometrium is totally shed and the subsequent repair and re-growth occurs by a process of re-epithelization from the outgrowth of glands deep in the basalis (Ferenczy 1976; Wynn 1984; Ferenczy *et al.* 1979). The other view is that most of the functionalis is conserved during menses and repair takes place from the remaining tissues by “dynamic physiological process” (Wilborn and Flowers 1984). It is one of the aims of the present study to evaluate the late secretory endometrial morphology which is likely to be the beginning of the sequence of events leading to the shedding of the human endometrium.

The morphology of the human endometrial glandular epithelium throughout the menstrual cycle has been well studied. However, despite their importance in implantation and maintenance of pregnancy, human endometrial stromal and vascular compartments have received less attention than they deserve. In order to obtain a better understanding of the sequential events that take place in the human endometrium during the menstrual cycle more reliable information on stroma and vascular tissue is needed. It is the aim of present study to fill this gap in human reproductive biology.

2.2. MATERIALS AND METHODS

2.2.1. SUBJECTS

Thirtyeight normal, healthy women volunteers of proven fertility, aged 20-40 years with regular menstrual cycles of 25-35 days, were used in this study. None of the women had used steroidal contraceptives during the previous two months. They were all non-pregnant and were able to communicate well with the investigators. If there was any clinically significant medical condition, including major uterine pathology, renal, hepatic, cardiovascular, gastrointestinal, psychiatric or endocrine disorders and hypothalamic pituitary failure, these volunteers were excluded from the study. All subjects were treated on an outpatient basis and were counselled and given instructions about the procedure of the experiment. All procedures had Ethics' Committee approval and all clinical procedures were carried out by Mr T.C. Li of the Jessop Hospital for Women, Sheffield.

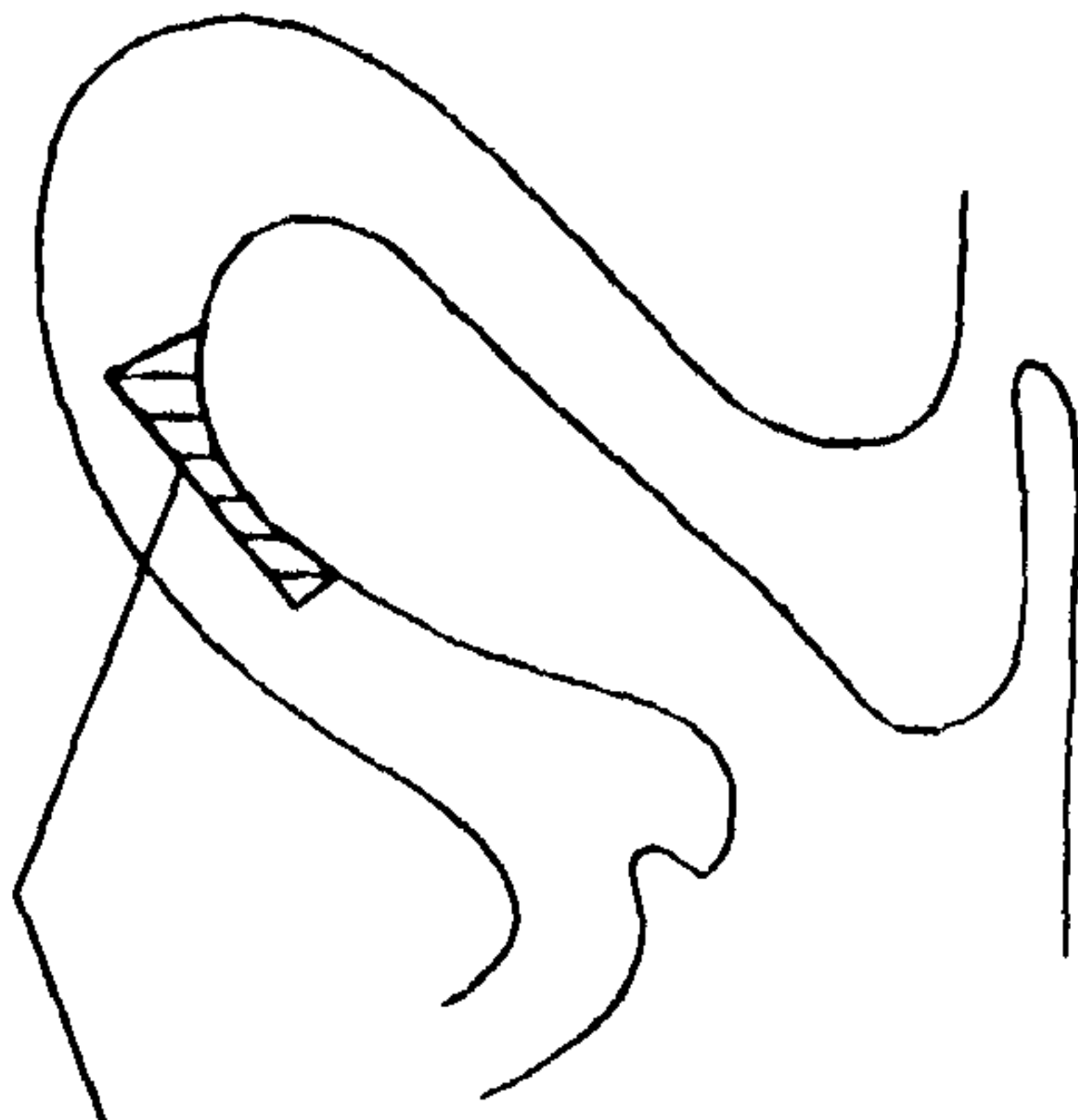
2.2.2. EXPERIMENTAL DESIGN

In order to examine the normal human endometrial structure from mid- to late-secretory phase of menstrual cycle, endometrial biopsies were obtained at 2 day intervals starting from day LH+6. Eighteen subjects were divided into four groups. The first group, consisting of 6 individuals, had biopsies taken at day LH+6 of the menstrual cycle and the other groups, each consisting of 4 individuals, were biopsied at days LH+8, LH+10 and LH+12 respectively. Chronological dating of the endometrial biopsies was carried out by determination of the luteinizing hormone (LH) peak by daily LH assay (Li *et al.* 1987).

2.2.3. BIOPSIES

Biopsies were obtained, with informed consent, as an outpatient procedure with or without Entonox analgesia. Using a Sharman's biopsy curette (Down's Surgical Ltd, Sheffield, UK), a single specimen was taken from the fundus and upper part of the body of the uterus (Figure 2.1). All of the biopsies were performed by a proficient gynaecologist to standardize the biopsy and sampling procedure.

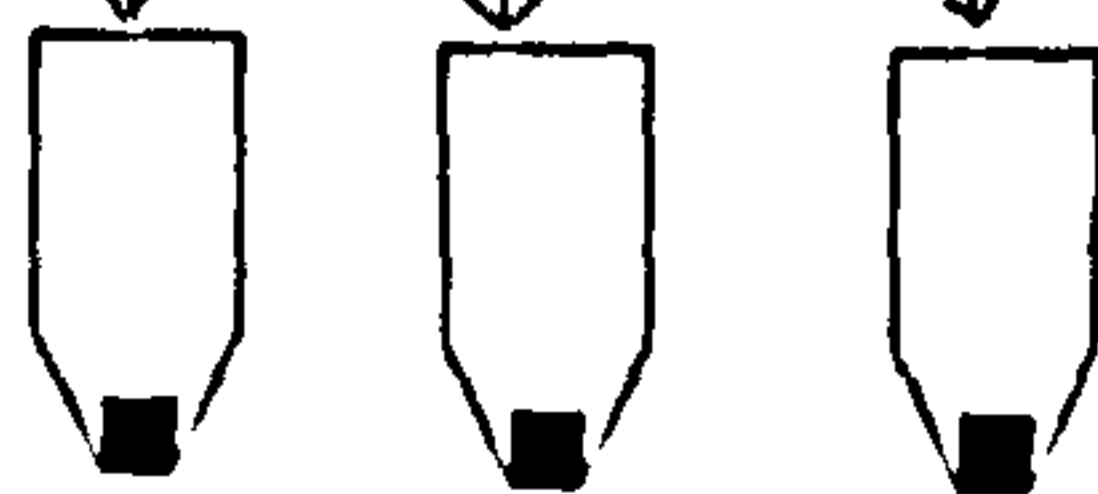
UTERUS



BIOPSY



JB 4 BLOCKS



EPON BLOCKS



SECTIONS

Figure 2.1: The tissue sampling design showing the various sampling levels used in the present study.

2.2.4. TISSUE PROCESSING FOR LIGHT MICROSCOPY

Each specimen was attached on a piece of dental wax at each end by pins and fixed by immersion in 2% glutaraldehyde in phosphate buffer (pH = 7.4) (see Appendix 1). After one hour, the tissue was cut up into smaller pieces in a standardized manner (See Figure 2.1). The tissue was fixed for a further 3 hours in 2% glutaraldehyde in phosphate buffer solution. After washing in phosphate buffer solution, tissue was dehydrated through the series of 70%, 80%, 90% and 95% ethanol (1.5 hr each step). The tissue was further dehydrated in absolute ethanol, two changes of 1hr each. Following dehydration, tissue was put in catalysed JB4 solution A (Appendix 1) overnight and next day tissue was embedded in JB4 embedding solution (Polysciences Inc., USA) and was kept in a refrigerator for 1-2 hours until the tissue had hardened. Two micrometer thick sections of this tissue were used for histological dating according to Noyes *et al.* (1950).

2.2.5. SECTIONING AND STAINING FOR LIGHT MICROSCOPY

Approximately 2 μm thick sections were cut from JB4 embedded endometrial tissues using an LKB 2218 Historange microtome (LKB Bromma, Sweden) and glass knives made with a LKB 2078 HistoKnifemaker (LKB Bromma, Sweden). Sections were collected on glass slides and stained with acid Fuschin-Toluidine blue staining. To do this, sections were stained in 1% aqueous acid Fuschin for 2 minutes. Then they were washed in distilled water and counter stained in 0.05% Toluidine blue (pH = 4.4) in acetate buffer for 2 minutes, washed in distilled water for 30 seconds, dried in air and mounted with a No.1 coverslip with DPX.

2.2.6. PERIODIC ACID-SCHIFF (PAS) STAINING FOR GLYCOGEN-LIKE MATERIAL AT LIGHT MICROSCOPE LEVEL

Other 2 μm thick JB4 sections, adjacent to those used for light microscopy, were used for PAS staining. They were put in 1% periodic acid for 5 minutes then washed in tap water. Sections were then put in Schiff's reagent for 15 minutes and washed well in running water for 15 minutes. Sections were stained in Haematoxylen 2 minutes for staining of the nuclei then rinsed in water, dehydrated through the ethanol series quickly, mounted with DPX after exposing slides to xylol. Glycogen-like material was stained

pink as a result of the PAS staining. A small intestine JB4 section was included with every set of endometrial sections for PAS staining as a control, since the small intestine is known to have large glycogen deposits in its surface epithelium.

2.2.7. TISSUE PROCESSING FOR ELECTRON MICROSCOPY

Glutaraldehyde fixation of the tissue was the same as described in the previous section on tissue processing for light microscopy. Following the first fixation step, tissue was post-fixed in 1% aqueous osmium tetroxide (OsO_4) for two hours and washed in 0.1M phosphate buffer several times until OsO_4 was totally removed from the tissue. Tissue was dehydrated through the series of 75% and 95% ethanol (20 minutes each step). Tissue was dehydrated in absolute ethanol (2 changes, 10 minutes each) and further dehydrated in absolute ethanol which was dried over anhydrous copper sulphate (20 minutes). Following the dehydration steps, tissue was put in propylene oxide (1,2-Epoxypropane) (Fisons Scientific Equipments, Loughborough, England) for 20 minutes (2 changes 10 minutes each). Then tissue was put in 50:50 propylene oxide / Epon resin mixture overnight. The following day, tissue was put in fresh Epon resin (two changes 2-3 hours each) and embedded in freshly prepared Epon resin and left to polymerise in an embedding oven (Taab, UK) at 60°C for 48 hours. Preparation of Epon resin is described in Appendix 1.

2.2.8. SEMI-THIN SECTIONING AND STAINING FOR ELECTRON MICROSCOPY

Two blocks of tissue were prepared from each biopsy. Approximately $0.5\ \mu\text{m}$ thick sections were cut from Epon blocks using a Reichert (OMU3) ultramicrotome and stained with 1% Toluidine blue in 1% borax for approximately 30 seconds at 60°C on a hotplate, differentiated in 50% ethanol, dried in air or on the hotplate and mounted with a No.1 coverslip using DPX. Glass knives were made using an LKB 7800 knifemaker (LKB Bromma, Sweden).

2.2.9. ULTRA-THIN SECTIONING AND STAINING FOR ELECTRON MICROSCOPY

The same blocks used for semi-thin sectioning were used for ultra-thin sectioning. Silver-gold interference coloured ultra-thin sections (about 70nm thick) were cut by the same ultra-microtome, collected on 3.05 mm 100 mesh copper grids (Agar Aids, Essex, UK) coated with 2% pyroxylyene (collodion) in amyl acetate. Sections were double stained with uranyl acetate (saturated in 50% ethanol) for 20 minutes in the dark and lead citrate for 5 minutes in a CO₂ free atmosphere. A few sodium hydroxide pellets were placed in the lead citrate staining dish in order to absorb the CO₂. Using the technically best parts of the sections, 10 electron micrographs per section (20 from each individual) in a systematic random manner (Figure 2.2). All electronmicrographs were taken using a Phillips 301 transmission electron microscope at an accelerating voltage of 60 KV. A grating replica of 2,160 lines per mm (Agar Ltd, Essex, UK) was used as a magnification standard on each film.

2.2.10. PERIODIC ACID THIOSEMICARBAZIDE - GELATINE METHENAMINE SILVER (PATSC-GMS) STAINING OF ENDOMETRIAL GLYCOGEN-LIKE CONTENT AND BLOOD VESSEL BASEMENT MEMBRANE

This staining procedure was performed according to Namimatsu (1992). Ultra-thin sections of endometrial biopsies were collected on nickel grids. The sections were pre-incubated in 25% aqueous ammonia water for 10 minutes, oxidized in 1% aqueous periodic acid for 20 minutes, reacted with 0.1% thiosemicarbazide (TSC) for 1 minute, post-incubated in 25% ammonia water for 5 minutes, and stained with gelatine methenamine silver (GMS) solution (Appendix 1) for 50 minutes at 50-55 °C in the oven. Observations were performed under a Philips 301 transmission electron microscope.

2.2.11. SAMPLING METHODS

The aim of the sampling design used in the present study was to obtain quantitative structural information on human endometrial stromal and vascular compartments in an efficient manner. It has been shown that the biological variation between different

individuals contributes most variation to the overall experimental variability, whereas the inter-block and feature to feature variations on sections are less important (Gundersen and Østerby 1981). Contribution of the variation at different sampling levels to the total variance in a biological study using experimental animals has been shown typically to be 70% - animals; 20% - blocks; and fields and intercepts combined - 10% (Gundersen and Østerby 1981). However, in the case of human endometrial biopsy, the inter-block variance contribution is similar to that between individuals (42% - individuals; 37% - blocks; 21% - fields) (Dockery *et al.* 1988a). This may reflect the variability of sampling during the biopsy procedure combined with real variation within the uterus. However, this is unavoidable since the endometrial biopsy is the only way to obtain tissue from living individuals for morphological study. The multilevel sampling strategy (sampling tree) used in the present study is shown in Appendix 6 and this was designed to minimize the effects of variation.

At the light microscope level, one section per block and two blocks per biopsy (individual subject) were taken. Systematic random samples of 10 fields of view were obtained from each section. Point counting (using a 1 cm square grid, 10 μm on the tissue) was used to determine the volume fractions of several endometrial features. The nuclear profile diameter measurements were performed at the light microscope level using a digitizer-light microscope (with a drawing tube)-computer device.

At the electron microscope level, 2 blocks per individual were selected by lottery and one section per block was cut. Ten electron micrographs per section were taken with a Philips 301 electron microscope in a systematic random pattern (Figure 2.2) and examined at 2800 times magnification for stromal cell volume fraction measurements using a 1 cm square grid (3.60 μm on the tissue).

In addition, for blood vessel observations, 2 blocks per individual were selected by lottery and 1 section per block was cut. Blood vessels were selected randomly and 10 electron micrographs per section were taken and examined at 5900 times magnification using a 1 cm square grid (1.70 μm on the tissue). A pilot study was performed to determine the optimum magnifications and sampling methods for morphometric evaluation of the endometrial tissue.

These electron micrographs were also used to determine the relationship between the thickness of the lipid vesicle membrane.

2.2.11 MORPHOMETRY

In order to obtain a full description of morphology of human red blood cells, a grid of random points (Wolfe, 1979) is superimposed on the electron micrograph. The grid is used to measure the area of the cell and the area of the membrane.

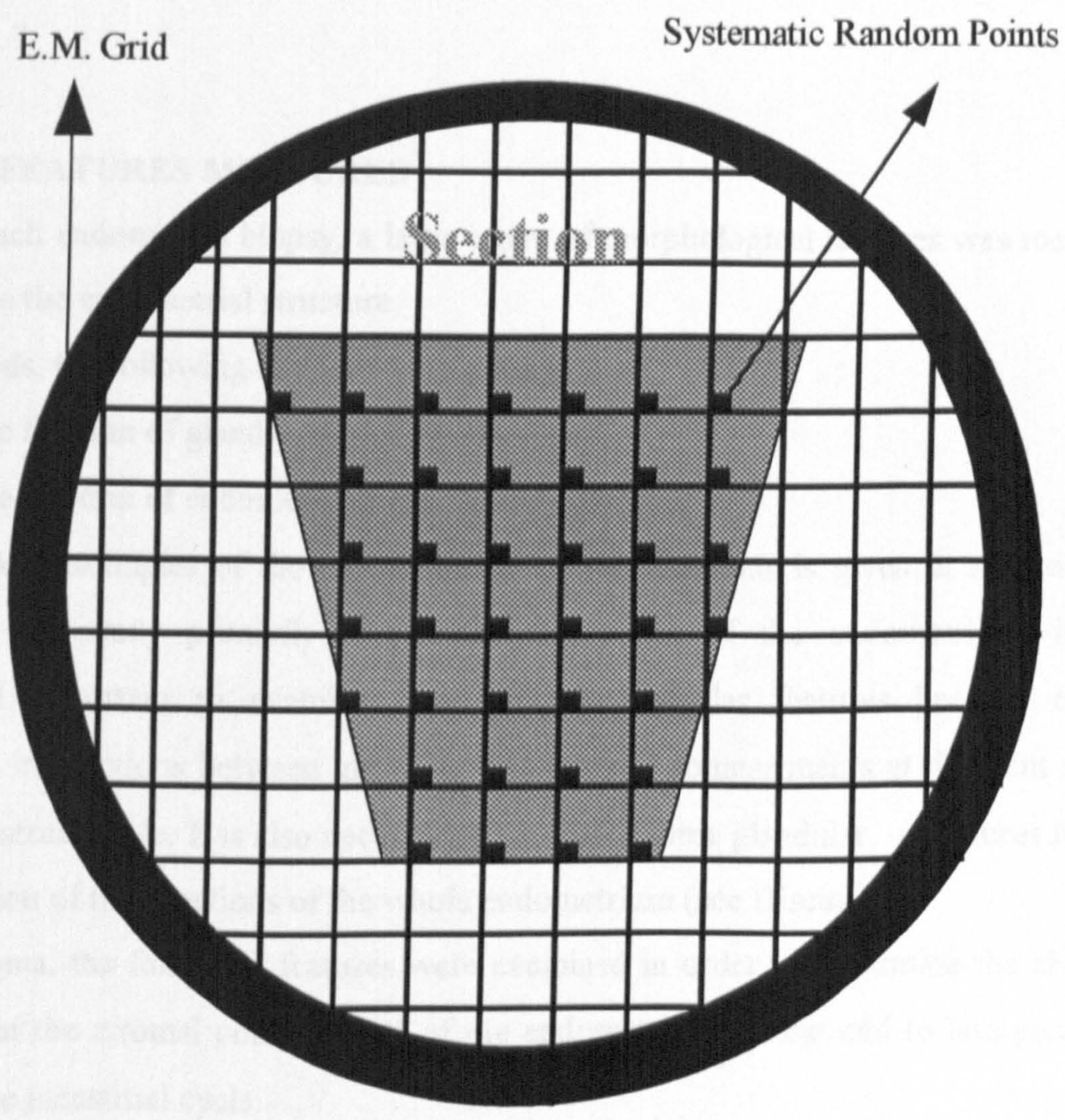


Figure 2.2: Sampling model for electron microscopical observations.

These electron micrographs were also used to determine the arithmetic and harmonic mean thickness of the blood vessel basement membrane.

2.2.12. MORPHOMETRY

In order to obtain a full descriptive morphology of human endometrium a wide range of features were evaluated using appropriate morphometric methods (Weibel 1979). A nested diagram of the morphometric model used in the present study is shown in Appendix 7.

2.2.12.1. FEATURES MEASURED

For each endometrial biopsy, a large range of morphological features was measured to evaluate the endometrial structure.

In glands, the following features were examined.

- a) Volume fraction of gland occupied by gland cell.
- b) Volume fraction of endometrium occupied by gland.

Worked examples of the estimation of volume fractions is given in Appendix 3. Although this study primarily involve stromal tissue of the endometrium, it was considered important to examine some of the glandular features because of the interaction in functions between glandular and stromal compartments at different stages of the menstrual cycle. It is also necessary to evaluate some glandular structures for the interpretation of the functions of the whole endometrium (see Discussion).

In stroma, the following features were examined in order to determine the changes occurring in the stromal compartment of the endometrium during mid to late-secretory phase of the menstrual cycle.

- a) Volume fraction of endometrium occupied by stroma.
- b) Volume fraction of endometrium occupied by stromal cell nucleus.
- c) Volume fraction of stromal cell occupied by nucleus.
- d) Volume fraction of nucleus occupied by euchromatin.
- e) Volume fraction of stromal cell occupied by mitochondria.
- f) Volume fraction of stromal cell occupied by “secretory apparatus” (Golgi complex, SER, vacuoles and vesicles were considered as “secretory apparatus”).

g) Volume fraction of stromal cell occupied by rough endoplasmic reticulum (RER).

In addition to the above features, stromal cell nuclear profile features were measured in order to determine the changes in shape of the stromal cell nucleus that occur in mid to late secretory phase of the menstrual cycle.

- a) Stromal cell nuclear profile major axis.**
- b) Stromal cell nuclear profile minor axis.**
- c) Stromal cell nuclear profile mean diameter.**
- d) Stromal cell nuclear axial ratio.**

In endometrial blood vessels including all types of blood vessels such as arteries, veins, arterioles, venules and capillaries, the following features were examined.

- a) Volume fraction of endometrium occupied by blood vessel lumen.**
- b) Volume fraction of endometrium occupied by blood vessel tissue.**
- c) Volume fraction of endometrium occupied by total blood vessel (obtained by the addition of points falling on lumen + tissue).**

The above features were chosen for the complete evaluation of the endometrial blood vessels in order to determine the contribution that each of these parts made to the vessels (See Discussion).

In order to evaluate the endothelial cell nuclear shape, the following features were measured .

- a) Endothelial cell nuclear profile major axis.**
- b) Endothelial cell nuclear minor axis.**
- c) Endothelial cell nuclear mean diameter.**
- d) Endothelial cell nuclear profile axial ratio.**

In the following endothelial cell features only the small endometrial capillaries with three or less endothelial cell profiles visible were chosen and evaluated. These capillaries were selected because they are the major site at which transport between blood and endometrium occurs.

- a) Volume fraction of endothelial cell occupied by nucleus.**
- b) Volume fraction of endothelial cell occupied by euchromatin.**
- c) Volume fraction of endothelial cell occupied by mitochondria.**
- d) Volume fraction of endothelial cell occupied by secretory apparatus.**

- e) Volume fraction of endothelial cell occupied by RER.
- f) Arithmetic mean basement membrane thickness of the endometrial blood vessels.
- g) Harmonic mean basement membrane thickness of the endometrial blood vessels.

2.2.12.2. ESTIMATION OF ENDOMETRIAL VASCULAR BASEMENT MEMBRANE THICKNESS

Human endometrial blood vessel arithmetic mean and harmonic mean basement membrane thicknesses were determined according to Jensen *et al.* (1978) and Hirose *et al.* (1982).

2.2.12.2.1. THE ARITHMETIC MEAN BASEMENT MEMBRANE THICKNES

Micrographs of randomly selected endometrial blood vessels were taken as described in Section 2.2.11. Electron micrograph negatives were projected on to a screen where a 5 cm grid was superimposed on the image. After enlarging the image at approximately X20,000 magnification, the exact magnification of the image was determined using a grating replica (2160 lines per millimetre) negative taken at the same magnification (X5900) as blood vessel electron micrographs. An ordinary ruler was used to measure the orthogonal intercepts of the blood vessel basement membrane in millimetres. Ten blood vessel electron micrographs per individual were used for basement membrane thickness measurements. The estimation of the true arithmetic mean basement membrane thickness was obtained using the formula below:

Formula 2.1. $Ta = \pi/4 \cdot l\alpha \cdot 10^{-6}$

Ta: Arithmetic thickness

$\pi/4$: Correction factor

$l\alpha$: Mean length of the orthogonal basement membrane intercepts

Formula 2.2. $l\alpha = 1/N \cdot \Sigma l$

N: Total number of observations

Σl : Total length of orthogonal intercepts

After estimating the arithmetic mean thickness, the resultant value is divided by the final magnification (M) of the electron micrograph for estimation of the true arithmetic basement membrane thickness in nanometers. (Worked example of arithmetic mean basement membrane thickness estimation is given in Appendix 5).

2.2.12.2.2. THE HARMONIC MEAN BASEMENT MEMBRANE THICKNESS

The distribution of true basement membrane thickness is markedly right-skewed (Gundersen and Østerby 1973), and the arithmetic mean is therefore not the most efficient estimator of thickness. Moreover, a certain bias is always present in estimations, due to curvature and unevenness of the basement membrane, and this bias can be significant in the arithmetic mean basement membrane thickness values. For these reasons, it is advantageous to also estimate the harmonic mean basement membrane thickness which is the reciprocal of the mean reciprocal thicknesses.

Sampling and measuring procedures for harmonic mean thickness measurements were the same as the arithmetic mean thickness measurements with one exception; a logarithmic ruler, instead of an ordinary ruler, was used to measure the thickness of the orthogonal intercepts of the blood vessel basement membrane. Estimation of the harmonic mean thickness used the following formula:

$$\text{Formula 2.3: } \ell h = N : \Sigma 1/\ell$$

ℓh = Mean length of the basement membrane orthogonal intercepts

N = Total number of observations

$$\text{Formula 2.4: } Th = 8 / (3\pi) \cdot \ell h \cdot 10^6$$

Th = Harmonic mean thickness

$8 / (3\pi)$ = Correction factor for estimation of the harmonic mean thickness

10^6 is used to convert the final thickness value from millimetres to nanometers.

Final harmonic mean value is divided by the real magnification (M) of the image which was projected on a screen. (Worked example of the estimation of harmonic mean basement membrane thickness is given in Appendix 4 section)

2.2.13. STATISTICAL ANALYSIS

Means and standard errors were calculated for each group and data were analysed using a one way analysis of variance (ANOVA) test where the conventional level of 5% was taken as the level of significance. In addition to the ANOVA test, mean values for each group were compared to others individually using Bonferroni's corrected t -test. Statistical comparisons were performed between day LH+6 group ($n=6$), day LH+8 group ($n=4$), day LH+10 ($n=4$) and day LH+12 group ($n=4$).

2.3. RESULTS

A wide range of endometrial features were evaluated morphometrically at cycle days LH+6, LH+8, LH+10 and LH+12 respectively and results of these evaluations were compared statistically using One Way Analysis of Variance (ANOVA) Test, paired t-test and unpaired Student's t-Test as appropriate. These results are presented in Tables 2.1-2.7.

2.3.1. QUALITATIVE EVALUATION OF THE ENDOMETRIAL GLYCOGEN CONTENT

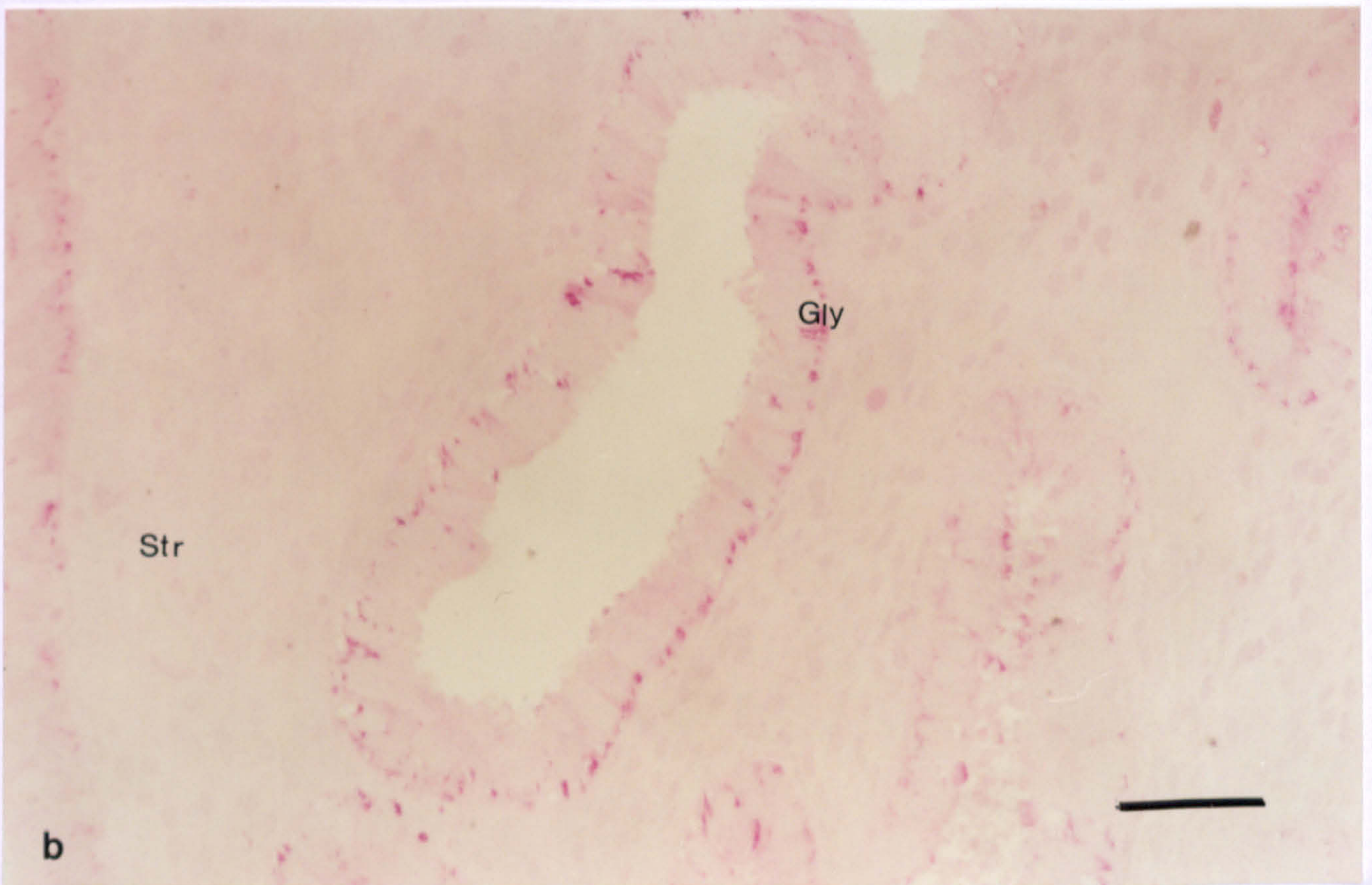
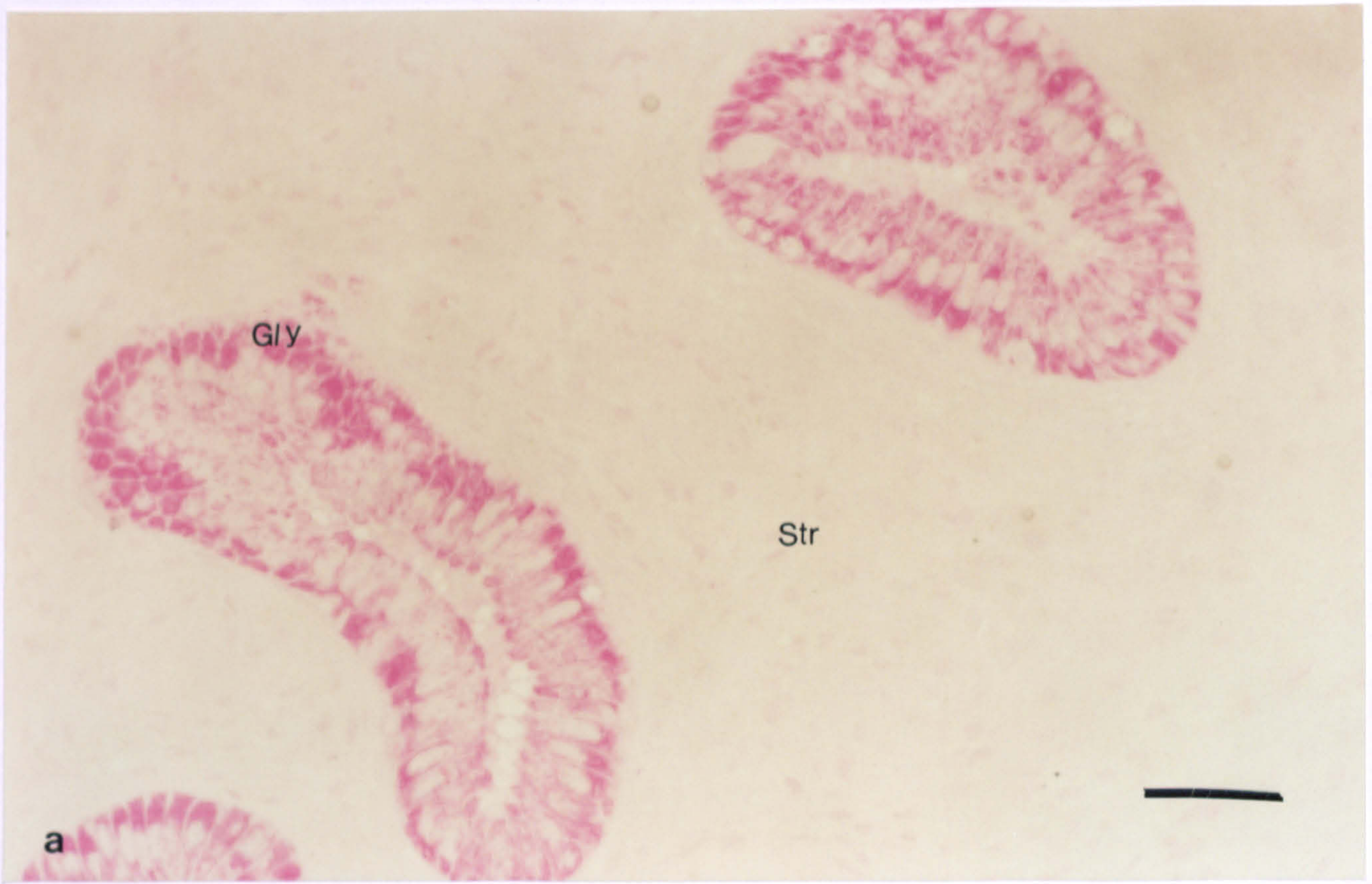
A qualitative evaluation of PAS-positive material in endometrium between days LH+2 and LH+12 at 2-day intervals was performed. Micrographs of some of this tissue are shown in Figures 2.3a,b. Much of the PAS-positive material was glycogen (See Discussion). At day LH+2, two out of six individuals had no glycogen deposits in their glandular epithelium. While one individual had relatively few glycogen deposits in glands, the remaining three individuals had more subnuclear glycogen deposits in the glandular epithelium. One individual had subnuclear glycogen material in some parts of the luminal epithelium. At day LH+4, there was more glycogen-like material in subnuclear regions of the glandular epithelial cells when compared to LH+2 (Figure 2.3a; Figure 2.5a). Two out of six individuals had glands full of PAS-positive glycogen-like material in the subnuclear region of the glandular cells. At this time of the cycle one individual had little glycogen whereas the remaining three had more glycogen in various regions of the gland cells. In addition, at day LH+4 stromal cells showed intracytoplasmic glycogen-like material whereas this material was not evident in stromal cells at day LH+ 10 (Figure 2.4a,b). By day LH+6 the intracellular glycogen levels in glandular cells decreased (Figure 2.3b). Three individuals had no glycogen anywhere, whereas one individual had a few glycogen deposits in the gland cells. The remaining two individuals still had some glycogen-like material in the subnuclear region. Luminal epithelia of three individuals at day LH+6 contained glycogen deposits in the subnuclear region of the cells. One individual that had no glycogen in the gland cells had glycogen-like material in luminal region of the endometrial glands. At day LH+8, few individuals had glycogen deposits in any of the endometrial structures. Only two individuals had a

Figures 2.3a,b: Shows the subnuclear glycogen-like material (Gly) of endometrial gland cells at day LH+4 (a) and at day LH+6 (b) of the menstrual cycle in normal fertile controls. While there is abundant PAS positive staining in the subnuclear region of the gland cells at day LH+4, positive glycogen staining decreases by day LH+6 of the cycle. (Str) stroma.

Staining: Periodic acid-Schiff (PAS).

(a)- Bar represents 34 μm

(b)- Bar represents 38 μm

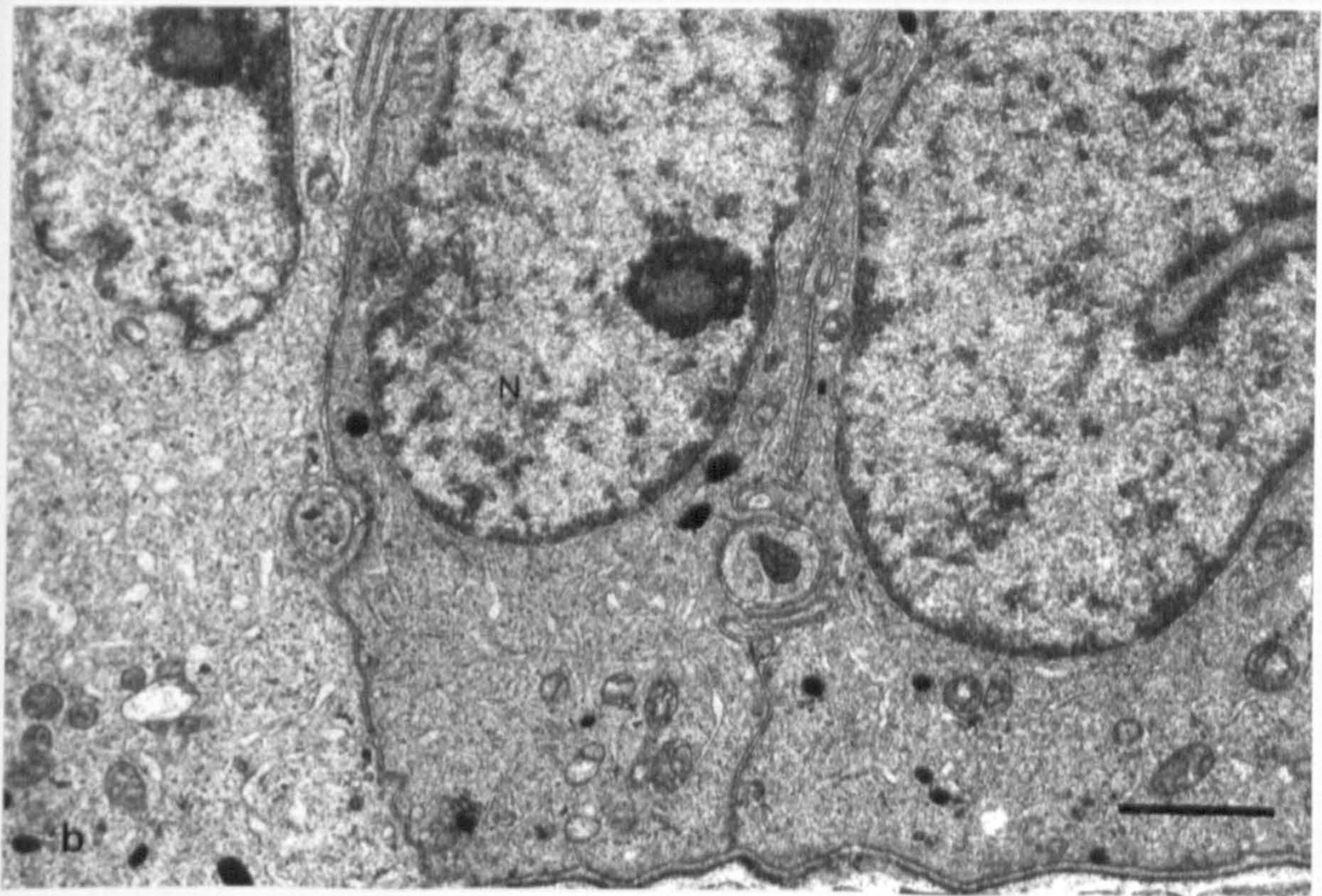
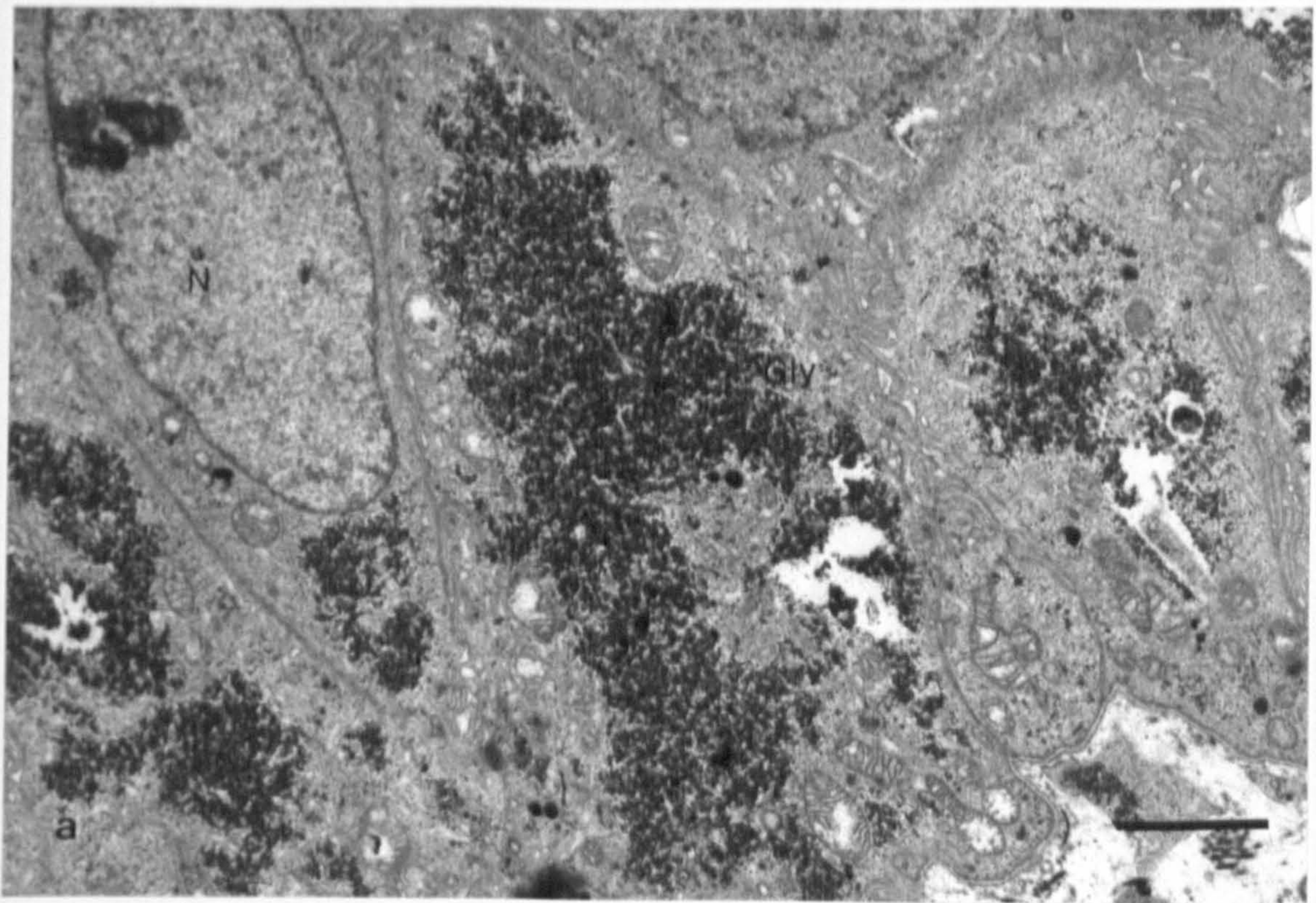


Figures 2.4a,b: Ultrastructural demonstration of glycogen-like content (Gly) in subnuclear region of the glandular cells at days LH+4 (a) and LH+10 (b) of the cycle. Day LH+4 gland cells show extensive glycogen-like material in the subnuclear region whereas cells from day LH+10 have no such material. (N) nucleus.

Staining: Periodic Acid Thiosemicarbazide - Gelatine Methenamine Silver (PATSC-GMS)

(a) Bar represents 2.30 μm

(b) Bar represents 1.35 μm



Figures 2.5a,b: Endometrial stromal cells and surrounding extracellular matrix at days LH+4 (a) and LH+10 (b) of the menstrual cycle in control subjects. Day LH+4 stromal cell cytoplasm contains glycogen-like material (Gly), stromal cells have a relatively regular shape and collagen fibres (arrow) are also evident. Day LH+10 cells show no glycogen-like material, however they demonstrate extensive collagen fibres (arrow) in the ECM and also inside the cell itself. (N) nucleus.

Staining: Periodic Acid Thiosemicarbazide - Gelatine Methenamine Silver Staining (PATSC-GMS)

(a) Bar represents 3.00 μm

(b) Bar represents 3.20 μm

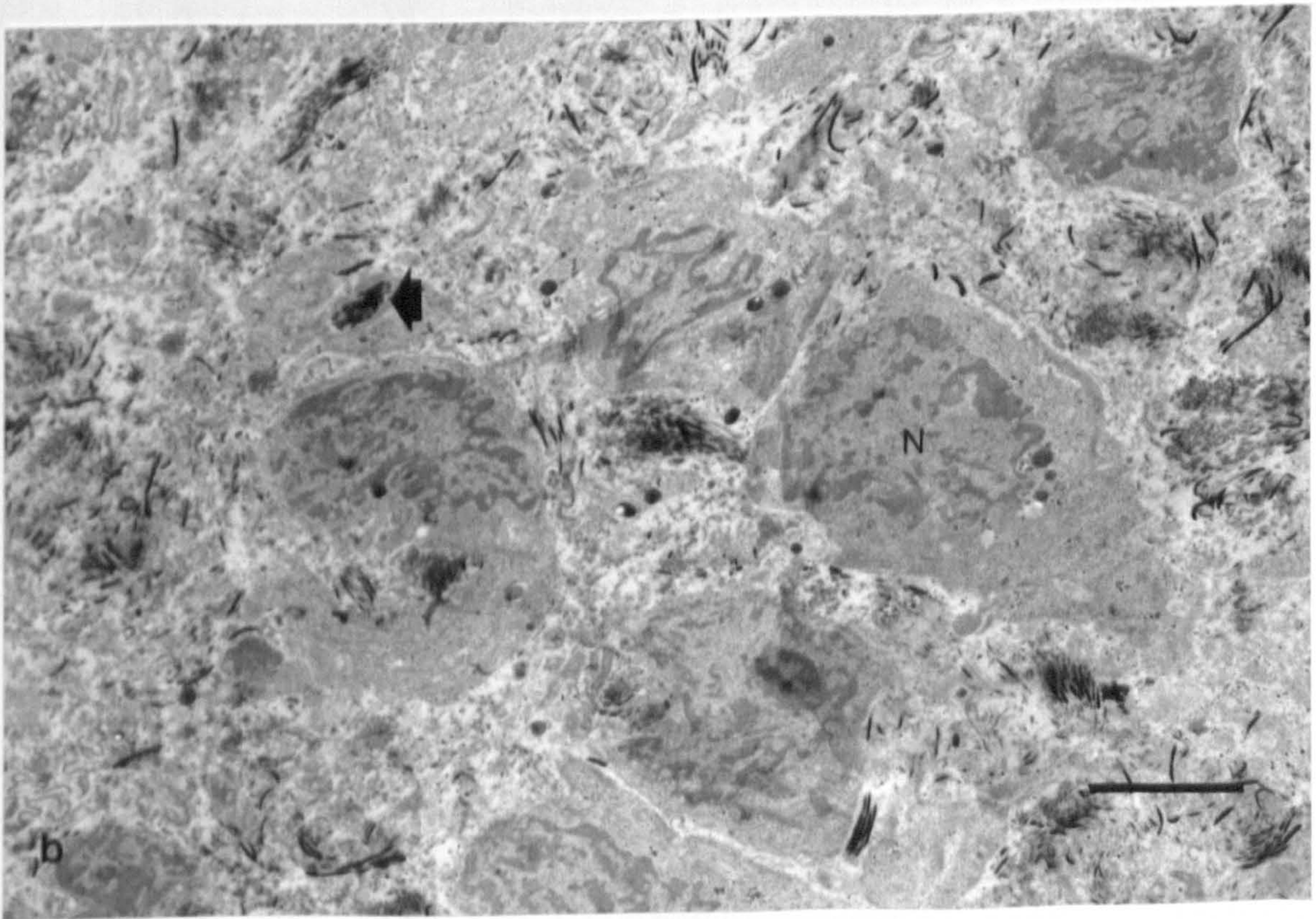
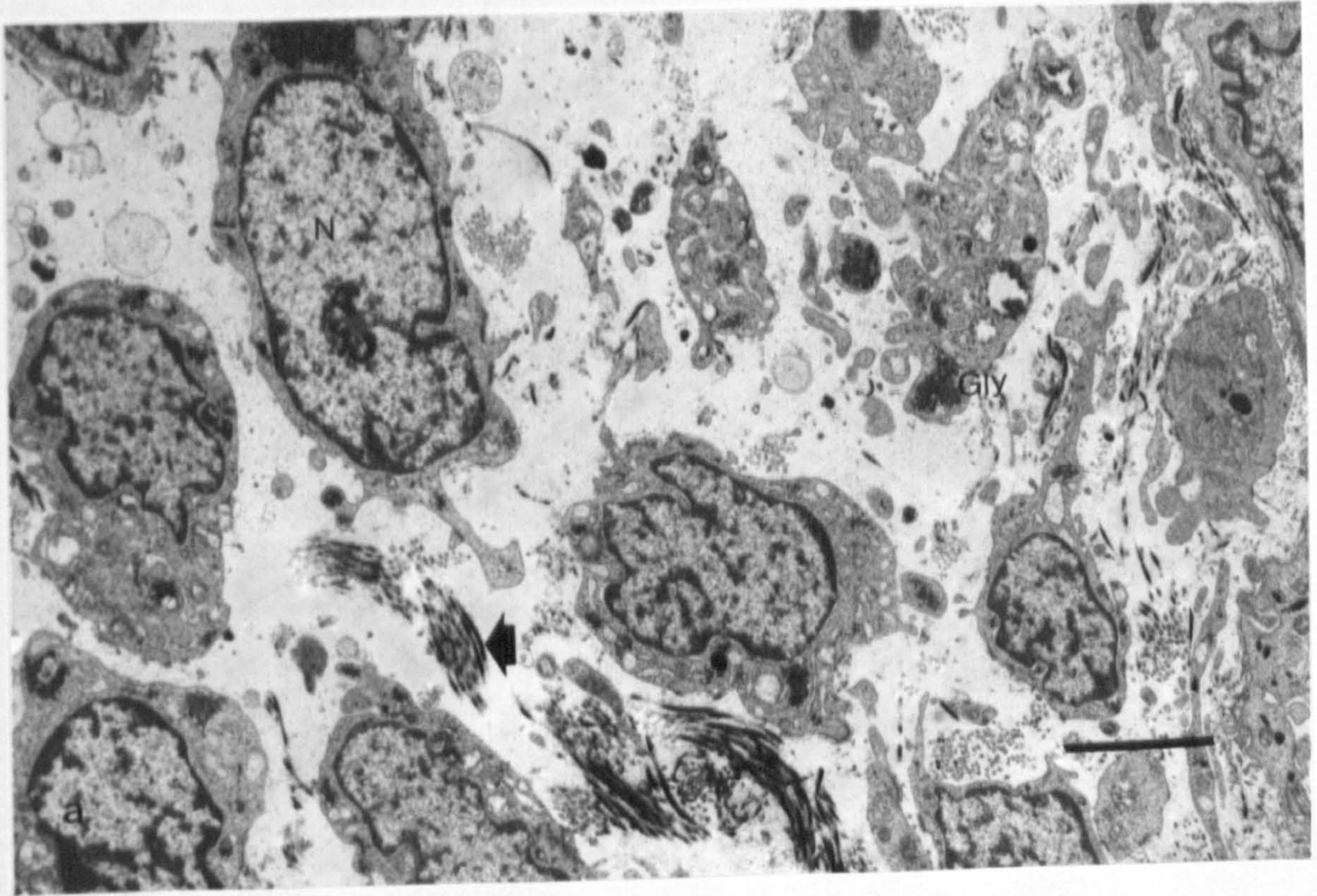


Table 2.1: Comparison of the volume fractions for glandular features in mid to late secretory phase of the menstrual cycle (days from LH+6 to LH+12 at 2 day intervals).

Features	Cycle Day LH+6 (n=8)	Cycle Day LH+8 (n=4)	Cycle Day LH+10 (n=4)	Cycle Day LH+12 (n=4)	ANOVA Test Result
Vv of Gland Occupied by Gland Cell	0.832 ± 0.021	0.832 ± 0.027	0.790 ± 0.015	0.767 ± 0.047	ns
Vv of Endometrium Occupied by Gland	0.223 ± 0.017	0.216 ± 0.023	0.196 ± 0.058	0.241 ± 0.048	ns

Results are means ± SE

n= number of individuals

ns: not significant ($p > 0.05$) (ANOVA).

very little amount of glycogen in gland cells whereas one individual had glycogen deposits in glandular lumen. LH+10 was similar to the LH+8 and no glycogen-like material in glandular epithelial cells was found (Figure 2.5b). At day LH+12 no individuals had any glycogen at any level.

2.3.2. GLANDULAR EPITHELIUM

The mean \pm standard error of volume fraction of gland occupied by gland cell did not differ among the groups and the variability between group means was not significantly different (Table 2.1). Similarly the volume fraction of endometrium occupied by gland was not significantly different between days LH+6 to LH+12 according to the ANOVA Test.

2.3.3. STROMA

Stromal cell nuclear profile diameter results showed significant differences among the groups (Table 2.3). Although stromal cell nucleus profile major axis did not differ between groups, the minor profile axis was significantly ($p= 0.0043$) different with time among the groups (Table 2.3) indicating that the nucleus was wider, and therefore rounder at day LH+12 than day LH+6 (Figures 2.6a,b,c,d). Although the stromal cell nuclear profile axial ratio did not differ between groups, there was a tendency of a decrease in axial ratio of the stromal cell nucleus which was 1.93 ± 0.10 at day LH+6 and 1.66 ± 0.10 at day LH+12. This decrease in axial ratio was consistent with the increase in profile minor axis suggesting that the nuclear shape was rounder towards the end of the secretory phase of the menstrual cycle. In addition, the stromal cell nuclear profile diameter was significantly ($p= 0.0037$) different with time gradually increasing from $4.82 \pm 0.14 \mu\text{m}$ at day LH+6 to $5.74 \pm 0.11 \mu\text{m}$ at day LH+12. t-test results showed that the difference between day LH+6 and day LH+12 values was significant ($p<0.05$).

Similar to the volume fraction of glandular epithelium to endometrium, the mean \pm standard error of the volume density of endometrium occupied by stroma was not significantly different between days LH+6 to LH+12. However, the volume fraction values of stromal nucleus to endometrium was significantly ($p= 0.0161$) different among

Table 2.2: Comparison of volume fraction data of the endometrial stromal histology and ultrastructure between the mid to late secretory phase of the menstrual cycle.

Features	Cycle Day LH+6 (n=6)	Cycle Day LH+8 (n=4)	Cycle Day LH+10 (n=4)	Cycle Day LH+12 (n=4)	ANOVA Test Result
Vv of Endometrium Occupied by Stroma	0.777 ± 0.017 (n=8)	0.784 ± 0.023	0.804 ± 0.058	0.759 ± 0.048	ns
Vv of Endometrium Occupied by Stromal Cell Nucleus	0.092 ± 0.010 (n=8)	0.138 ± 0.015	0.078 ± 0.009	0.102 ± 0.004	p=0.0161 †
Vv of Stromal Cell Occupied by Nucleus	0.454 ± 0.013	0.455 ± 0.013	0.451 ± 0.008	0.430 ± 0.029	ns
Vv of Stromal Cell Nucleus Occupied by Euchromatin	0.801 ± 0.034	0.741 ± 0.042	0.692 ± 0.050	0.770 ± 0.014	ns
Vv of Stromal Cell Occupied by Mitochondria	0.036 ± 0.004	0.031 ± 0.004	0.038 ± 0.003	0.046 ± 0.003	ns
Vv of Stromal Cell Occupied by "secretory apparatus"	0.054 ± 0.004	0.046 ± 0.003	0.052 ± 0.006	0.065 ± 0.002	p=0.0518#
Vv of Stromal Cell Occupied by RER	0.033 ± 0.004	0.034 ± 0.003	0.053 ± 0.007	0.055 ± 0.007	p=0.0144 †

Results are means ± SE

n= number of individuals

ns= not significant (p>0.05)

difference is marginally significant (ANOVA)

† difference is significant (ANOVA)

Table 2.3: Shows the stromal cell nuclear dimension data (micrometers) between the mid to late secretory phase of the menstrual cycle.

Features	Cycle day LH+6 (n=8)	Cycle Day LH+8 (n=4)	Cycle Day LH+10 (n=4)	Cycle Day LH+12 (n=4)	ANOVA Test Result
Stromal Cell Nuclear Profile Major Axis (µm)	6.59 ± 0.27	7.06 ± 0.21	6.69 ± 0.23	7.28 ± 0.24	ns
Stromal Cell Nuclear Profile Minor Axis (µm)	3.62 ± 0.12*	4.22 ± 0.17	4.40 ± 0.26	4.60 ± 0.14*	p= 0.0043 ††
Stromal Cell Nuclear Profile Mean Diameter (µm)	4.82 ± 0.14*	5.41 ± 0.15	5.38 ± 0.24	5.74 ± 0.11*	p= 0.0037 ††
Stromal Cell Nuclear Profile Axial Ratio	1.93 ± 0.10	1.76 ± 0.10	1.60 ± 0.08	1.66 ± 0.10	ns

Results are means ± SE

n= number of individuals

ns: not significant (p>0.05)

* p<0.05 (paired t-test corrected for multiple testing by the Bonferroni procedure)

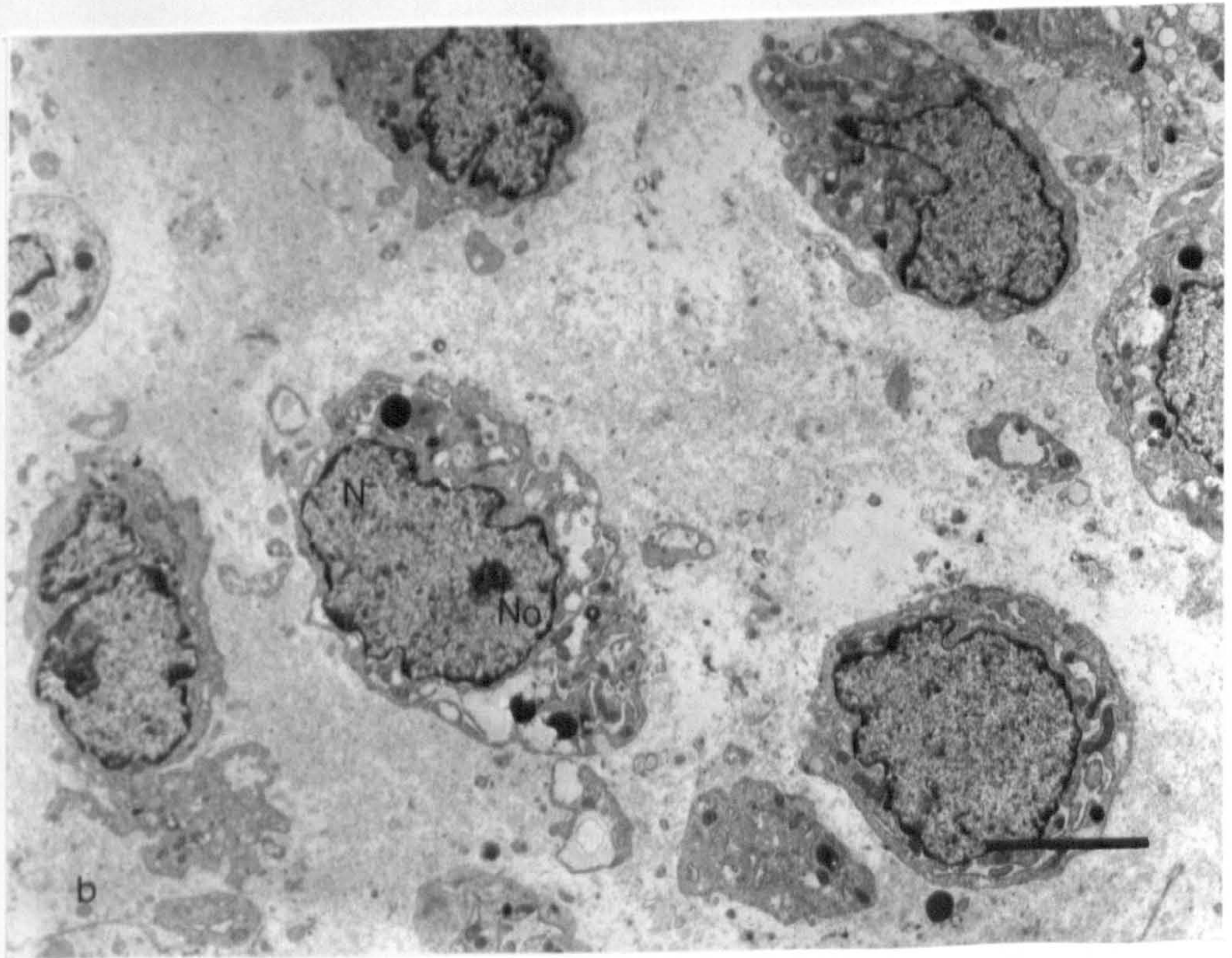
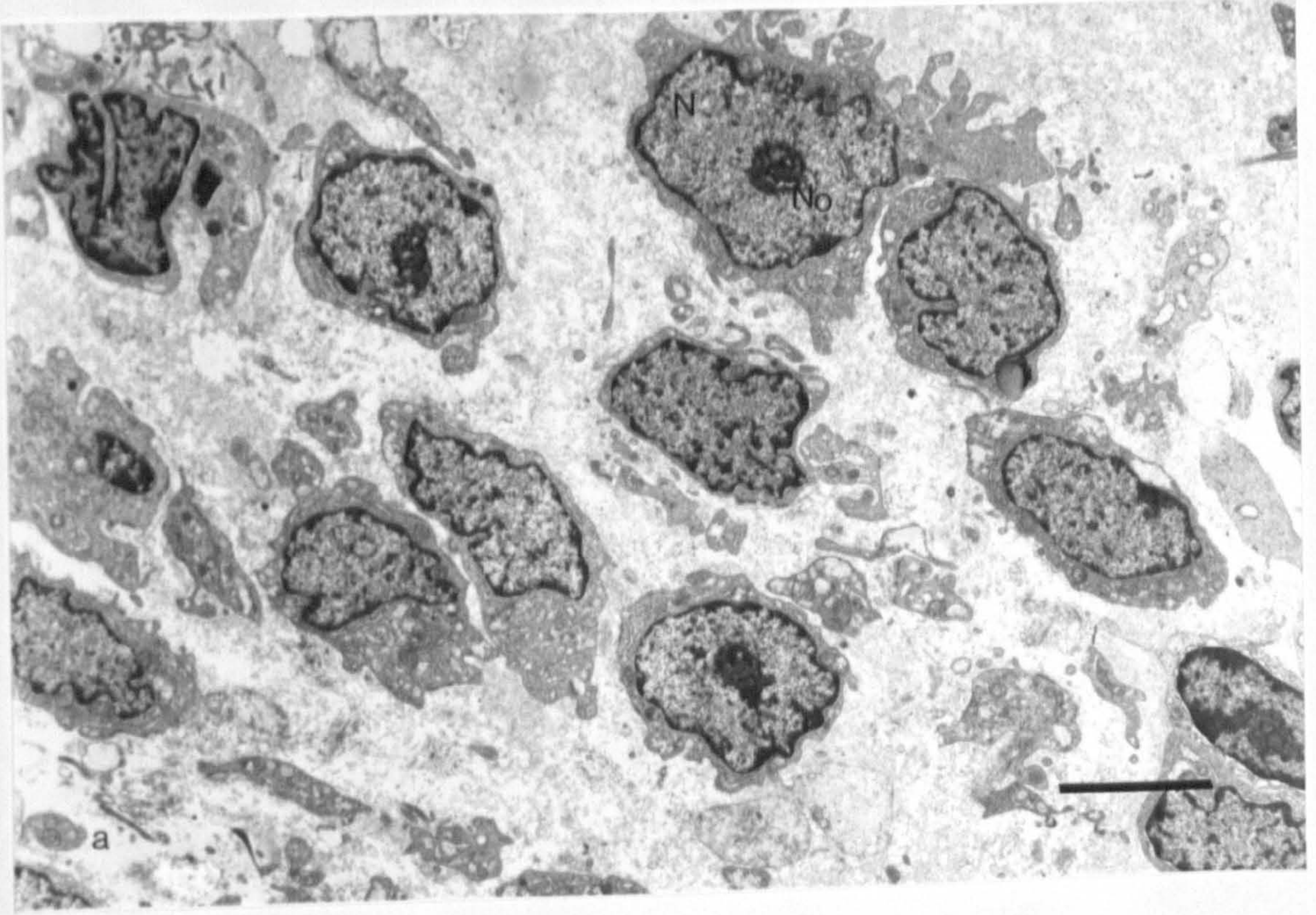
††p<0.01 (ANOVA)

Figures 2.6a,b: Endometrial stromal cells at days LH+6 (a) and LH+8 (b). Both groups of stromal cells show similar features such as euchromatic nuclei (N) and prominent nucleoli (No) with a similar amount of cellular organelles.

Staining: Uranyl Acetate - Lead Citrate

(a) Bar represents 3.80 μm

(b) Bar represents 4.30 μm

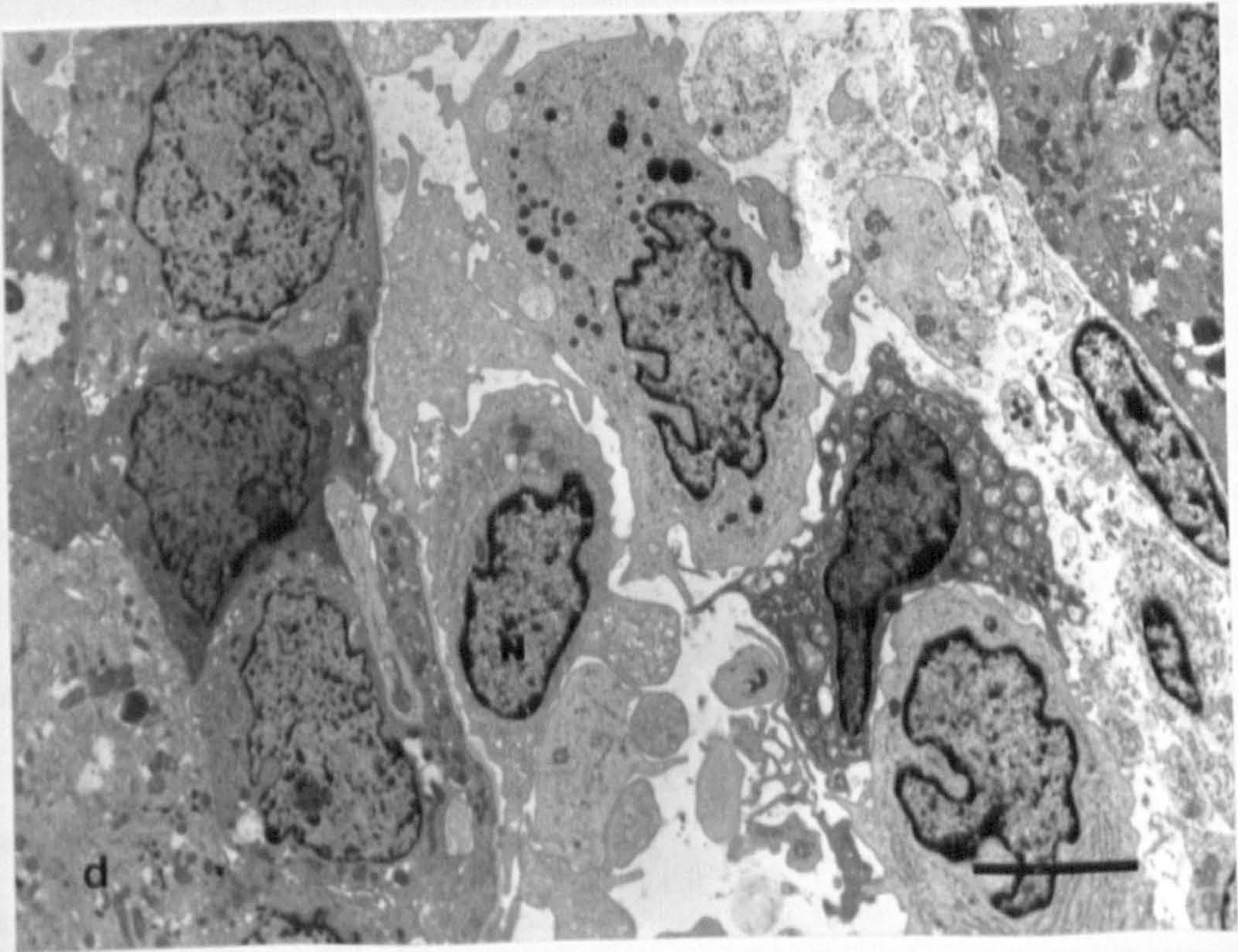
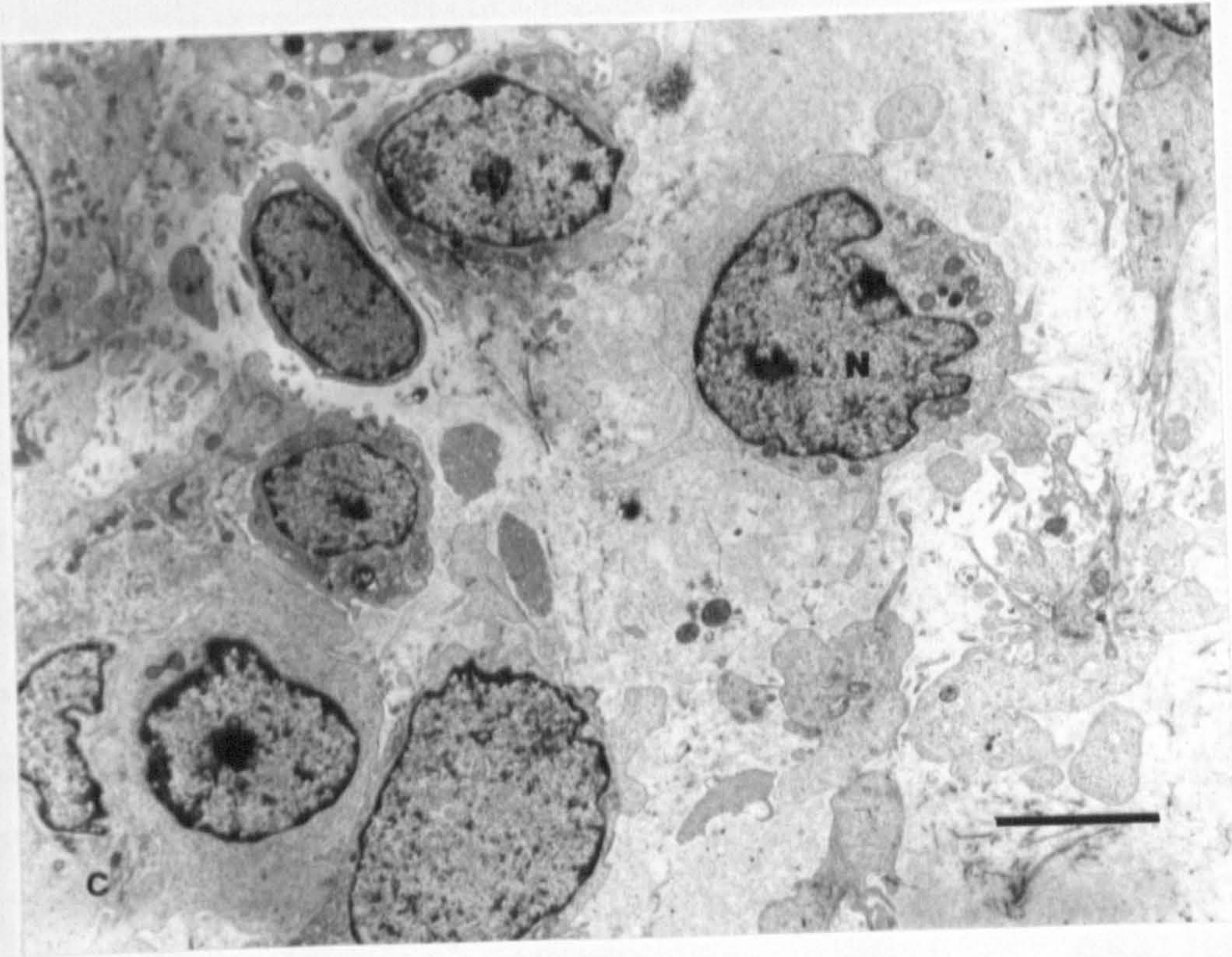


Figures 2.6c,d: Stromal cells at days LH+10 (c) and LH+12 (d). These cells are closely grouped together and the relative amount of cytoplasm is higher in these cells than the day LH+6 and LH+8 stromal cells. Nuclei (N) at day LH+12 are beginning to become irregular in profile.

Staining: Uranyl Acetate - Lead Citrate

(c) Bar represents 4.25 μm

(d) Bar represents 4.10 μm



the groups (Table 2.2). At day LH+6 it was 0.092 ± 0.010 with an increase at day LH+8 (0.138 ± 0.015) followed by a large decrease at day LH+10 (0.078 ± 0.009). The coefficients of variation within the groups was consistently low for this feature. The mean \pm standard error of the volume density of stromal cell occupied by nucleus, and stromal cell nucleus occupied by euchromatin, did not differ among the groups (Table 2.2) (Figures 2.6a,b,c,d). In addition, the mean \pm standard error of the volume fraction of mitochondria to cell was not significantly different among the groups with time indicating no change in metabolic activity of the stromal cells during the late luteal phase. However, the difference in the volume fraction of “secretory apparatus” (consisting of SER, Golgi complex, vacuoles and vesicles) to stromal cell was marginally significant ($p= 0.0518$) showing a tendency of an increase in activity of these organelles in stromal cells in the late secretory phase. The volume fraction of stromal cell occupied by secretory apparatus was 0.054 ± 0.004 at day LH+6, 0.046 ± 0.003 at day LH+8, 0.052 ± 0.006 at day LH+10 reaching its peak of 0.065 ± 0.02 at day LH+12. The volume density of rough endoplasmic reticulum to stromal cell was significantly ($p=0.0144$) different among the groups ranging from 0.033 ± 0.004 at day LH+6 to 0.055 ± 0.007 at day LH+12 suggesting an increased synthetic activity of the stromal cells towards the end of the secretory phase (Figure 2.6e). This quantitative data were consistent with qualitative data which showed an increased collagen secretion and accumulation in endometrial stroma at the later stages of the menstrual cycle (Figure 2.4b).

2.3.4. BLOOD VESSELS

At the light microscope level, the mean \pm standard error of the volume density of endometrium occupied by blood vessel lumen, tissue and both lumen and tissue did not significantly differ with time (Table 2.4). Similarly, endothelial cell nucleus profile diameter evaluations showed no significant differences in any of these features with time (Table 2.5). However, there was a tendency for endothelial cell nuclear profile major axis, minor axis, mean diameter and nuclear axial ratio to increase during the cycle.

On the other hand, endothelial cell features seems to be changed later in the cycle (Table 2.6) (Figures 2.7a,b,c.). According to ANOVA Test, the mean \pm standard error of the volume fraction of the endothelial cell occupied by nucleus significantly

Figure 2.6c: Shows a stromal cell at day LH+12 of the menstrual cycle. The nucleus contains abundant euchromatin material (Eu) with prominent nucleoli (No). The cytoplasm demonstrates abundant “secretory apparatus” including a Golgi complex (arrow) and vacuoles (V).

Staining: Uranyl Acetate - Lead Citrate

Bar represents 1.90 μm

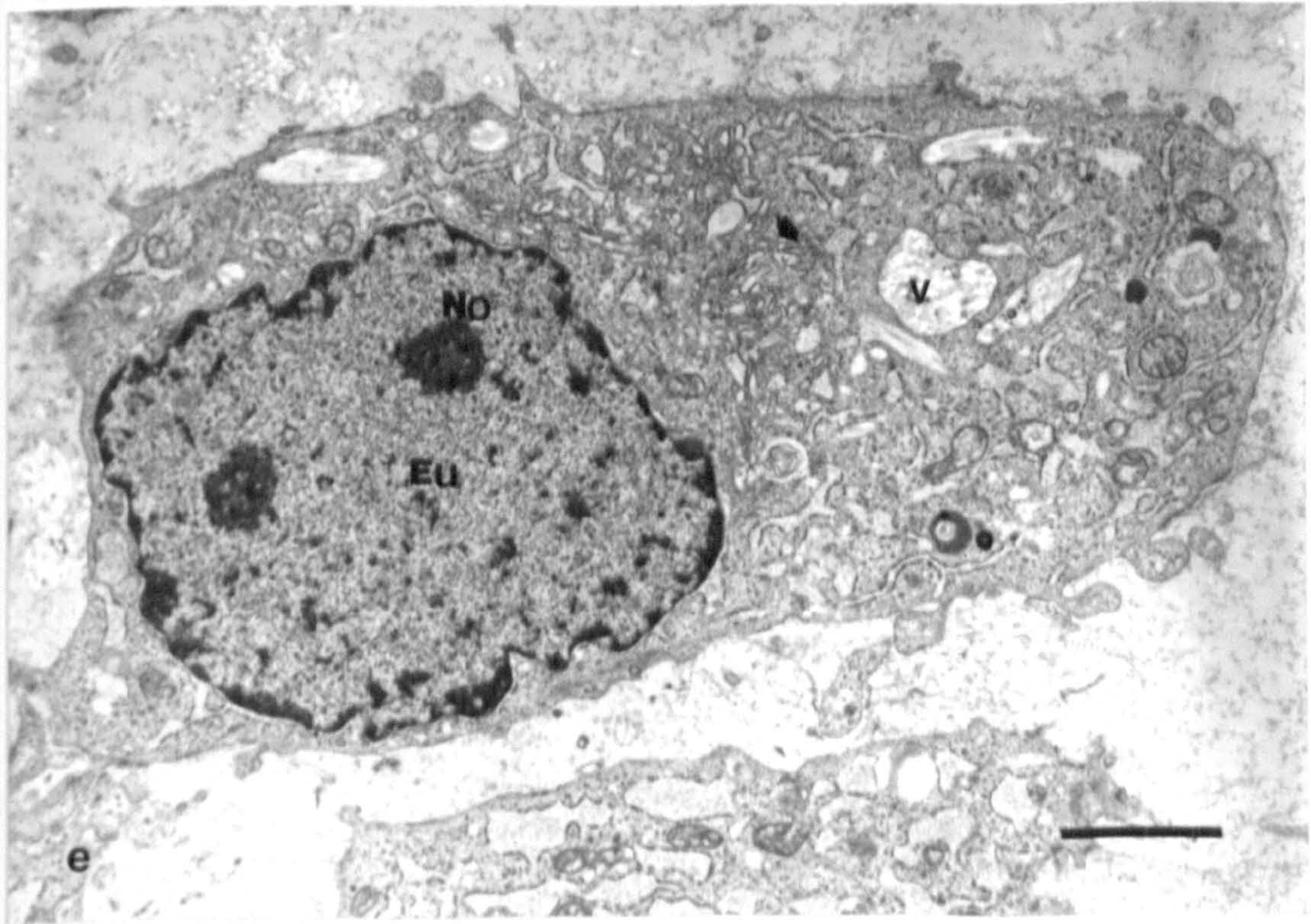


Table 2.4: The comparison of volume fractions of endometrial blood vessel features in mid to late secretory phase of the menstrual cycle.

Features	Cycle Day LH+6 (n=8)	Cycle Day LH+8 (n=4)	Cycle Day LH+10 (n=4)	Cycle Day LH+12 (n=4)	ANOVA Test Result
Vv of Endometrium Occupied by Blood Vessel Lumen	0.011 ± 0.003	0.011 ± 0.001	0.013 ± 0.003	0.013 ± 0.004	ns
Vv of Endometrium Occupied by Blood Vessel Tissue	0.028 ± 0.002	0.030 ± 0.004	0.036 ± 0.001	0.034 ± 0.003	ns
Vv of Endometrium Occupied by Blood Vessel Total (Vessels + Lumen)	0.039 ± 0.003	0.041 ± 0.005	0.049 ± 0.003	0.047 ± 0.007	ns

Results are means ± SE

n= number of individuals

ns: not significant ($p > 0.05$) (ANOVA)

Table 2.5: The cyclical changes in endothelial cell nuclear profile dimensions (micrometers) during the mid to late secretory phase of the menstrual cycle.

Features	Cycle Day LH+6 (n=6)	Cycle Day LH+8 (n=4)	Cycle Day LH+10 (n=4)	Cycle Day LH+12 (n=4)	ANOVA Test Result
Endothelial Cell Nuclear Profile	6.90 ± 0.35	7.84 ± 0.39	8.09 ± 0.43	8.21 ± 0.33	ns
Major Axis (µm)					
Endothelial Cell Nuclear Profile	4.33 ± 0.10	4.42 ± 0.13	4.66 ± 0.20	4.68 ± 0.14	ns
Minor Axis (µm)					
Endothelial Cell Nuclear Profile	5.41 ± 0.13	5.81 ± 0.20	6.07 ± 0.27	6.14 ± 0.22	ns
Mean Diameter (µm)					
Endothelial Cell Nuclear Profile	1.66 ± 0.10	1.89 ± 0.11	1.82 ± 0.10	1.83 ± 0.02	ns
Axial Ratio					

Results are means ± SE

n= number of individuals

ns: not significant ($p > 0.05$) (ANOVA)

Table 2.6: The volume fraction results of endothelial cell features throughout the mid to late secretory phase of the menstrual cycle.

Features	Cycle Day LH+6 (n=6)	Cycle Day LH+8 (n=4)	Cycle Day LH+10 (n=4)	Cycle Day LH+12 (n=4)	ANOVA Test Result
Vv of Endothelial Cell Occupied by Nucleus	0.595 ± 0.027*	0.467 ± 0.023	0.466 ± 0.043	0.442 ± 0.033*	p=0.0097
Vv of Endothelial Cell Nucleus Occupied by Euchromatin	0.854 ± 0.019	0.740 ± 0.032	0.749 ± 0.038	0.788 ± 0.030	p=0.0359
Vv of Endothelial Cell Occupied Mitochondria	0.025 ± 0.005	0.035 ± 0.002	0.033 ± 0.003	0.047 ± 0.008	p=0.0535#
Vv of Endothelial Cell Occupied by “secretory apparatus”	0.039 ± 0.003*	0.049 ± 0.007	0.057 ± 0.009	0.067 ± 0.002*	p=0.0138
Vv of Endothelial Cell Occupied by RER	0.031 ± 0.004	0.034 ± 0.007	0.052 ± 0.004	0.048 ± 0.004	p=0.0180

Results are means ± SE

n= number of individuals

* p<0.05 (paired t-test corrected for multiple testing by the Bonferroni procedure)

difference is marginally significant (ANOVA)

Figures 2.7a,b: Endometrial blood vessels at days LH+6 (a) and LH+8 (b) of the menstrual cycle. Both vessels contain endothelial cells with rounded, euchromatic nuclei (N), regular basement membranes (arrow) and cytoplasm with apparently normal organelle structure. At the electron microscope level, capillaries containing no more than 2 endothelial cell profiles were selected for study since most of the transport between blood and endometrium occurs in these vessels. (L) lumen, (Str) stroma.

Staining: Uranyl Acetate - Lead Citrate

(a) Bar represents 1.85 μm

(b) Bar represents 1.95 μm

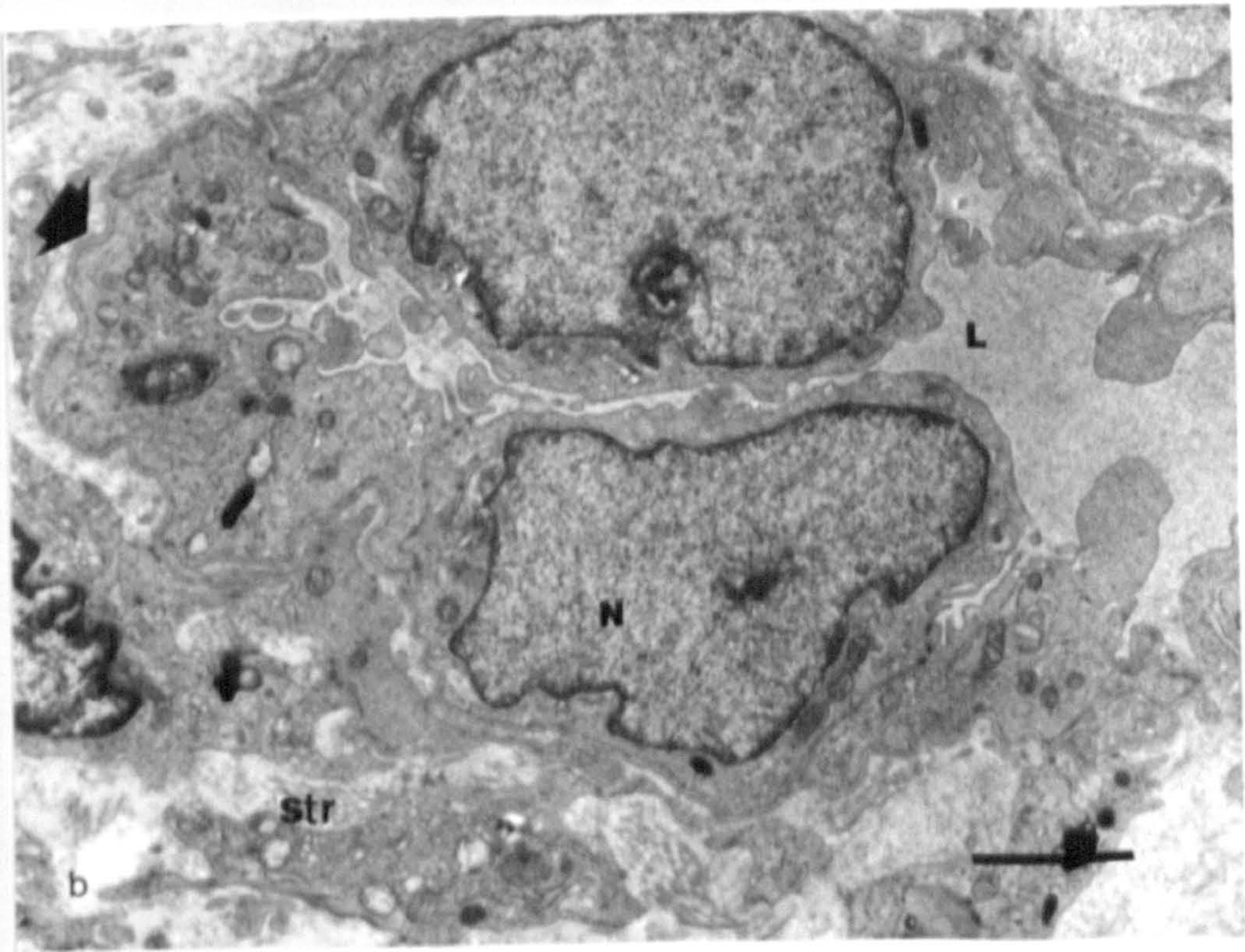
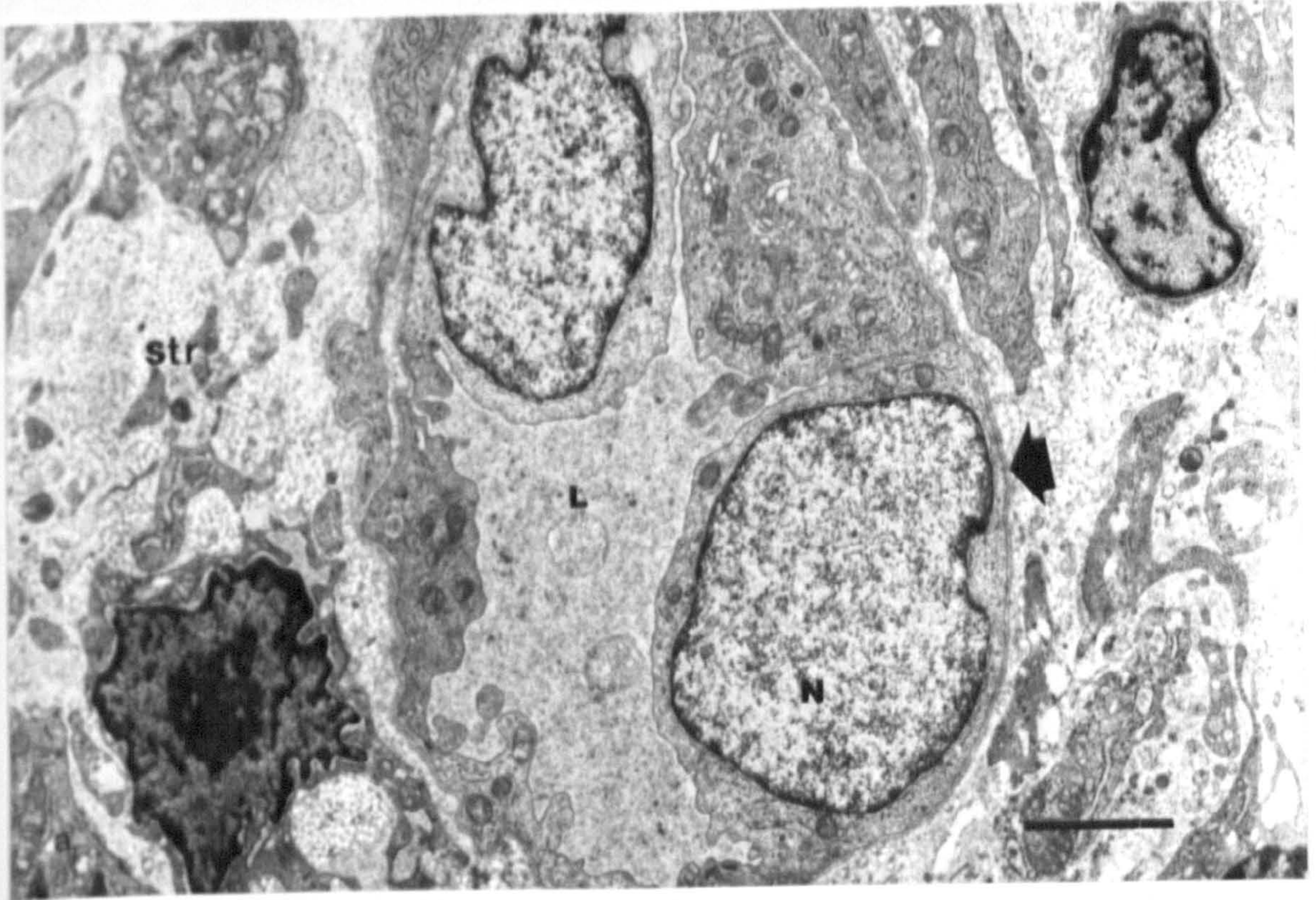
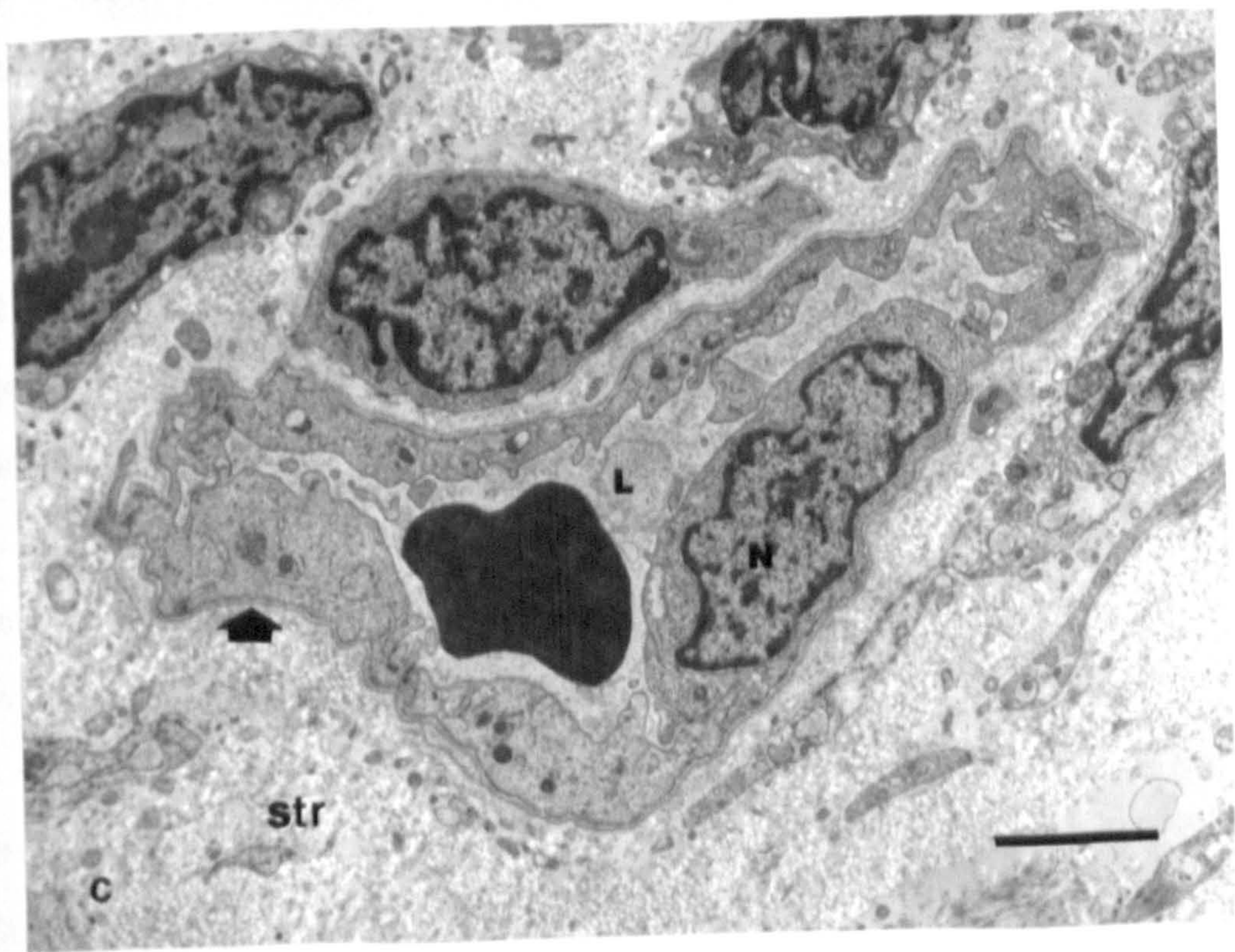


Figure 2.7c: An endometrial blood vessel at day LH+10 of the cycle. Although endothelial cell nucleus (N) seems less euchromatic and irregular shaped here than the cells from days LH+6 and LH+8, morphometric evaluation showed no difference among groups. (L) lumen, (arrow) basement membrane, (Str) stroma.

Staining: Uranyl Acetate - Lead Citrate

Bar represents 1.95 μm



($p=0.0097$) decreased towards the end of the cycle (Table 2.6). The volume density of nucleus to endothelial cell was 0.595 ± 0.027 at day LH+6 and it was reduced to 0.442 ± 0.033 at day LH+12. In addition, Bonferroni-corrected t-Test results showed that the difference between LH+6 and LH+12 was significant ($p<0.05$), confirming the gradual decrease in volume density of the endothelial cell occupied by nucleus. The mean \pm standard error of the volume density of euchromatin to nucleus was significantly ($p=0.0359$) different with time. This decreased euchromatin ratio from days LH+6 to LH+12 is likely to indicate a decrease in synthetic and metabolic activities in endothelial cells. The volume fraction of endothelial cell occupied by mitochondria was considered significantly different over the same time period with a significance level of $p=0.0535$ (ANOVA). The mean \pm standard error of the volume density of "secretory apparatus" to endothelial cell significantly ($p=0.0138$) increased during the late secretory phase of the menstrual cycle, ranging from 0.039 ± 0.003 at day LH+6 to 0.067 ± 0.002 by day LH+12. t-Tests result showed that the difference between days LH+6 and LH+12 was significant ($p<0.05$) indicating increased membrane turnover, possibly due to an increased secretory activity in endothelial cells or an increased transport between blood and endometrial stroma via pinocytosis by the endothelial cells. Similar to the other organelle volume densities, the mean \pm standard error of the volume density of endothelial cell occupied by rough endoplasmic reticulum (RER) was significantly ($p=0.0180$) different with time. It was relatively low at day LH+6 (0.031 ± 0.004) but increased on day LH+12 (0.048 ± 0.004). This may suggest an increased synthetic activity in endothelial cells towards the end of the secretory phase.

The arithmetic and harmonic mean basement membrane thicknesses of the endometrial blood vessels were also determined in order to complete the morphometric evaluation of blood vessels during the late secretory phase. However, after inspection of other data it was decided to investigate only LH+6 and LH+10 tissues as these were representative days of mid and late secretory phase of the menstrual cycle (Figures 2.8a,b). The mean \pm standard error of blood vessel arithmetic mean basement membrane thickness was significantly ($p<0.001$) different with time being 81.17 ± 1.92 nanometers at day LH+6 and 104.64 ± 2.63 nanometers at day LH+10 (Table 2.7). Since coefficients of variation in both groups were low, the difference was highly significant.

Table 2.7: Comparison of blood vessel arithmetic and harmonic mean basement membrane thicknesses between days LH+6 and LH+10 of the normal menstrual cycle.

Features	Cycle Day LH+6 (n=6)	Cycle Day LH+10 (n=4)
Endothelial cell Arithmetic Mean Basement Membrane Thickness (nanometers)	81.17 ± 1.92	104.64 ± 2.63***
Endothelial cell Harmonic Mean Basement Membrane Thickness (nanometers)	82.74 ± 1.87	102.00 ± 3.12***

Results are means ± SE

n= number of individuals

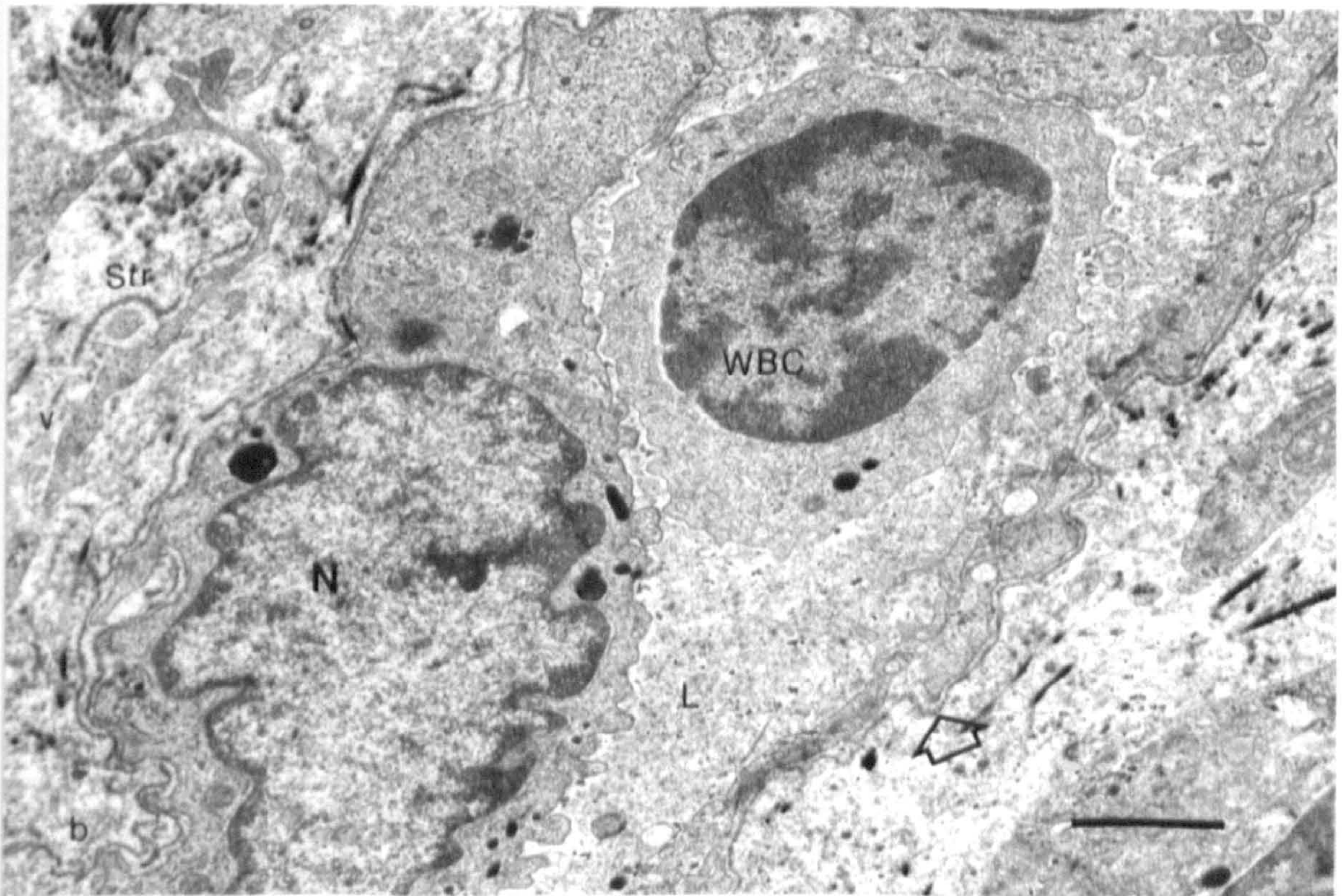
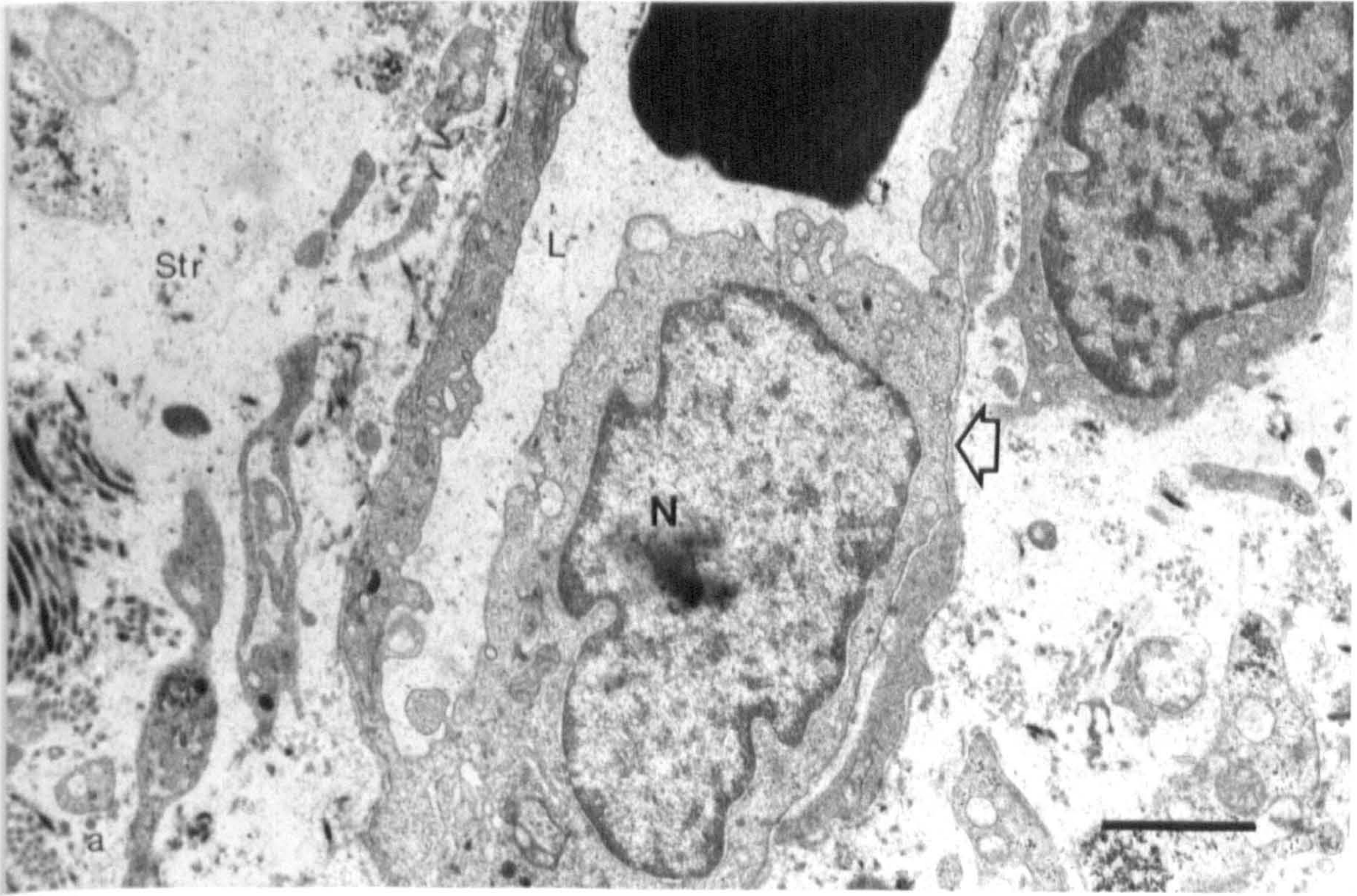
*** p<0.001 Student's t-Test Result

Figures 2.8a,b: Endometrial vessels at days LH+4 (a) and LH+10 (b) of the cycle. Vessels were stained with a modified procedure of periodic acid staining to demonstrate the basement membranes (arrow) (see text for details). Basement membrane thickness of the vessels at day LH+10 appear thicker than those at day LH+4. Morphometry results have also confirmed this qualitative observation. (N) nucleus, (L) lumen, (Str) stroma, (WBC) white blood cell.

Staining: Periodic acid Thiosemicarbazide - Gelatine Methenamine Silver (PATSC-GMS).

(a) Bar represents 1.70 μm

(b) Bar represents 1.80 μm



Similarly the mean \pm standard error of blood vessel harmonic mean basement membrane thickness differed significantly ($p < 0.001$) between groups. It was 82.74 ± 1.87 nanometers at day LH+6 and 102.00 ± 3.12 nanometers at day LH+10. Both arithmetic and harmonic mean basement membrane thickness results suggest a changed vascular structure and function in the late secretory phase of the menstrual cycle. This increase in blood vessel basement membrane thickness was consistent with the increase in synthetic activity of the endothelial cells in the late secretory phase of the menstrual cycle.

2.4. DISCUSSION

This study has investigated the morphological changes that occur in human endometrial stromal and vascular compartments during mid to late secretory phase of the menstrual cycle. Some of the glandular and luminal epithelial features were also examined quantitatively and qualitatively in order to determine the overall changes that occur during this period of the cycle in humans.

Morphometric methods used in this study mainly involved volume fraction measurements, using point counting techniques, at light and electron microscope levels. The stromal and endothelial cell nuclear profile dimensions were evaluated using a digitizer-computer-light microscope device. In addition, endometrial blood vessel arithmetic and harmonic mean basement membrane thicknesses were evaluated. Morphometric methods used in the present study were chosen in order to obtain comprehensive descriptive data for the endometrial stromal and vascular morphologies.

2.4.1. DATING THE ENDOMETRIAL BIOPSIES

In the present study, dating of the endometrial biopsies was done using chronological dating according to the luteinizing hormone (LH) surge that occurs just before ovulation (Li *et al.* 1987). The histological dating was performed conventionally by the criteria of Noyes *et al.* (1950) on JB4 - embedded tissue stained with acid Fuschin and Toluidine blue. For many years, the chronological date has been assigned retrospectively from the onset of the next menstrual period (NMP) or according to the last menstrual period (LMP) (Cooke *et al.* 1972; Wentz 1980). However, it is now well established that the menstrual cycle may vary considerably in length from cycle to cycle, mainly due to variation of length in the proliferative phase (Lenton *et al.* 1984), so that the use of the onset of the NMP or the LMP as a guide to dating may introduce undesirable errors. Some investigators have also used basal body temperature (BBT) to identify the time of ovulation (Gautray *et al.* 1981), or by combining the BBT with the onset of the NMP (Noyes and Haman 1953). However, menstrual cycles may range between 25 and 35 day in normal healthy women and so using either NMP or LMP can lead to considerable error. On the other hand, LH rise reproducibly occurs about 38 hours before ovulation (Li *et al.* 1987). Also LH is easily measured by radio-immunoassay in either blood or

urine (Li *et al.* 1987). Since the luteal phase is of relatively constant length (Lenton *et al.* 1984), dating endometrial biopsies according to the LH peak seems to be most accurate method currently available (Tredway *et al.* 1973; Koninckx *et al.* 1977; Li *et al.* 1987).

The accuracy and reliability of two methods of chronological dating (LH peak and the onset of the NMP) and their correlation with the traditional histological dating were examined by Li *et al.* (1987). They concluded that the correlation between histological and chronological dating is much more accurate and reliable using the LH peak than when using the retrospective method of dating from the onset of the NMP. It therefore appears that the most accurate and reliable method for dating the endometrial biopsy material is by determination of the LH peak by daily LH assay, and so this method was used in the present study.

2.4.2. GLANDULAR EPITHELIUM

While there were no changes in the glandular components of the endometrium examined, some of the stromal and vascular features (see later) differed significantly during the cycle. Most of the changes occurred later in the secretory phase around days LH+10 and LH+12 of the cycle.

Taking all stages overall (days LH+6 - LH+12), the groups did not differ significantly throughout the cycle when the volume fraction of endometrium occupied by gland was compared statistically using an ANOVA Test. Similarly the volume density of gland occupied by gland cell was not significantly different during the cycle. These features indicate the secretory functions of glands and amount of glandular epithelium in the whole endometrium. That they did not differ significantly across the luteal phase may seem unusual, since dramatic changes in glandular epithelium can be seen qualitatively (see later). However, these changes occur in a precisely co-ordinated pattern over a short time scale (discussed below) and are masked when examined across days LH+2 - LH+12.

In the normal cycle around day LH+4 deposits of glycogen are seen in glandular epithelial cells (Noyes *et al.* 1950; Dockery *et al.* 1988a, 1988b; Dockery and Rogers 1989). This increases the overall volume of gland cell cytoplasm from (mean \pm SE)

877.3 ± 77.5 μm^3 at day LH+2 to 1198.1 ± 74.7 μm^3 at day LH+4 (Dockery *et al.* 1993). Cytoplasmic accumulation of glycogen also reduces the gland lumen so the ratio of gland cell to gland values also increase. So a change in the volume fraction of gland cell to gland may indicate the accumulation glycogen in the glandular epithelial cells. Qualitative observations were performed in the present study using a modification of the PAS method for electron microscopy (Namimatsu 1992). The presence of glycogen-like material in the endometrium throughout the secretory phase showed a peak at day LH+4, in agreement with the literature published on this field (Verma 1983; Johannisson *et al.* 1987; Dockery *et al.* 1988a, 1988b; Dockery and Rogers 1989). The peak amount of glycogen-like material in gland cells seen around day LH+4, decreased by day LH+6 and disappeared completely towards the end of the secretory phase. The secretion passes apically out of the cell into the gland lumen (Smith *et al.* 1989; Graham *et al.* 1990). These changes are widely believed to prepare the endometrium for implantation and since the present study focuses on the stromal cell changes that occur later in the secretory phase, when most glandular changes have already taken place, it was not unexpected to find that the volume densities of “gland cell to gland” and “gland to endometrium” were unchanged when the whole luteal phase was considered together.

Previous reports published on the histology of the glandular epithelium deal with the early secretory phase and peri-implantation periods (Verma 1983; Johannisson *et al.* 1987; Dockery *et al.* 1988a; Li *et al.* 1991a). Dockery *et al.* (1988a) reported separately glandular cell cytoplasmic and nuclear volumes. While there was an unchanged nuclear volume, the cytoplasmic volume increased by 37% between days LH+2 and LH+4 followed by a 23% decrease from day LH+4 to day LH+5. This is consistent with an increase in glycogen accumulation in glandular cells until day LH+4 followed by a rapid loss. Their quantitative data agree with the qualitative data of the present study that the maximum glycogen accumulation in endometrial glandular cells is seen at day LH+4.

Kim-Bjorklund *et al.* (1991) performed a quantitative study of normal human endometrial morphology in postovulatory cycles. It may be expected that glandular cell height would increase when intracytoplasmic glycogen accumulation was high. Glandular cell height was maximum around days LH+4 and LH+7 and decreased around days LH+8 and LH+11. However, their recruitment of the LH dated endometria put

specimens into broader groups than in the present study. For example they grouped LH+0 - LH+3, LH+4 - LH+7 and LH+8 - LH+11 biopsies together, therefore changes which occur between days LH+2 and LH+3 and between days LH+4 and LH+5 could not be obtained. Nevertheless their results broadly agree with those above.

Johannisson *et al.* (1987) reported an increased diameter of the endometrial glands between days LH-1 and LH+12. This increased gland diameter is likely to be the result of several things. For example, the accumulation of glycogen-like material occurs in gland cells around day LH+4. After this material is secreted into the lumen at day LH+6, and glandular cell height is decreased (Johannisson *et al.* 1987), the presence of secretion in the lumen could still cause the overall gland diameter to be larger than before ovulation. In addition in Johannisson's study, the basal vacuoles (thought to be glycogen containing) in gland cells disappeared after day LH+6 and no regular basal vacuolation appeared after that day of the cycle. This is also consistent with the reduced epithelial cell height and increased gland lumen volume density noted above.

2.4.3. STROMA

Stromal cell nuclear profile diameters showed significant differences during the cycle. While stromal cell nuclear profile major axis was not significantly different the minor profile axis differed significantly, being greater in the late secretory phase than the mid secretory phase. This significant difference in minor profile axis combined with unchanged major profile diameter indicates a wider and rounder stromal nuclear shape towards the end of the cycle. This change was consistent with the axial ratio of the nucleus being greater in the mid-luteal phase than the late-luteal phase suggesting that the stromal cell nuclear shape was rounder at the end of the cycle. The average profile diameter of the stromal cell nuclei differed significantly throughout the cycle, gradually increasing from $4.82 \pm 0.14 \mu\text{m}$ at day LH+6 to $5.74 \pm 0.11 \mu\text{m}$ at day LH+12. Overall change in stromal cell nuclear profile dimensions suggests that the stromal cell nucleus was larger and rounder towards the end of the menstrual cycle.

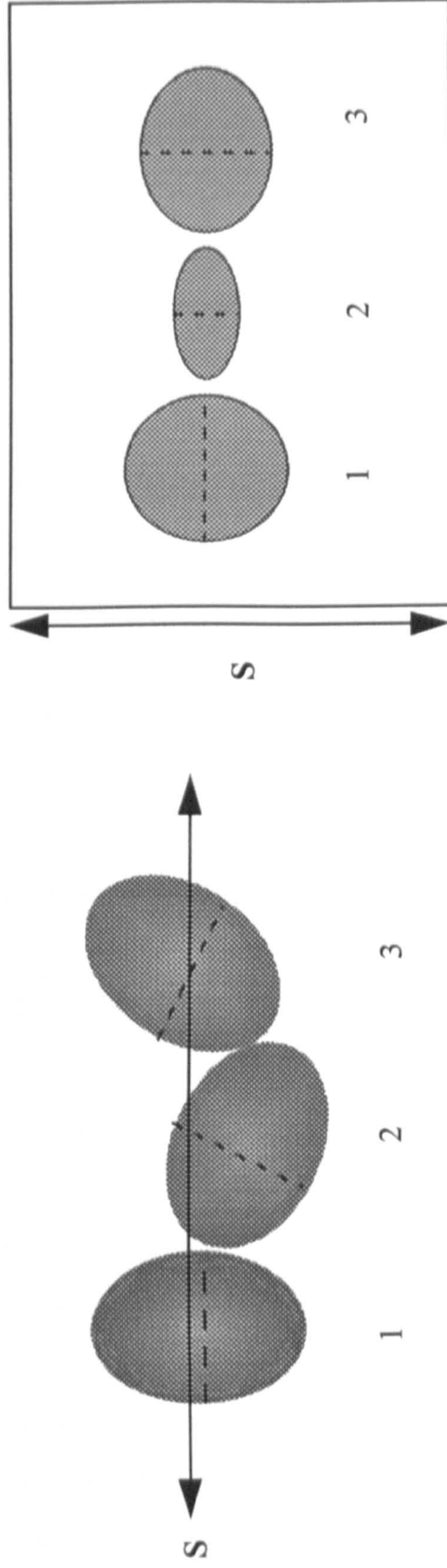
The nuclear profile dimensions may be useful indicators of the functional state of the cells. Increased nuclear profile diameter is reflected by increased transcriptional activity in the nucleus. The chromatin pattern of a nucleus may be considered a guide to the

cell's activity. In general, cells with euchromatic nuclei are more active than those with condensed, heterochromatic nuclei (Junqueira *et al.* 1992). In euchromatic nuclei, more DNA surface is available for the transcription of genetic information, however, in heterochromatic nuclei, the coiling of DNA makes less surface available. As a result euchromatic nuclei are rounder and wider than heterochromatic nuclei (Junqueira *et al.* 1992).

The nuclear profile diameters are used to determine the prognosis of some diseases such as marked atypical endometrial hyperplasia (Ausems *et al.* 1985). In their study, the profile major and minor axis, profile mean diameter and axial ratio of the endometrial glandular cell nuclei were measured to determine whether the endometria were cancerous or not. However, the sampling technique used in their study was different from the present study. They selected the most atypical areas and nuclei in order to determine the prognosis of the disease. Whereas in the present study systematic random areas of the human endometria were used to evaluate the overall changes that occur in stromal nuclear shape and thus nuclear function in normal menstrual cycles. Determination of the true stromal cell nuclear diameter is almost an impossible task because of the random orientation of the cells and the random sectioning through the irregular cell nuclei (vertical, horizontal or tangential) (Figure 2.9). However, determination of the profile nuclear diameter was used in combination with Vv estimates to make comparisons about the changes that occur in the other parts of the cell such as changes in relative cytoplasmic volume or changes in overall cell volume. While it is recognised that the nuclear profile diameter has its limitations as a direct estimator of nuclear dimensions, it was used in the present study to help interpret Vv data.

Dockery *et al.* (1990) reported increased stromal cell nuclear dimensions during the peri-implantation period of the normal human endometrium. In their study, stromal cell nuclear major and minor profile diameters significantly increased between days LH+2 and LH+8. Furthermore, stromal cell nuclear profile axial ratio significantly decreased and mean nuclear profile diameter increased between the same days of the cycle. Although their study involved only the peri-implantation period, changes in stromal nuclear diameters were similar to those reported here. The present study has confirmed and extended their data to show that a further increase in stromal cell nuclear diameters

Figure 2.9: The effects of sectioning on profile size and shape.



A - Simplified diagram represents 3 stromal cell nuclei of similar size and shape cut by a random section (S).

B - Profiles resulting from section of particles in A. In all cases the mean diameter is an underestimate of mean particle diameter.

occurred in the late secretory phase of the menstrual cycle. During the late secretory phase, More *et al.* (1974a) reported that endometrial stromal cells had rounded nuclei and prominent nucleoli, which also agrees with the present study (Figure 2.6c,d,e).

Overall, the volume density of endometrium occupied by stroma did not significantly differ during the secretory phase. From the day of ovulation to the end of the cycle, Johannisson *et al.* (1987) suggested a linear decrease in the volume density of endometrium occupied by stroma and this decrease was accompanied by a linear increase in the volume fraction of endometrial glandular compartment. Their findings are in contrast with the findings of the present study where there was no overall change in the volume density of stromal compartment throughout the secretory phase. It is well established that the total glandular volume is increased between days LH+2 and LH+6 because of the massive accumulation of glycogen material in the glandular cell cytoplasm (see earlier). This increase in gland volume is largely reversed after glycogen is secreted and a decrease in the volume density of glands occurs (Dockery *et al.* 1988a). However, the volume fraction of stromal cell nucleus to endometrium did significantly differ with time when comparisons were made between individual sampling points rather than overall changes across the cycle. There was a large increase in the volume density of endometrium occupied by stromal cell nucleus between days LH+6 and LH+8 followed by a dramatic decrease at day LH+10. Stromal oedema is at its maximum level around days LH+8 and LH+10 (Noyes *et al.* 1950, Dockery and Rogers 1989) and this dramatic increase then decrease in the volume density of the endometrium occupied by stromal cell nucleus around these days of the cycle may be due to changed levels of stromal oedema, and thus changed packing density of the stromal cells. Dockery *et al.* (1990) reported an increased packing density of the stromal cells between days LH+2 and LH+6, and a decrease in packing density occurred after day LH+6. They suggested a possible increase in stromal oedema caused this decrease in packing density of the stromal cells. The results of the present study agree with their data that the large decrease of the volume density of stromal cell nucleus to endometrium around day LH+10 may be due to increased stromal oedema during this time of the menstrual cycle and this was confirmed qualitatively.

On average, the volume densities of stromal cell nucleus to cell, and nucleus occupied by euchromatin did not differ during the secretory phase. Although the stromal cell nuclear dimensions increased towards the end of the cycle, an unchanged stromal cell nuclear volume density indicates that stromal cell cytoplasm increased in proportion to the nucleus and that the same proportion was euchromatin. Qualitative ultrastructural observations of the stromal cells confirmed this increased stromal cell cytoplasm volume during the late secretory phase of the menstrual cycle.

The average volume fraction of mitochondria to cell in stromal cells did not differ significantly over the secretory phase indicating that the metabolic demand of the stromal cells was unchanged throughout the late secretory phase. However, the volume density of stromal cell occupied by “secretory apparatus”, including smooth endoplasmic reticulum (SER), Golgi apparatus, vacuoles and vesicles, did differ significantly at the times studied. The “secretory apparatus” was considered as a separate organelle in the present study because of the difficulties of identifying and so measuring its components individually. The “secretory apparatus” plays very important roles in cellular functions such as membrane re-cycling, vesicle transport, absorption and secretion (Junqueira *et al.* 1992). The significance level was often marginal for this feature, indicating only a tendency of a change in secretory function of the stromal cells in the late luteal phase. Any such changes in this feature may not be related directly to “secretion” but to membrane turnover or internal transport. Similar to the “secretory apparatus”, the volume density of stromal cell occupied by rough endoplasmic reticulum (RER) significantly increased in the late luteal phase, suggesting an increased protein synthetic activity in the stromal cells during this period of the cycle. Qualitative ultrastructural evaluation of the stromal cells in the late secretory phase confirmed increased amounts of RER, SER, Golgi apparatus and vesicles in stromal cell cytoplasm and also showed an apparent increase in collagen fibre accumulation in the extracellular matrix of the endometrial stroma. These qualitative observations were consistent with the quantitative data of the present study.

In the normal menstrual cycle, the stromal cells undergo distinctive changes which correlate with proliferation, differentiation and maturation (Weinke *et al.* 1968; More *et al.* 1974a). While there are few reports of stromal cell ultrastructure in the human, More

et al. (1974a) demonstrated diffused chromatin and occasional nuclear bodies in stromal cell nuclei during the late luteal phase (from day 24 of the traditionally dated cycle onwards - about day LH+10). In their study, cytoplasm was abundant, mitochondria were simple in shape and scattered in the cytoplasm in small numbers, the rough endoplasmic reticulum was represented by a few irregularly oriented cisternae, the smooth endoplasmic reticulum was extensive and often associated with a prominent Golgi apparatus during the late secretory phase. Their findings mainly agreed with the quantitative findings of the present study that the cytoplasmic organelles, especially SER and Golgi apparatus increased towards the end of the cycle. However, their qualitative study suggested an unchanged RER level during the late luteal phase, which contrasts with the findings of the present study. In addition, they suggested an increased collagen secretion in the early luteal phase, whereas an increase in collagen secretion was seen in the present study in the late secretory phase. The findings presented by More *et al.* (1974a) were obtained by qualitative observations. In addition, they do not mention the dating of the endometrial biopsies and the accuracy and reliability of the technique of dating they used. Since in the present study, objective morphometric methods were used to evaluate the endometrial stromal morphology during the mid and late secretory phase of the cycle from many subjects and endometrial biopsies were dated according to the LH peak, the present data may be regarded as more reliable.

2.4.4. BLOOD VESSELS

On average, the volume density of endometrium occupied by blood vessel did not differ significantly when different days of mid and late secretory phase of the menstrual cycle were compared using the ANOVA test. In addition, blood vessel lumen to endometrium and surrounding blood vessel tissue to endometrium were not significantly different with time. Therefore vessels grow proportionately with the whole endometrium. The capillary lumen has been described as progressively increasing in the late luteal phase (Sheppard and Bonner 1980). In a morphometric study of human endometrial capillaries, Peek *et al.* (1992) reported a significantly increased dilatation of the sub-epithelial capillary plexus in the postovulatory phase, and they concluded that the dilatation might be the result of increased stromal oedema during the peri-implantation

stage. However, in the present study, despite clear oedema no change was found in the volume density of blood vessel lumen to endometrium. In the secretory phase, growth of the vascular tree accompanies an increase in endometrial thickness (Dockery and Rogers 1989), and the unchanged blood vessel-endometrium proportions observed in the present study may be the result of a proportional increase in endometrial thickness in the luteal phase. Estimates of blood vessel diameter may be required to determine the changes in blood vessel dimensions. Unfortunately biopsies inevitably damage the endometrial vessels, and any statement on their diameter in biopsy material should be regarded with caution.

There were no significant differences in blood vessel endothelial cell nuclear profile dimensions with time. Although they were not significantly different, there was a tendency of an increase in some of these features, such as profile major axis, minor axis and mean diameter during the menstrual cycle. On the other hand, according to the ANOVA test, the average proportions of nucleus to endothelial cell significantly decreased in the late secretory phase (from about 60% at day LH+6 to 45% at day LH+12). This decrease in the volume fraction of endothelial nucleus to cell might be either due to a decreased nuclear volume or due to an increased cytoplasmic volume. The tendency of the increase in endothelial cell nuclear dimensions observed in the present study suggests the latter possibility, that there is an increase in the cytoplasmic volume greater than the increase in nuclear volume. This was supported by qualitative observations.

The average volume fraction of endothelial cell nucleus occupied by euchromatin significantly decreased in the late luteal phase suggesting decreased levels of nuclear activities in endothelial cells during this time of the cycle. However, investigation of the volume density of mitochondria to endothelial cell did not show a similar effect, but an increase towards the end of the cycle ($p=0.054$). This increased mitochondrial volume density should be viewed with the already noted large increase in cytoplasm. Mitochondrial volume has increased more than cytoplasmic volume has increased and therefore it is likely to indicate a much increased metabolic demand in endothelial cells.

The average volume fraction of endothelial cell occupied by “secretory apparatus” significantly increased in the late luteal phase, possibly indicating an increased membrane

turnover in endothelial cells. This increased membrane turnover may be the result of either an increase in secretory activity or an increase in transport between blood and endometrial stroma via pinocytosis by the endothelial cells. Additionally, the volume density of endothelial cell occupied by RER significantly increased in the late secretory phase indicating an increased protein synthetic activity in the endothelial cells during this period of the cycle. This increase in secretory activity was consistent with the increase in secretory function and the increased membrane turnover may well be the reflection of increased synthetic activity in endothelial cells. Qualitative investigation of the late secretory stage endothelial cells agreed with these increased synthetic and secretory activities in blood vessel endothelial cells. However, it may be considered that there is a discrepancy between the decreased levels of euchromatin (which means a lower transcriptional activity in endothelial cell nucleus) and increased levels of metabolic, synthetic and secretory activities in endothelial cell cytoplasm. However, this may simply reflect a temporal sequence with transcription finishing before translation and secretion. All of this activity may explain the previous increase in mitochondrial volume density.

To complete evaluation of the endometrial blood vessel morphology and function, arithmetic and harmonic mean basement membrane thicknesses were also measured using appropriate morphometric methods. However, after consideration only days LH+6 and LH+10 were selected since they represent the mid and late luteal phase. The average arithmetic mean basement membrane thickness significantly differed between days LH+6 and LH+10 with a 30% increase (81.17 ± 1.92 nm at day LH+6 and 104.64 ± 2.63 nm at day LH+10). Similarly, the average harmonic mean basement membrane thickness was significantly different between day LH+6 and day LH+10 with a 25% increase (82.74 ± 1.87 nm at day LH+6 and 102.00 ± 3.12 nm at day LH+10).

Basement membranes are complex, morphologically identifiable structures physically separating particular cell types in various tissues and organs. Basement membranes have been implicated in the attachment, spreading, migration, growth, repair and differentiation of the overlying cell populations as well as in the physical phenomena of fluid filtration and gas exchange (Wracko 1982). At the ultrastructural level, the basement membrane is composed of a basal lamina (consisting of lamina densa and lamina rara) and a reticular lamina (Madri *et al.* 1984). The lamina densa is make up of a

continuous sheet of densely packed fibrillar arrays, with the lamina rara which is a less electron-dense layer, separating the lamina densa from the cell processes above (Madri *et al.* 1984). While the complete composition of basement membranes is still unknown, several components have been identified, including collagen types IV and V, laminin and heparan sulphate proteoglycan (Kleinman *et al.* 1982; Foellmer *et al.* 1983).

Arithmetic mean basement membrane thickness represents the average anatomical thickness whereas the harmonic mean thickness represents the “physiological” thickness of the diffusion barrier aspect of basement membranes. Evaluation of the arithmetic basement membrane thickness can give undesirable errors in interpretation of the function of blood vessel while harmonic mean thickness provides an indicator of the membrane as a barrier to diffusion. Arithmetic mean is useful, but a certain bias is always present in arithmetic mean basement membrane estimates due to curvature and unevenness of the basement membranes. For these reasons, it is advantageous also to estimate the harmonic mean basement membrane thickness (which is the reciprocal of the mean of reciprocal thicknesses; Hirose *et al.* 1982). For example, if we have a 10 nm thick membrane and a 100 nm thick membrane, using the reciprocals of these thicknesses gives values of 0.1 and 0.01 respectively. When averaged the anatomically thicker membrane makes an appropriately smaller contribution, reflecting its poorer diffusing capacity. In the present study, blood vessel arithmetic and harmonic mean basement membrane thicknesses were consistently similar in both groups. In addition, the coefficients of variation for each group were consistently low suggesting that the thickness of the basement membranes for individual blood vessels were very consistent for each group. The thickness of the blood vessel basement membrane is one feature which can affect diffusion between blood and surrounding tissue. Others include distance from a vessel, type of vessel, intervening tissues and many others. In the present study, increased basement membrane thickness may indicate a decreased level of diffusion or transport through the endothelial tissue and the basement membrane.

The anatomy of the endometrial vessels during the normal menstrual cycle has been described in detail (Schmidt - Matthiesen 1963). During the last 30 years, only a few studies have focused on the morphological changes in the endometrial vessels and their potential relationship to sex steroid production during the normal menstrual cycle

(Sheppard and Bonner 1980; Johannisson and Redard 1984; Peek *et al.* 1992). The vascularization of the endometrium may play an important role in implantation (Akerlund 1991) and a direct effect of the sex steroids on the capillary endothelial cells cannot be excluded (Johannisson 1986). The influence of circulating sex steroids on the vascularization of uterine tissues seems to vary according to the location. The vessels of the myometrium do not seem to be significantly affected by sex hormones; however, the vessels of the upper functional layer of the endometrium are likely to be highly sensitive to steroid hormones and to undergo significant changes during the normal menstrual cycle (Schmidt-Matthiesen 1963). This sensitivity has been reported to occur in the most distal parts of the spiral arteries, the arterioles, the capillaries, the venous lakes and the veins of the functional layer (Schmidt - Matthiesen 1963). Since the present study involves the changes that occur in the blood vessels of the functional layer and especially the capillaries, it is possible that the sex steroids may have significant effects on those endometrial blood vessels. Peek *et al.* (1992) have shown a significant correlation between plasma progesterone levels and the mean area of the endometrial capillary lumen, however, there was no significant correlation between plasma levels of oestrogen and the mean area of the capillary lumen. The changes in the endometrial blood vessels during the mid and late secretory phase observed in the present study may be related to the amounts of circulating sex steroids. However, the interaction between the production of ovarian steroids, the dynamics of the endometrial blood vessels of the functional layer, the permeability of the capillary endothelial cells and the development of oedema in the endometrial stroma is likely to be a highly complex phenomenon which is still incompletely understood. Further studies are required to describe this relationship.

In summary, there were no significant differences in any of the glandular epithelial features during mid to late luteal phase evaluated morphometrically in the present study. However, qualitative observations of the glandular glycogen content showed differences. Maximum glycogen - like material in the glandular epithelial cells was observed around day LH+4 and a decrease occurred after this time.

In contrast to the glandular features, statistically significant changes occurred in stromal cells and blood vessel endothelial cells. Profile dimensions of the stromal cell nuclei significantly increased towards the end of the menstrual cycle. In addition, the

volume density of endometrium occupied by stromal cell nucleus significantly increased at day LH+8 and a remarkable decrease occurred in this feature at day LH+10 indicating the effect of stromal oedema on the packing density of the stromal cells. Furthermore, the volume fraction of stromal cell occupied by cytoplasmic organelles increased towards the end of the cycle, suggesting increased synthetic and secretory functions during this time of the cycle. Qualitative ultrastructural observations of the stromal cells confirmed that collagen secretion from the stromal cells was increased and abundant collagen fibre accumulation in the extracellular matrix was evident (Figure 2.5a,b).

Although the endothelial cell nuclear profile diameters did not differ significantly, there was a tendency of an increase in this feature in the late luteal phase of the cycle. A linear decrease occurred in the volume fraction of nucleus to endothelial cell indicating (with nuclear profile data) that the cytoplasmic volume was greater towards the end of the cycle. The volume density of euchromatin was decreased around days LH+8 and LH+10 however, it increased at day LH+12. The increase in endothelial cell cytoplasmic volume was consistent with the increased organelle volume densities. In addition, the arithmetic and harmonic mean basement membrane thicknesses increased in the late luteal phase.

In conclusion, while there was no significant change in endometrial glandular features in mid to late luteal phase of the menstrual cycle, endometrial stroma and blood vessels underwent remarkable changes during this period of the cycle.

CHAPTER 3

**EFFECTS OF AN ANTI-OESTROGEN (CLOMIPHENE CITRATE)
ON NORMAL HUMAN ENDOMETRIAL STROMA AROUND THE
TIME OF IMPLANTATION**

3.1. INTRODUCTION

Clomiphene citrate (CC) is a widely prescribed anti-oestrogen therapeutic agent used for ovulation induction. CC was first synthesized in 1956 (Palopoli *et al.* 1967) and first used for induction of ovulation in the early 1960s by several investigators (Greenblatt *et al.* 1961; Roy *et al.* 1963; Kistner 1965).

At low doses, CC acts as an oestrogen antagonist by blocking oestrogen receptors. Previous studies in normal cycling women performed in the early follicular phase (Kerin *et al.* 1985) and luteal phase (Marauic and Casper 1987) of the menstrual cycle have identified a hypothalamic site of action for CC where it appears to compete with oestrogen receptor sites (Huppert 1979). The blockade of the oestrogen receptors has the effect of inhibiting the negative feed back of endogenous oestrogens, resulting in an elevation in gonadotrophin-releasing hormone (Gn-RH) secretion (Speroff *et al.* 1989). The Gn-RH surge causes increased pituitary gonadotrophin secretion which stimulates and enhances the follicular growth and subsequent ovulation (Vandenberg and Yen 1973; Fritz *et al.* 1991). CC-treatment of women with unexplained infertility is followed by increased plasma levels of oestrogen and progesterone (Koike *et al.* 1991), supporting the theory that the inhibition of negative feed back of endogenous oestrogens causes the elevation of serum LH, FSH, oestrogen and progesterone levels. CC-induced ovulation in women with polycystic ovarian syndrome (PCOS) is accompanied by increased secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) with enhanced oestrogen and progesterone secretion, indicating an effect at the hypothalamic site (Fedele *et al.* 1989; Kettel *et al.* 1993). Similarly, FSH levels are increased after induction of ovulation with CC in women with ovarian endometriosis (Tanbo *et al.* 1992). Ovulation occurs 13-15 days after CC initiation (Wu 1984).

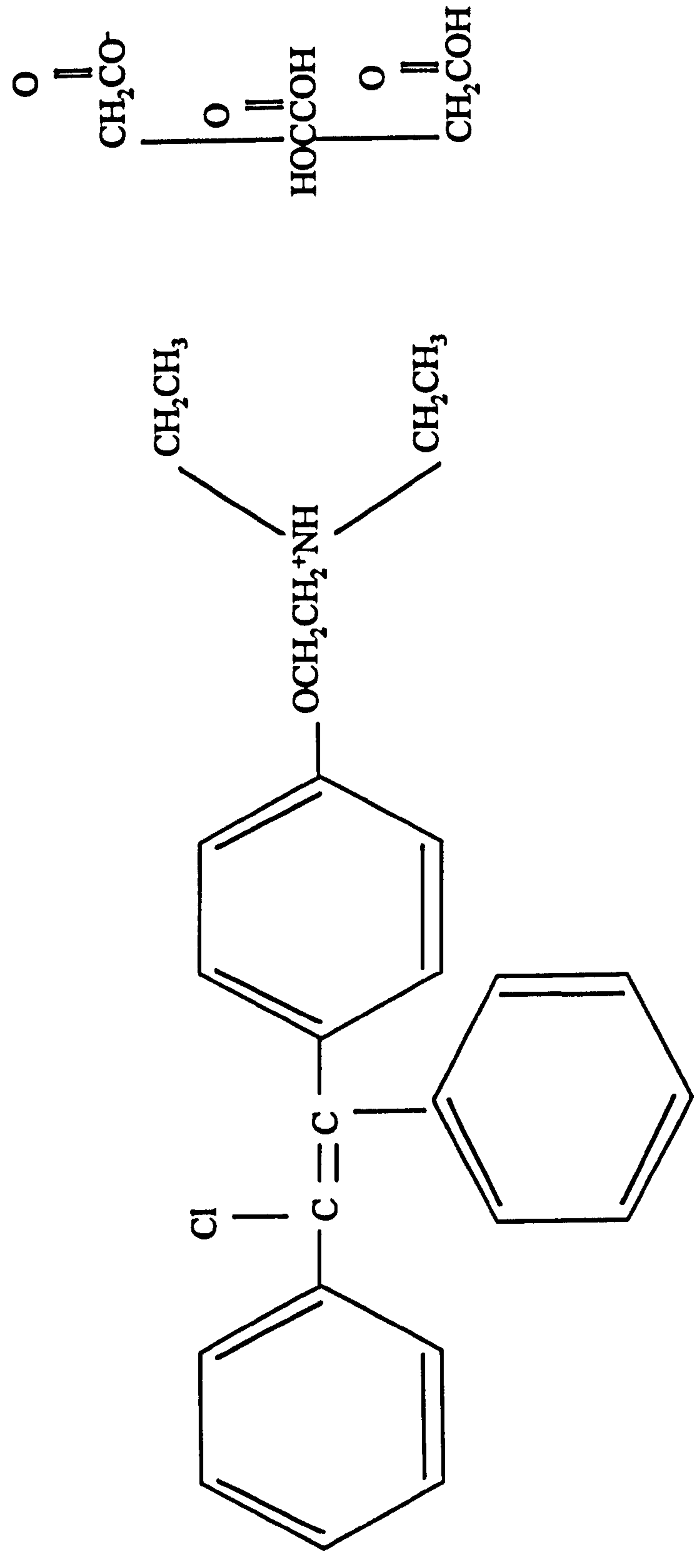
At high doses, CC is thought to act as an oestrogen agonist by directly blocking gonadotrophin secretion in the pituitary (Clark and Markaverich 1982) and possibly also by affecting the steroidogenic activity in ovaries (Glasier 1990). CC is also used for the treatment of luteal phase defects in anovulatory women (Li and Warren 1993). However, the efficacy of CC in the treatment of luteal phase defect has been studied and success in treatment was achieved in only 56% of cases, suggesting that CC does not consistently correct this condition (Guzick and Zeleznik 1990). Therefore, most

recently, Rodin *et al.* (1994) investigated the effect of CC therapy on cyst formation and luteal phase defects in unexplained infertility patients. They have shown that the treatment with CC reduces the incidence rates of cyst formation from 40 percent to 9 percent and the incidence of luteal phase defects from 31 percent to 3 percent indicating that CC therapy must be considered as a useful treatment. It has also been suggested that the response of the endometrium to ovarian stimulation by CC is likely to be variable (Li and Warren 1993).

Structurally CC, a non-steroidal oestrogen, is a triphenylethylene derivative (Figure 3.1a) which is capable of interacting with oestrogen receptor binding proteins just like the native oestrogens (Clark and Markaverich 1982). However, the nature of the interaction of CC with oestrogen binding proteins may display qualitative differences compared to the naturally occurring ligand. Most importantly, CC is best characterized by the tendency to display prolonged nuclear receptor occupancy (Adashi *et al.* 1980; Clark and Markaverich 1982). Indeed, CC can occupy nuclear receptor proteins for weeks and this phenomenon sharply contrasts with the rather brief interaction of native oestrogens, known to clear the cell within 24 hours (Adashi 1993).

In clinical use, CC is provided as a racemic mixture of its two stereochemical isomers, *en* clomiphene (trans isomer) and *zu* clomiphene (cis isomer) citrate (Ernst *et al.* 1976). While the *en* clomiphene molecule has its two unsubstituted phenyl rings on opposite sides of a hypothetical plane, dividing the clomiphene citrate molecule through its ethylene core, the two unsubstituted phenyl rings of *zu* clomiphene molecule are found on the same side of this hypothetical plane (Figure 3.1b). Studies in humans have shown that the *en* clomiphene isomer is responsible for the anti-oestrogenic action of the drug, while *zu* clomiphene is responsible for its oestrogenic activity (Clark and Markaverich 1982; Glasier *et al.* 1989). However, the currently marketed formulations of CC consist of a mixture of *en* and *zu* clomiphene, each of which has a different ratio of agonist-antagonist properties (Clark and Markaverich 1982) so that the ratio of oestrogenic and anti-oestrogenic effects may vary from preparation to preparation. It has been suggested that the number of follicles and follicular phase oestrogen secretions were not changed after treatment with *zu* clomiphene but were significantly increased after *en* clomiphene treatment. This suggests that *en* clomiphene may be the isomer active in inducing

Figure 3.1: Structural formula of clomiphene citrate. From AMA Drug Evaluations (1980).



follicular development (Glasier *et al.* 1989). The racemic mixture administered to induce ovulation in women is generally made up of 38% *zu* clomiphene and 62% *en* clomiphene citrate. Two common proprietary preparations of CC are Clomid (Merrel Dow Pharmaceuticals) and Serophene (Serono Labs UK Ltd). The initial dose of CC is typically 50mg administered orally for 5 days, between days 2 and 6 of a traditionally dated cycle. This dose is gradually increased to a maximum of 200-250mg/d with the therapy usually continued for 3-6 cycles (Thorneycroft 1984; Wu 1984). However, it has also been suggested that there are no significant differences between CC-treated groups who started CC therapy on the 2nd, 3rd, 4th or 5th day of cycle respectively in terms of anovulation rates, luteal dysfunction and normal ovulation rates (Wu and Winkel 1989).

Despite its positive effects on the induction of ovulation, there are discrepancies between ovulation and conception rates after treatment with CC, possibly suggesting it has negative side effects on the reproductive tract. The pregnancy rate (30%) achieved following CC treatment is significantly lower than the induced ovulation rate (70%) (Wu 1984). Such a discrepancy between ovulation and conception rates may be due to adverse effects of the anti-oestrogen on endometrial development (Scialli 1986). However, it has been shown that pregnancy rates are significantly ($p < 0.004$) higher (19%) in CC treated patients with unexplained infertility than both placebo treated and hCG treated ones (Fisch *et al.* 1989). The authors concluded that CC is useful in treating unexplained infertility and is a reasonable initial therapy.

Several studies on the effects of CC on endometrial development have suggested that CC treatment significantly decreases the endometrial thickness in humans (Saito *et al.* 1991; Yagel *et al.* 1992; Dickey *et al.* 1993). However, in all of these studies infertile subjects were used and it is not possible to exclude underlying pathology from the results. Wolman and co-workers (1994) have also examined the effect of CC on endometrial thickness in women with unexplained infertility using transabdominal ultrasonography. In this study, 8 women who conceived after CC treatment had a significantly thicker endometrium than those 38 women who did not conceive, and the authors concluded that CC has an adverse effect on endometrial growth. However, it has also been suggested that CC does not produce an effect on either the concentrations

or the binding affinities of either oestrogen or progesterone nuclear receptors in luteal phase peri-implantation endometrium, indicating that CC has no adverse effect on these steroid receptors (Hecht *et al.* 1989; Fritz *et al.* 1991). Such apparently conflicting data are difficult to interpret, however it may indicate a range of different sub-groups in the population, each responding in a different way. One aim of the present study was to determine the precise changes in normal endometrium due to CC by using objective criteria.

CC has significant effects on other regions of the female reproductive tract. For example, the production of cervical mucus (under the influence of oestrogen and progesterone) can be substantially affected by CC (Thompson *et al.* 1993; Assad *et al.* 1993; Massai *et al.* 1993; Gelety and Buyalos. 1993). Cervical mucus plays a crucial role in the successful passage of sperm into the uterine cavity and any effects of CC on mucus could influence fertility rates. In several recently published studies, cervical mucus volume was reported to be significantly decreased after treatment with CC, suggesting that at least some of the discrepancies between ovulation and conception rates may be due to the local anti-oestrogenic effect of CC at the level of the cervix.

The relevance of recently published studies to the efficacy of ovulation induction by CC and to its presumed adverse effects on the other parts of the reproductive tract remains uncertain. Most previous studies on the effect of CC on human endometrium have been performed on women being treated for infertility and it is not possible to exclude any underlying pathology from their results. However in the present study, normally ovulating women of proven fertility were used both as control and for CC treatment in order to overcome this problem. Therefore the aim of this study was to investigate the effects of CC treatment on normal fertile human endometrial morphology around the time of implantation by examining epithelial, stromal and vascular components using quantitative microscopical methods.

3.2. MATERIALS AND METHODS

3.2.1. SUBJECTS

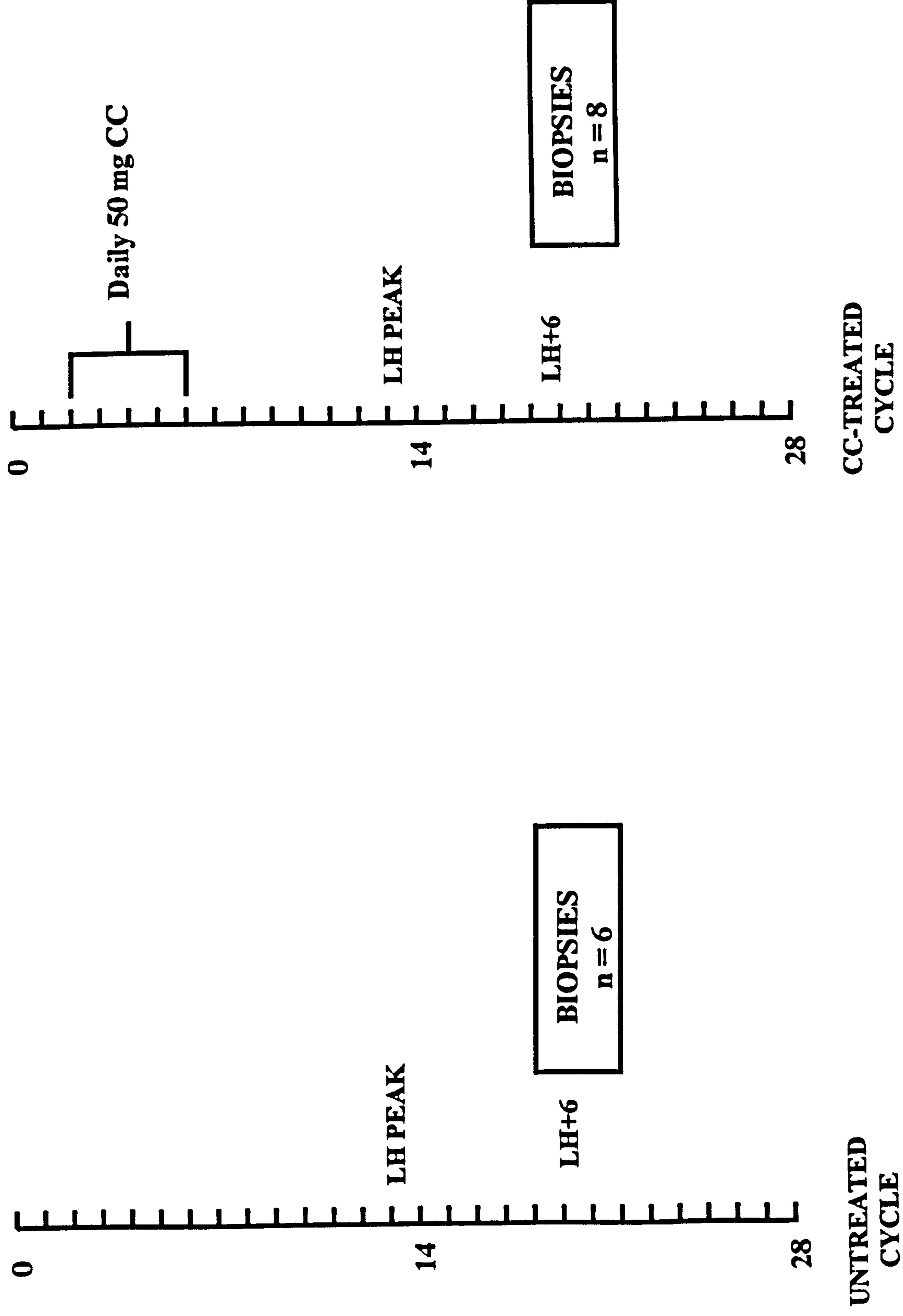
Eight normal, healthy women volunteers with proven fertility, aged 20-40 years with regular menstrual cycles of 25-35 days, were used in this study. None of the persons had used steroidal contraceptives during the previous two months. They were all non-pregnant and able to communicate well with the investigators. If there was any clinically significant medical condition, including major uterine pathology, renal, hepatic, cardiovascular, gastrointestinal, psychiatric or endocrine disorders and hypothalamic pituitary failure, these volunteers were excluded from the study. All subjects were treated on an outpatient basis and were counselled and given instructions about the procedure of the experiment. All procedures had Ethics' Committee approval and all clinical procedures were carried out by Mr T.C. Li of the Jessop Hospital for Women, Sheffield.

3.2.2. EXPERIMENTAL DESIGN

This was an open, random ordered and controlled study to find out the effects of clomiphene citrate (CC) on normal human endometrial stromal morphology. Each volunteer had a cycle without CC treatment and control group biopsies were obtained at day LH+6 [6 days after the luteinizing hormone (LH) peak which occurs just before the ovulation] of the untreated cycle. After a "washout" period of one cycle when no treatment was given, volunteers were given daily 50 mg CC tablets (Scrophene, Serono, UK) between days LH-11 and LH-7 (11 and 7 days respectively before the LH peak) during the proliferative phase in the menstrual cycle following the "washout" cycle. Clomiphene citrate treatment group biopsies were also obtained at day LH+6 of the menstrual cycle (Figure 3.2).

Biopsy procedure, LH dating, histological processing, sectioning and staining of the endometrial tissue for light and electron microscopy and morphometric methods used in this study were the same as described in Chapter 2.

Figure 3.2: Protocol of the experiment showing the untreated and CC-treated cycles with drug dosage and times.



3.2.3. STATISTICAL ANALYSIS

Every feature was calculated on a 'per individuals' basis. Means and standard errors were calculated for each group and data were analysed using Student's unpaired *t*-test where the conventional level 5% was taken as the level of significance. Same normal fertile subjects were used for both groups and comparisons were made before and after CC administration. While untreated group consisted of 6 individuals, CC-treated group contained 8 individuals.

3.3. RESULTS

3.3.1. GLANDULAR EPITHELIUM

The mean \pm standard error of the volume fraction of gland occupied by gland cell showed that there was no significant difference between untreated (0.832 ± 0.021) and CC treated (0.827 ± 0.021) groups (Table 3.1). The coefficient of variation for both groups was small (7%). The volume fraction of endometrium occupied by gland again was not significantly different between the groups, being 0.223 ± 0.017 in the untreated group and 0.279 ± 0.030 in the CC treated group (Table 3.1). The coefficient of variation of gland to endometrium for controls was 3 times larger (22%) than for gland occupied by gland cell, even larger (30%) in the CC treated group indicating its greater variability.

3.3.2. STROMA

The mean \pm standard error of the volume fraction of stroma to endometrium was 0.777 ± 0.017 in untreated group and 0.721 ± 0.030 in CC treated group which was not significantly different (Table 3.3). The volume fraction of stromal cell nucleus to endometrium was 0.092 ± 0.010 in untreated group and 0.111 ± 0.006 in CC-treated group, however this numerical difference was not significant (Table 3.3).

Similarly the mean \pm standard error values of stromal cell nuclear profile measurements showed that the major axis, minor axis, mean diameter and axial ratio of the stromal cell nuclei did not significantly differ between groups (Table 3.2).

The mean \pm standard error for volume fraction of stromal cell occupied by nucleus was 0.454 ± 0.013 in untreated group, 0.487 ± 0.026 in CC treated group and the difference was not significantly different between groups (Table 3.3) (for comparison see Figure 2.6a and Figure 3.3a). However, once again the variation in the CC-group was about twice that seen in controls. The volume fraction of stromal cell nucleus occupied by euchromatin was not significantly different between groups and neither were the volume fractions of stromal cell occupied by mitochondria, "secretory apparatus" and RER different (Table 3.3). On the other hand, the coefficient of variation of "secretory

Table 3.1: Comparison of volume fractions for glandular features between untreated and anti-oestrogen (CC) treated groups.

Feature	Untreated Group (n=8)	CC-Treated Group (n=8)
Vv of Gland Occupied by Gland Cell	0.832 ± 0.021	0.827 ± 0.021
Vv of Endometrium Occupied by Gland	0.223 ± 0.017	0.279 ± 0.030

Results are means ± SE

n= number of individuals

Table 3.2: Shows the stromal cell nucleus dimension data in micrometers comparing the untreated and CC-treated groups.

Feature	Untreated Group (n= 8)	CC-Treated Group (n=8)
Stromal Cell Nucleus Profile Major Axis (μm)	6.59 ± 0.27	6.37 ± 0.17
Stromal Cell Nucleus Profile Minor Axis (μm)	3.62 ± 0.12	3.71 ± 0.08
Stromal Cell Nucleus Profile Mean Diameter (μm)	4.82 ± 0.14	4.80 ± 0.08
Stromal Cell Nucleus Profile Axial Ratio	1.93 ± 0.10	1.84 ± 0.08

Results are means \pm SE

n= number of individuals

Table 3.3 : The comparison of the volume fraction type data of the endometrial stromal and ultrastructural stromal cell features between untreated and CC-treated groups.

Features	Untreated Group (n=6)	CC Treated Group (n=7)
Vv of Endometrium Occupied by Stroma	0.777 ± 0.017 (n=8)	0.721 ± 0.030 (n=8)
Vv of Endometrium Occupied by Stromal Cell Nucleus	0.092 ± 0.010 (n=8)	0.111 ± 0.006 (n=8)
Vv of Stromal Cell Occupied by Nucleus	0.454 ± 0.013	0.487 ± 0.026
Vv of Stromal Cell Nucleus Occupied by Euchromatin	0.801 ± 0.034	0.806 ± 0.023
Vv of Stromal Cell Occupied by Mitochondria	0.036 ± 0.004	0.035 ± 0.002
Vv of Stromal Cell Occupied by "Secretory Apparatus"	0.054 ± 0.004	0.075 ± 0.012
Vv of Stromal Cell Occupied by RER	0.033 ± 0.004	0.041 ± 0.006

Results are means \pm SE

n= number of individuals

apparatus" feature was much higher (41%) in the CC-treated group than the untreated group (18%) indicating a higher inter-subject variation within the CC group. In contrast the variation in mitochondria to cell was higher (28%) in controls than the CC-treated group (11%).

3.3.3. BLOOD VESSELS

While there were no significant differences in the mean \pm standard error of the volume fractions of blood vessel lumen to endometrium, and blood vessel tissue to endometrium, the volume fraction of blood vessel total (consisting of the lumen and the tissue) to endometrium was significantly ($p < 0.05$) higher (0.049 ± 0.003) in the CC-treated group than the untreated group (0.039 ± 0.003), indicating a possible effect of the clomiphene citrate on the endometrial blood vessels (Table 3.4). However, the coefficient of variation of the blood vessel lumen to endometrium was high and was twice as high (82%) in controls as in the CC-treated group.

While the volume fraction of endothelial cell occupied by nucleus was not changed by CC treatment (for comparison see Figure 2.7a and Figure 3.3b), the volume fraction of endothelial cell nucleus occupied by euchromatin was significantly ($p < 0.05$) decreased after CC treatment being 0.854 ± 0.019 in controls and 0.787 ± 0.008 in CC-treated group (Table 3.5). This may indicate reduced transcription of DNA in the CC group. There was no significant change in the volume fraction of mitochondria to cell following the CC treatment. However, the coefficient variation in this feature in the control group (44%) was much higher than the CC-treated group (25%). Although the volume fraction of "secretory apparatus" to cell was greater in the CC-treated group (0.049 ± 0.004) than the untreated group (0.039 ± 0.003), the significance level was not within the conventional 5% limits ($0.05 < p < 0.1$) due to the high coefficient of variations within the groups, indicating only a tendency of an increase after the CC treatment (Table 3.5). The volume fraction of endothelial cell occupied by RER again was not significantly different between groups but there was a tendency of reduction following the CC treatment with the significance level of 10% ($0.05 < p < 0.1$) being 0.031 ± 0.004 in the untreated group and 0.021 ± 0.003 in CC-treated group. Same result for the volume

Table 3.4: Shows the comparison of endometrial stromal blood vessel features between the untreated and CC-treated group.

Features	Untreated Group (n=8)	CC-Treated group (n=8)
Vv of Endometrium Occupied by Blood Vessel Lumen	0.011 ± 0.003	0.018 ± 0.003
Vv of Endometrium Occupied by Blood Vessel Tissue	0.028 ± 0.002	0.031 ± 0.003
VV of Endometrium Occupied by Blood Vessel Total (Vessels+Lumen)	0.039 ± 0.003	0.049 ± 0.003*

Results are means ± SE

n= number of individuals

* p< 0.05

Table 3.5: The comparison of ultrastructural endothelial cell features between groups.

Features	Untreated Group (n=6)	CC Treated Group (n=8)
Vv of Endothelial Cell Occupied by Nucleus	0.595 ± 0.027	0.560 ± 0.013
Vv of Endothelial Cell Nucleus Occupied by Euchromatin	0.854 ± 0.019	0.787 ± 0.008*
Vv of Endothelial Cell Occupied by Mitochondria	0.025 ± 0.005	0.024 ± 0.002
Vv of Endothelial Cell Occupied by “Secretory Apparatus”	0.039 ± 0.003	0.049 ± 0.004†
Vv of Endothelial Cell Occupied by RER	0.031 ± 0.004	0.021 ± 0.003†

Results are means ± SE

n= number of individuals

* p < 0.05

† 0.05 < p < 0.1

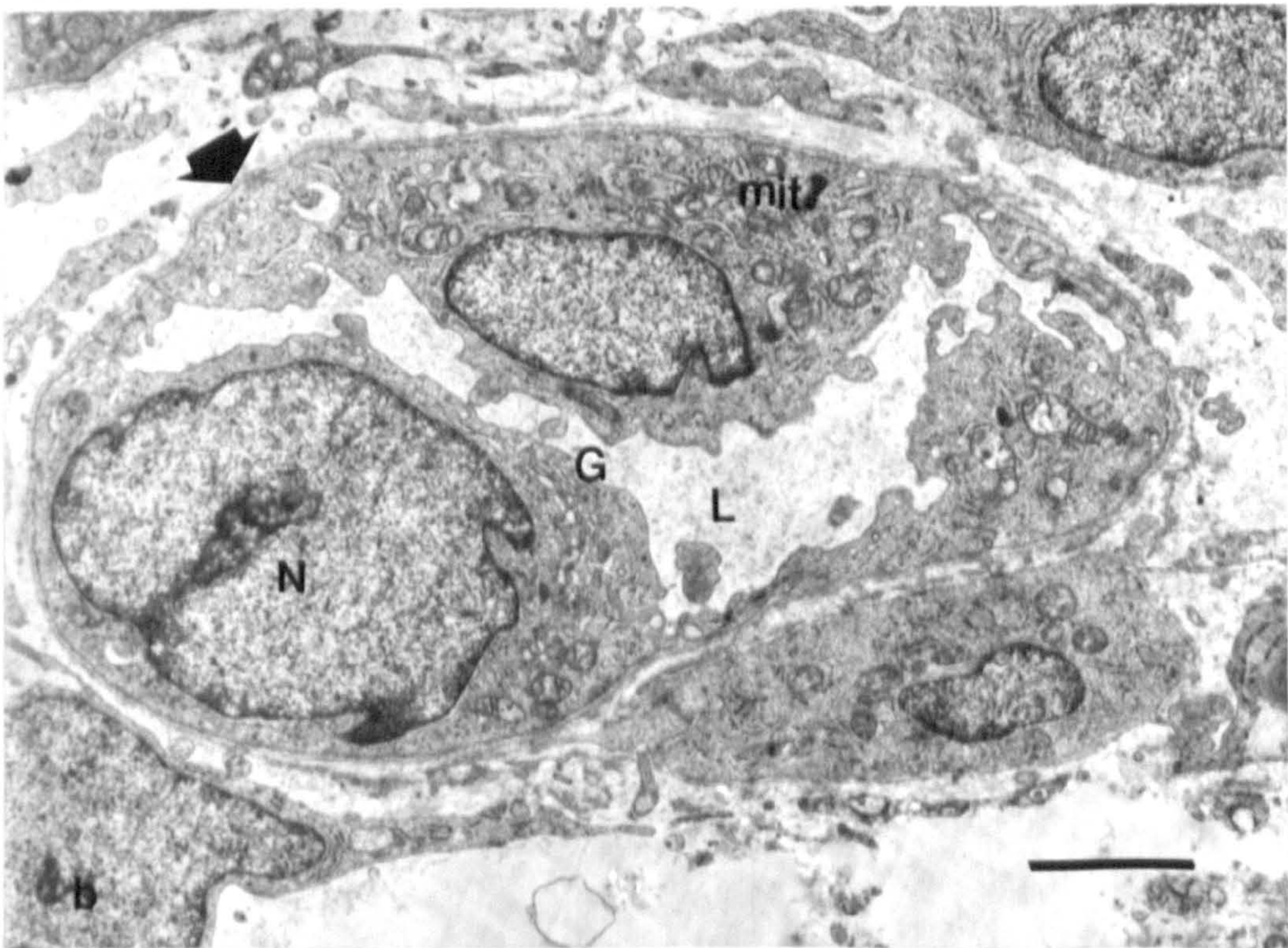
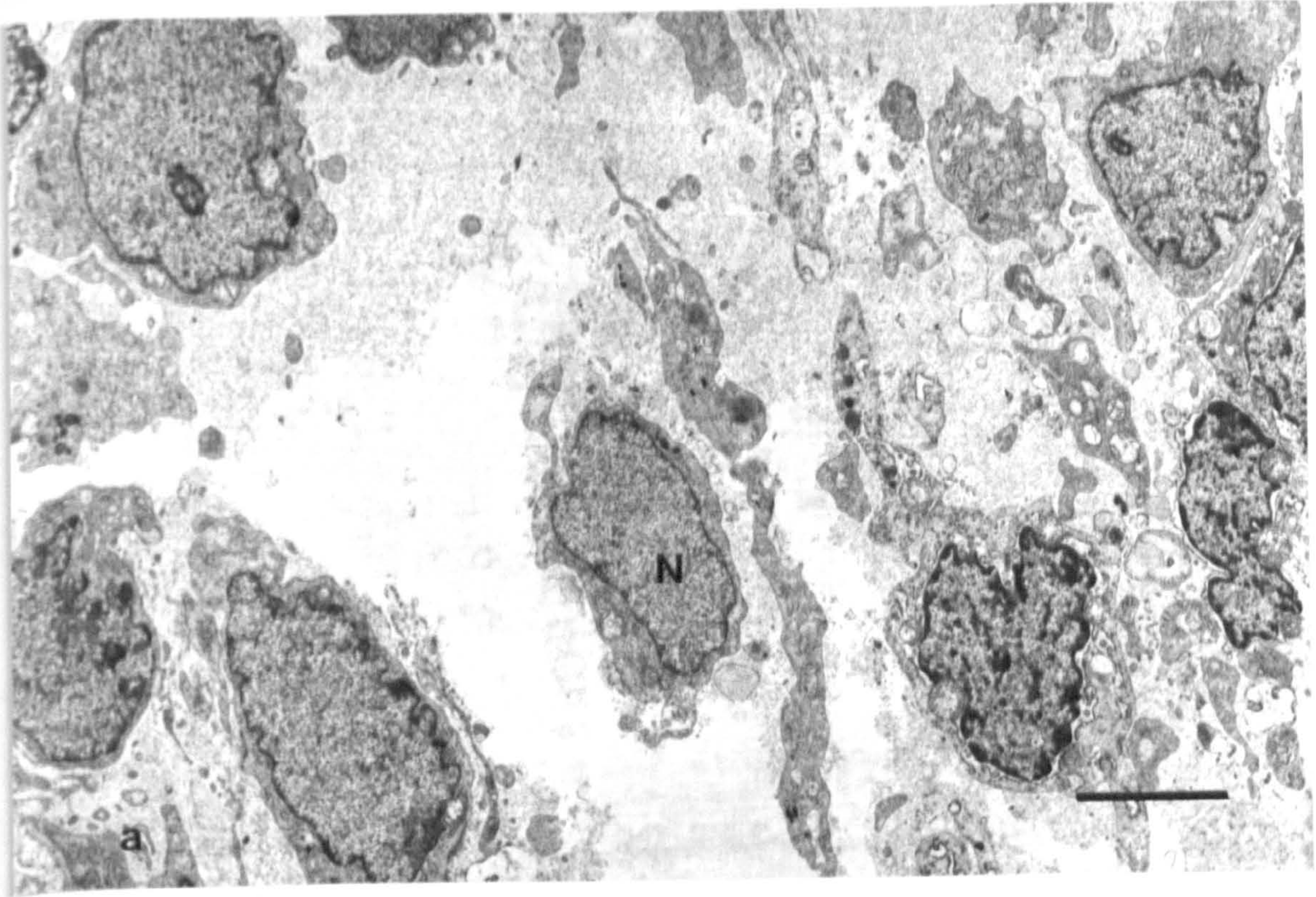
fraction of mitochondria to cell has been observed in endothelial cells as seen in stromal cells that the coefficient of variation was higher (44%) in controls than the CC treated group (25%) indicating an unknown effect of CC on the metabolic activity of both stromal and endothelial cells.

Figures 3.3a,b: Clomiphene citrate-treated human endometrial stroma (a) and blood vessel (b) at day LH+6 of the menstrual cycle. (a) Stromal cells contain generally oval, euchromatic nuclei (N) and regular cytoplasmic features which look similar to the day LH+6 control group stromal cells (see Figure 2.6a for comparison). (b) Blood vessel have round-shaped euchromatic nuclei (N) with organelles and a basement membrane (arrow) which are similar to normal day LH+6 vessels (for comparison also see Figure 2.7a). (L) lumen, (G) Golgi apparatus, (mit) mitochondria.

Staining: Uranyl Acetate - Lead Citrate

(a) Bar represents 4.00 μm

(b) Bar represents 1.80 μm



3.4. DISCUSSION

The major aim of this study was to investigate the effect of clomiphene citrate on the quantitative morphology of the stromal and vascular components of the normal fertile human endometrium. Some glandular features were also examined to determine the overall effect of the anti-oestrogen on endometrial structure. Normally ovulating fertile, rather than infertile, women were examined because the latter may be anovulatory or have abnormal endometrial development. Also, any changes observed in the endometrium of infertile women treated with anti-oestrogens may be associated with a pre-existing abnormality, or be the effects of anti-oestrogen treatment, or both. However, any change seen in normal fertile women receiving anti-oestrogen treatment must be the result of CC treatment.

While there were no major changes in glandular and stromal morphological features, blood vessels were slightly affected after treatment with CC. However, the coefficient of variations in some of the glandular, stromal and vascular features were greater in the CC-treated group than the untreated one, indicating a variable response of the subjects to the drug treatment.

3.4.1. GLANDULAR EPITHELIUM

On average, the control group was not different from the CC-treated group when the volume density of gland cell to gland was compared. In the normal cycle around day LH+4 deposits of glycogen are seen which increase the overall volume of gland cells (Dockery *et al.* 1988b). This has the effect of closing the gland lumen and so increases the ratio of gland cell to gland values. This lasts only 1 or 2 days since the glycogen is transported apically and secreted into the gland lumen, thereby returning the cell volume to its earlier proportions. This rapid change makes gland cell to gland volume density a useful feature to determine advances or delays in uterine development compared to controls. In the present experiment there was no significant difference in the Vv of gland cell to gland between CC-treated and control subjects suggesting that treatment with anti-oestrogens had no effect on endometrial glandular development at the light microscopical level. Similarly, the volume fraction of endometrium occupied by total

gland did not differ between groups indicating no retardation or delay in endometrial development after CC treatment. On the other hand, the coefficient of variation in gland cell to gland volume density was smaller than the variation of gland to endometrium volume fractions. This indicates that while the response of glands to CC treatment were consistent at the level of individual glandular glycogen production and secretion, across the whole endometrium glands may show subtle changes in response to CC-treatment.

Bonhoff *et al.* (1993) have reported that CC treatment causes impaired glandular maturation. In that study, different types of ovarian stimulation protocols (combined hMG/hCG, combined CC/hMG/hCG and CC alone) were performed on infertile women undergoing infertility treatment and those results were compared to results from normal fertile women (22 Caucasian women, 20-40 years of age with a history of one or more pregnancies and with regular menstrual cycles and normal hormonal profiles). A total of 65 subjects were used in their study and CC dosage regime was 50 to 150 mg/day between days 3-7. However, the sample size for CC-treatment experiment was small with only 12 subjects being used for all three CC-dosage regimes. Bonhoff and co-workers have analysed the endometrial tissue by using semi-quantitative morphometry, and stereological analyses were performed by using a computerized cell image analyzer. They found relatively large glands, being $62.9 \pm 18.8 \mu\text{m}$ in diameter in CC-treated group, $47.0 \pm 10.4 \mu\text{m}$ in diameter in CC/hMG/hCG treated group, $53.3 \pm 12.7 \mu\text{m}$ in the fertile group, and decreased epithelial cell height, being $17.7 \pm 4.2 \mu\text{m}$ in CC-treated group, $19.4 \pm 4.8 \mu\text{m}$ in CC/hMG/hCG treated group, and $23.2 \pm 3.3 \mu\text{m}$ in the fertile group, after treatment with CC in the early secretory phase. While the sub-nuclear vacuolation was not changed in the early secretory phase, it was increased in mid-luteal and late-luteal phase after treatment with CC. In addition, they reported a decreased volume fraction of glandular epithelium to endometrium, being 12.4 ± 3.2 in CC-treated group and 16.6 ± 3.8 in the fertile group, following CC administration. They suggested that the CC-treatment impaired the glandular maturation in human endometrium.

In contrast, in the present study objective stereological techniques were used to determine the volume densities of glandular and stromal features. In Bonhoff's study, infertile subjects were used and results from these subjects were compared with data from fertile women and it is not possible to exclude underlying pathologies from their

results. In addition, the experimental groups used by Bonhoff *et al.* were not grouped appropriately. For example biopsies between days LH+2 and LH+6 and between days LH+7 and LH+10 were grouped together. It is not good practice to group the features from LH+2 and LH+6 endometrium because many structural changes occur normally at this time (Li *et al.* 1991a). For example, glandular sub-nuclear glycogen content is maximum about day LH+4 and secretion of this glycogen content is completed by the end of day LH+6. Pooling groups as done by Bonhoff *et al.* (1993), would be likely to obscure such changes. In contrast, in the present study all biopsies were taken at day LH+6 in both untreated and CC-treated groups. In addition normally ovulating fertile women rather than infertiles were used in both groups and so any change in endometrial structure was most likely the result of CC treatment.

In a similar study, Hosie *et al.* (1991) compared the endometrial glandular features morphometrically using a fully automated image analysis system, between different types of ovarian stimulation protocols and suggested that the CC-hMG treatment has a suppressive effect on glandular development and growth. Other drug regimes in their study were CC/hMG + progesterone and busarelin/gnRH/hMG treatments. All biopsies obtained in their study were dated as day 17 using traditional histological criteria. However, all subjects used in their study were infertile and all comparisons were made between these drug treated infertile groups. Also they have not used normal fertile subjects as a control group. Comparison of three different superovulatory drug regimes without an unsupplemented control group should not be considered as a controlled, comparative experiment.

Yeko *et al.* (1992) have reported the histology of the mid-luteal corpus luteum and endometrium in normally ovulating fertile women after gradually increasing CC treatment. They suggested that the corpus luteum histology was not affected by CC treatment in any subject and that 83% of 12 mid-luteal phase endometria were “in-phase” and only 17% were “out of phase”. These 17% exhibited a 3-day lag in endometrial development. Apart from these 17% (2 out of 12 subjects) the results shown in their study generally agreed with those of the present study that the CC treatment has no adverse effect on endometrial morphology. Interestingly, in an ultrastructural study on the effects of CC on mid-proliferative phase human endometrium, Fedele *et al.* (1988)

have reported that the CC taken in a dose not exceeding 100 mg/day does not impair the endometrial development in anovulatory infertile women. These authors have suggested that “the discrepancy between the proportion of ovulatory cycles and pregnancy rate in women undergoing treatment with CC cannot be explained by endometrial modifications”. Similarly, in a semi-quantitative morphological study, Dehou *et al.* (1987) demonstrated that neither CC nor the other hyperstimulatory drugs, such as hMG and hCG, have adverse effects on endometrial glandular morphology. Interestingly, the Dehou study was performed on infertile women who were treated for IVF. In contrast, in a similar type of study, Birkenfield *et al.* (1986) suggested a direct effect of CC on the anovulatory human endometrium indicating advanced secretory changes in glands and low progesterone levels during late proliferative phase of the menstrual cycle. They have commented that such a premature epithelial secretory pattern may cause a functional desynchronization which may have its implication during the peri-implantation period. However, these two studies were again performed on anovulatory women who may have different underlying pathologies and may react to CC treatment in different ways.

In a qualitative morphological study, Benda (1992) reported straight, narrow and less tortuous glands than those in infertile women after treatment with CC when compared to the results those from untreated patients. In their study the gland to stroma ratio was decreased and glandular secretion was scant throughout the secretory phase. Late secretory epithelium was low cuboidal and rarely showed hypersecretory change. The author has commented that the less mature glandular morphology and reduced function are due to the hypo-oestrogenic activity of the CC by directly altering the oestrogen action on the endometrium. However, first of all the results of their study were made using subjective qualitative evaluation and secondly, like most of the other studies on the effect of CC on endometrium, infertile subjects were used in their study.

In a prospective, randomized, cross-over study, Li *et al.* (1992) reported that neither CC nor cyclofenil (another anti-oestrogen) has adverse effects on human endometrial morphology. 10 healthy, regularly cycling and previously fertile women were used in their study. A 50 mg daily dosage of CC were given to subjects between days 2-6 and endometrial biopsies were obtained at day LH+6 of the menstrual cycle and the same subjects were used both for control and CC treatment. There were no significant

differences between untreated and CC-treated groups in any of the following features; glandular mitosis (per 1000 cells), subnuclear vacuoles (per 100 gland cells), supranuclear vacuoles (per 100 gland cells), the volume fraction of gland to stroma and the volume fraction of gland cell to gland. Results of the Li study fully agreed with the results from the present study.

3.4.2. STROMA

The main aim of the present study was to investigate the effects of CC on endometrial stroma, and no stromal features appeared to be affected by treatment. Similar to the volume fraction of endometrium occupied by glands, the volume density of stroma to endometrium was not changed after treatment with CC. Several stromal nuclear features were measured to determine whether or not CC has adverse effects on stromal cell function. The volume density of stromal cell nuclei to endometrium was measured to determine the relative proportion of total stromal cell nuclei following treatment with CC. A change in this feature could indicate changes in nuclear size or number, however it was unaffected by CC treatment. Nuclear profile size was measured by direct digitization and average diameter and ratio of maximum to minimum diameter used to obtain axial ratio, a crude indicator of profile shape. Active nuclei are often larger and rounder than inactive ones (Dockery *et al.* 1990). However, in the present study no difference in nuclear size or shape was seen indicating CC had no effect on nuclear activity seen at the light microscopical level. In addition, the volume density of nucleus to cell was not significantly different between untreated and CC-treated groups, although the coefficient of variation in the CC-treated group was twice as much as that observed in the untreated group. This may indicate a greater inter-subject variation within CC-treated group. The major morphological feature used to indicate nuclear transcription is the euchromatin-heterochromatin distribution, with an increased euchromatin ratio within the nucleus suggesting increased transcription of DNA. In the present study, the volume density of nucleus occupied by euchromatin did not differ between untreated and CC-treated groups. All these stromal cell nuclear measurement results indicate that the CC treatment did not change either the morphology or the function of the stromal cell nuclei.

By examining the relative proportion of cell made up by organelles it is possible to infer functional changes. For example, increased Vv of mitochondria to cell would indicate increased oxidative phosphorylation, increased RER suggests elevated translation and protein secretion. In order to determine whether or not CC had effects on any of these functions various organelle Vvs were estimated. At the stromal cell organelle level, CC treatment had no effect on the volume densities of mitochondria to cell, "secretory apparatus" to cell and RER to cell suggesting that neither the metabolic activity nor the protein synthesis and the secretory function of the stromal cells were affected by treatment. These results were consistent with the results of the nuclear euchromatin Vv measurements which were also unchanged after the treatment. However, the coefficient of variation of "secretory apparatus" to cell in CC-treated group was much higher (41%) than in the controls (18%) showing a large inter-subject variation in that group. Oddly, the coefficient of variation of mitochondria to cell was higher in controls than the CC-treated group, although the reason for this remains unclear.

Bonhoff *et al.* (1993) reported an increased volume density of stromal components shortly after ovulation and during mid to late luteal phase after treatment with CC and they have suggested a "disturbed" transformation of the secretory endometrium. However, as it was discussed in this chapter before, sample size in that study was too small and a very wide range of LH dated biopsies were grouped together. In addition, in contrast to the present study, there was no further morphometric investigation of stromal cell features such as stromal cell nucleus and cytoplasmic organelles. Therefore in the present study, a wide range of stromal cell features were evaluated morphometrically and any effect of CC on endometrial stromal cell morphology was investigated. None of the stromal and stromal cell features, including volume density of stroma to endometrium, seemed to be affected by treatment with CC in the present study.

Dehou *et al.* (1987) have reported an increased endometrial stromal oedema around days 21 and 22 of the cycle after sequential stimulation with CC, hMG and hCG. However, they have commented that "all biopsies of stimulated cycles were found "in-phase" and no advanced or delayed endometrium being diagnosed".

Similarly, Li *et al.* (1992) reported no changes in normally ovulating fertile human endometrium after treatment with CC. None of the endometrial stromal features, such as stromal mitosis (per 1000 cells), volume fraction of stromal nucleus to endometrium, mean stromal nuclear diameter and axial ratio of the stromal cell nucleus, were affected by CC treatment. The authors commented that anti-oestrogens do not have a major adverse effect on endometrial development which is the main outcome of the present study.

In a qualitative morphological study, Fedele *et al.* (1988) investigated the effect of clomiphene citrate on anovulatory human endometrial structure during mid-proliferative and late-proliferative phases and postovulation days 1 to 3. Stromal cells had an eosinophilic cytoplasm and mitosis were abundant during the mid-proliferative phase after treatment with CC. At the electron microscope level during that time, stromal cells were spindle shaped with various digitations and elongated nuclei which are the characteristics of the stromal cells in proliferative phase. In the late-proliferative phase, stromal oedema was observed at the light microscope level. Three days after ovulation, stromal oedema were increased and at the ultrastructural level, stromal cells were rounded and had numerous glycogen particles and lipid droplets in their cytoplasm. Yet again, these are the characteristics of the postovulatory endometrial stroma (Li *et al.* 1991a). The authors concluded that "CC, when taken at doses not exceeding 100 mg/day from day three of the cycle, is able to induce endometrial maturation that is almost identical with that of spontaneous cycle" and this conclusion fully agrees with the results of the present study.

Benda (1992) reported a progressive decidualization of stroma in the late luteal phase of the infertile human endometrium after treatment with clomiphene citrate. In some cases, the predecidual cells were smaller than expected, with less cytoplasm, and the overall amount of decidua was decreased. As discussed earlier in this chapter their study was performed on infertile women using subjective morphological evaluation. The results of the present study have clearly shown that there was no major effect of CC on endometrial morphology of fertile, normally ovulating women.

Although the endometrial stroma has as important a function as the endometrial glandular epithelium in the maintenance of pregnancy, it has been poorly investigated by

previous studies and there appear to be very few previous published studies which involve a full morphometric evaluation of the human endometrial stroma; the present report aimed to fill this gap.

3.4.3. BLOOD VESSELS

Apart from glandular epithelium and stroma, endometrial blood vessels are another important compartment of the endometrium which play a crucial role for the transport of hormones and other essential compounds into the endometrium as well as part of the placenta . Any effect of CC on endometrial blood vessels may cause severe defects in endometrial function. To evaluate this possibility, in the present study, several endometrial blood vessel features were studied morphometrically at both light and electron microscopical levels. At the light microscope level, the volume density of blood vessel plus lumen to endometrium was significantly ($p < 0.05$) increased (from 0.039 ± 0.003 to 0.049 ± 0.003) after CC treatment. This result could indicate several possible reactions of blood vessels to drug treatment. First is an increase in blood vessel lumen volume, second is an increase in blood vessel tissue volume and/or there could be an increase in both. However, the volume densities of blood vessel lumen to endometrium and blood vessel tissue to endometrium were evaluated separately and there were no significant changes in either of these individual features after treatment with CC. Therefore, while the increase in each component was small, combined the overall effect was significant. This may have been due to either an increase in number of the endometrial blood vessels following CC treatment or by hypertrophy of existing cells. In blood vessel lumen to endometrium feature, the coefficient of variation was considerably higher in the control group (82%) than the CC-treated group, being the most variable effect of the clomiphene's effect on endometrial structure and function.

The volume density of endothelial nucleus to cell was not changed by treatment. However, the volume fraction of endothelial cell nucleus occupied by euchromatin was significantly ($p < 0.05$) decreased after treatment with CC. Euchromatin-heterochromatin ratio is one way of evaluating cell function. Increased euchromatin amount is the indicator of the increased transcriptional rate of DNA in the nucleus and subsequent increased metabolic and secretory activity in the cytoplasm. In the present study,

reduced euchromatin amount in the endothelial cell nucleus may indicate an adverse effect of CC on endometrial blood vessels. However, further investigation of the endothelial cell morphology disagrees with this possibility. There was no significant difference in the volume fraction of mitochondria to cell, and similar to stromal cells, the coefficient of variation of this feature was much higher in the untreated group than the CC-treated group indicating the same unknown effect of the clomiphene citrate treatment on the metabolic activity of both cell types. Although the volume density of endothelial cell occupied by "secretory apparatus" was numerically greater in the CC-treated group than the untreated group, the significance level was not within the conventional 5% limits, indicating only a tendency of an increase after treatment with clomiphene citrate. This result may indicate two possible interpretations; it is either an increase in the secretory activity of the cell, or more likely an increase in transportation of substances between blood and stromal compartment. The volume fraction of endothelial cell occupied by RER again was not significantly different between groups but there was a tendency of a reduction after CC treatment with the probability level of 10 percent. It could be suggested that the tendency of this reduction in the volume density of RER may be the reflection of the decrease in volume density of euchromatin to endothelial cell nucleus. However, neither of these results were significant and so such comment remains speculative.

Although a very small number of studies have dealt with the effect of CC on endometrial stroma, there was not a single study published on the potentially important effects of CC on endometrial blood vessel and endothelial cell morphology.

Eicosanoids, including prostaglandins, leukotrienes and related substances, are potent biologic mediators which are produced by a variety of tissues including those in the endometrium and decidua (Boura and Walters 1991). These substances act as local hormones with a major influence on vasomotor tone, as mediators of vasoconstriction, vasodilatation and vessel permeability (Shaw *et al.* 1994). Eicosanoid production by the endometrium may be important in the regulation of the menstrual cycle and possibly conception and implantation (Friedman 1988). In recent years, the abnormal production and action of these substances have been implicated in the pathophysiology of a number of disorders complicating pregnancy, including preterm labour and pregnancy loss

(Mitchell *et al.* 1990; Bennet *et al.* 1987). Prostaglandin (PG) dehydrogenase inactivates PGs by oxidising the 15-hydroxyl group and thus plays a key role in controlling their effective local concentrations (Kelly *et al.* 1994). This enzyme is thought to be under the control of steroid hormones and is found in human endometrium at high levels in the secretory phase of the menstrual cycle, but is absent during the proliferative phase (Downie *et al.* 1974). It has been shown that the amount of PG dehydrogenase is increased during the secretory phase of the menstrual cycle after treatment with clomiphene citrate (Kelly *et al.* 1994). It could be speculated that in the present study, slightly changed endometrial blood vessel morphology after CC treatment may be related to prostaglandin inactivation by CC in endometrium which plays an important role in the permeability of the vessels and has both vasoconstrictor and vasodilator functions. However, this aspect of the human endometrium needs more consideration. As is seen in the present study, function and morphology of the endometrial compartments including epithelium, stroma and blood vessels are closely related to each other and must be considered as a complete structure when examining the endometrium and the effects of drug treatment on it.

In summary, there were no significant differences in any of the glandular epithelial features evaluated in the present study. Stromal cell nuclear profile values and none of the volume fraction values differed between groups. On the other hand, the coefficients of variation in some features such as “secretory apparatus to cell” and stromal nucleus to cell were greater in CC-treated group than the untreated group. In addition, interestingly the coefficient of variation in the volume fraction of stromal cell occupied by mitochondria was higher in the untreated group than the CC-treated group, indicating some unknown effect of anti-oestrogen on the metabolic activity of the stromal cells. The volume density of blood vessels to endometrium was increased following the treatment and there were no change in the volume fraction of endometrium occupied by blood vessel lumen and blood vessel tissue, suggesting that the increase in total volume of the blood vessels might be the result of either an increase in number of blood vessels or an increase in existing endothelial cell size after CC treatment. In addition there was a tendency of an increase in the volume fraction of “secretory apparatus to cell”, indicating an increased secretory function of the endothelial cells and/or an increase in

transportation between blood and stromal compartment. Furthermore, there was a tendency of the volume fraction of RER to cell to decrease, being consistent with the reduction in the volume density of euchromatin to endothelial cell nucleus. Similar to the volume density of mitochondria to cell in stromal cells, the coefficient of variation of this feature in endothelial cells again was much higher in the untreated group than the CC-treated group, showing the same unknown effect of the anti-oestrogen on the metabolic activity of both cell types.

It could be concluded that the CC administration had no adverse effect on glandular epithelial and stromal morphology of the normally ovulating fertile human endometrium. Therefore, blood vessels seemed to be slightly affected by treatment with clomiphene citrate.

CHAPTER 4

EFFECTS OF GROWTH HORMONE ON HUMAN ENDOMETRIAL STROMA AROUND THE TIME OF IMPLANTATION

4.1. INTRODUCTION

For many years, growth hormone (GH) has been used to treat children with hypopituitarism and short stature. More recently, it has also been used to treat patients with Turner's syndrome to improve the final height achieved (Wilton 1987; Massa *et al.* 1993; Naeraa *et al.* 1994). In addition, GH has been used to augment the response of the human ovary to stimulation by human menopausal gonadotrophins (Homburg *et al.* 1988; 1990). A growing number of reports clearly indicate the possible involvement of GH in the regulation of ovarian follicle growth and development in mammals (Jia *et al.* 1986; Manson *et al.* 1990; Ando *et al.* 1994). It is likely that GH will be used more often in the future for the treatment of infertility, however relatively little is known about exactly how it works and whether or not it has effects on reproductive organs such as the endometrium as well as the ovary.

It is the aim of the present study to investigate the effects of GH supplementation on human endometrial stromal and vascular morphologies in two groups of patients undergoing hormone replacement therapy (HRT); the 2 groups are patients with Turner's syndrome and patients with idiopathic premature ovarian failure (POF). The results from the infertile groups (Turner's syndrome and idiopathic POF compared both separately and as a combined HRT group) will also be compared to the results from a group of well-characterized normal fertile individuals in order to provide a better understanding of the effects of GH on endometrial structure and function. In addition to the stromal and vascular evaluations, some glandular features will also be examined for a more complete picture of the endometrial morphology during GH treatment and hormone replacement therapy.

4.1.1. PREMATURE OVARIAN FAILURE (POF)

Premature ovarian failure (POF) is defined as the cessation of menses as a result of failing gonads, indicated by elevated gonadotrophins before the age of 40 years (de Morales-Ruehsen and Jones 1967; La Barbera *et al.* 1988) and affects about 1% of women (Coulam *et al.* 1986). The absence of follicles differentiates POF from the potentially treatable disorder (in terms of restoring fertility) termed the resistant ovary syndrome (Aiman and Smentek 1985; Mehta *et al.* 1992). POF may occur as a result of

an autoimmune destruction of ovarian tissue analogous to the destruction of the thyroid in chronic thyroiditis. In this type of POF, sensitised lymphocytes invade the gonad and destroy ovarian tissue with consequent failure of hormone production (La Barbera *et al.* 1988). The presence of associated autoimmune disorders in patients with POF suggests that up to 40% may have their ovarian failure mediated by an immune mechanism (Coulam 1983; Alper and Garner 1985). More commonly the ovaries may fail prematurely without evidence of infiltration by lymphocytes or circulating ovarian antibodies, and the cause of this premature ovarian failure is unknown and it is called idiopathic POF. In these disorders, the ovary is unable to respond to appropriate gonadotrophin stimulation. POF can be differentiated from other causes of anovulation as the serum and urinary levels of FSH will be raised. Ovarian disorders may also be responsible for the failure of ovulation by producing abnormal quantities of hormone which disrupt the hypothalamic control of gonadotrophins (Philipp and Carruthers 1981).

4.1.2. TURNER'S SYNDROME

Two normal X chromosomes are necessary for the maturation of female germ cells. If one X chromosome is absent or deficient, ovarian follicles do not develop. In this case the gonad is represented by a ridge on the broad ligament in the normal position of the ovary, and it is composed of connective tissue without follicles (Philipp and Carruthers 1981). The XO gonad has follicles at birth though reduced to only 20% of the normal quantity. In the syndrome described by Turner (1938), these features of ovarian dysfunction were associated with shortness of stature, webbing of the neck and cubitus valgus. Although development of the ovary is influenced to a varying degree in different individuals, most patients with this syndrome are of short stature and rarely reach a height of more than 5 ft (Lippe 1991). Most women with this condition have only 45, instead of the normal 46, chromosomes and this is due to non-disjunction of the sex chromosome occurring during meiosis and consequently their sex chromosome constitution is XO instead of XX. These patients are chromatin negative on nuclear sexing, because the chromatin body of the cell is due to the second X chromosome normally present in the female cell. However, one-quarter of these patients have 46 chromosomes and so are chromatin positive. These patients with Turner's syndrome

have the normal number of chromosomes but one of the X chromosomes is structurally abnormal. The other cause of the Turner's syndrome with a normal number of chromosomes is the result of what is called mosaicism. In this condition some of the cells have 46 chromosomes and others have 45 and this is due to non-disjunction occurring after fertilization during one of the early divisions of the zygote. Such patients may have normal stature and some secondary sex development due to the presence of the XX cell line, and may even appear quite normal.

4.1.3. GROWTH HORMONE: ITS RELEVANCE TO THE FEMALE REPRODUCTIVE SYSTEM

Growth hormone (GH) is the most abundant hormonal element in the human pituitary gland and its role in early growth is well-documented (Wilton 1987; Massa *et al.* 1993; Naera *et al.* 1994). Growth hormone is a small protein which, in human, consists of 191 amino acids with the molecular weight of 27,000 and it has two disulphide bridges (Lee and Laycock 1978). It is estimated that as much as 5 to 10 mg of GH is present in adult pituitary somatotrophic cells (Daughaday 1985). About 35% to 45% percent of the total cellular mass of the adult pituitary gland is made of the somatotrophic cells (Daughaday 1985). Despite this apparent abundance, the precise role of growth hormone in adult life remains uncertain and while it markedly increases during puberty, it declines progressively thereafter (Daughaday 1985; Katz *et al.* 1993).

GH may enhance gonadotropin action, synergizing in the promotion of ovarian function. It may be viewed as a co-gonadotropin, capable of enhancing gonadotropin hormonal action but possibly incapable of acting by itself (Katz *et al.* 1993). It is also possible that GH may affect ovarian function via the hypothalamic-pituitary axis.

4.1.3.1. THE OVARY AS A SITE OF GH-RELEASING HORMONE (GHRH) PRODUCTION, RECEPTION AND ACTION

The presence of immunostainable growth hormone releasing hormone (GHRH) positive cells in human ovarian tissue was recently demonstrated (Moretti *et al.* 1990a) using a highly specific anti - GHRH serum. Specifically, GHRH immunoreactivity is localized in the corpora lutea of normally cycling women. Particularly strong and

specific staining is evident in theca-derived cells of the corpora lutea (Moretti *et al.* 1989). Human GHRH reactivity is also detectable in oocytes (Moretti *et al.* 1990b). The above mentioned findings suggest the existence of GHRH or a GHRH-related protein in both the somatic (thecal) and germ cell (oocyte) components of the ovary. Although GHRH constitutes a central component of the somatotrophic hypothalamic-pituitary axis, it is also possible that ovarian GHRH may play intraovarian roles distinct from its GH releasing property regardless of the absence or presence of ovarian GH-secreting cells (Katz *et al.* 1993). This possibility is supported by recent observations where GHRH has been shown to augment FSH-supported cAMP accumulation by cultured rat granulosa cells (Moretti *et al.* 1990b). However, it must be considered that the modulatory action of GHRH at the level of the ovary is FSH-dependent and that locally derived GHRH may synergize with FSH in the promotion of follicular development (Katz *et al.* 1993).

4.1.3.2. THE OVARY AS A SITE OF GH PRODUCTION, RECEPTION AND ACTION

Immunoreactive human GH has been found in human ovaries both by radioreceptor and radio-immunoassays (Kaganowicz *et al.* 1992). The first demonstration of the apparent dependence of rat ovarian function on GH were Advis *et al.* (1981) whose observations revealed that the *in vitro* ovarian progesterone response to both hCG and human FSH was distinctly increased by *in vivo* GH pre-treatment. Moreover, concomitant GH treatment increased the FSH-inducible granulosa cell LH/hCG receptor binding capacity by enhancing the action of low doses of FSH (Jia *et al.* 1986). The gonadotropic property of GH was investigated in rats by Jorgensen *et al.* (1991). They examined the influence of systemic treatment with human growth hormone on the length of oestrus cycle and the number of implantation sites and corpora lutea in the rat. A significant increase in the length of oestrus cycle occurred when compared to the placebo-treated controls. In addition, the number of implantation sites and corpora lutea was significantly higher in GH-treated rats (Jorgensen *et al.* 1991).

Taken together, the published information strongly suggest that the ovary is in fact a site of GH reception and action. However, additional formal demonstration of ovarian

GH receptor gene expression and cellular localization remains to be shown. Although the exact role of GH in ovarian physiology remains to be determined, it may well act as a co-gonadotropin, i.e., as an amplifier of gonadotropin hormonal action.

4.1.4. INSULIN-LIKE GROWTH FACTOR (IGF) SYSTEM

Recently, the significance of intraovarian regulators has become increasingly recognized and much of the attention has focused on IGFs. A large number of evidence suggests the existence of an intraovarian IGF system complete with ligands, receptors and binding proteins (Adashi *et al.* 1985). IGF-I and IGF-II show structural homology to pro-insulin and have effects on cell proliferation, growth, differentiation and metabolism (Daughaday and Rotwein 1989). IGFs are bound to specific insulin-like growth factor binding proteins (IGFBPs) which play an important role in IGF action. Six different IGFBPs have been identified, cloned and sequenced (Shimasaki and Ling 1991). IGFBPs regulate the transport and presentation of IGFs to their receptors and modulate the biological actions of IGFs. IGFs have been shown to exert a variety of effects at the level of murine (Davoren *et al.* 1985; Adashi *et al.* 1986), porcine (Veldhuis *et al.* 1986; Maruo *et al.* 1988) and human (Erickson *et al.* 1989, 1990) somatic ovarian cells. Their most important role is to synergize with pituitary gonadotropins and to amplify their impact (Katz *et al.* 1993). Immunoreactive IGF-I and IGF-II gene expressions have been clearly demonstrated at the level of ovarian granulosa cells in several species (Murphy *et al.* 1987; Hsu and Hammond 1987). It has also been shown that the ovary is not only a site of IGF production but that there exist high-affinity, low-capacity type-I IGF binding sites in murine (Adashi *et al.* 1988), porcine (Baranao and Hammond 1984), ovine (Monget *et al.* 1989) and human (Gates *et al.* 1987) ovarian granulosa cells.

The action of GH at the ovarian level may potentially involve two separate pathways. On the one hand, GH may interact with GH receptors, resulting a signal transduction directly modulating gonadotropin hormone action. Alternatively, GH may be acting (via its receptor) indirectly by stimulating ovarian IGF gene expression, which could result in the augmentation of the gonadotropin-supported growth and differentiation of somatic ovarian cells (Katz *et al.* 1993).

The endometrium also synthesizes both IGF-I and IGF-II (Seppala *et al.* 1994). Type-I and type-II IGF receptors have been demonstrated in tissues, including the endometrium and placenta (Marshall *et al.* 1974; Rutanen *et al.* 1988). In rat uterus, oestrogen stimulates both IGF-I and IGF-II as well as expression of type-I IGF receptor mRNA, indicating that IGFs mediate the proliferative action of the oestrogen (Murphy and Ghahary 1990). In human endometrium, IGF-I mRNA is abundant in the late proliferative phase and IGF-II mRNA in the early proliferative phase (Boehm *et al.* 1990), or in the secretory phase (Giudice *et al.* 1991).

4.1.5. GH SECRETION DURING THE MENSTRUAL CYCLE

GH is released from the pituitary in a pulsatile fashion (Miller *et al.* 1982), the secretion pattern being fairly constant for each individual subject (Parker *et al.* 1969). Zadik *et al.* (1985) analyzed the menstrual pattern of GH release by 24-hour sampling and found no difference in the integrated concentrations of GH between the follicular and luteal phase of 23 normally cycling women studied once each phase of the cycle. More recently, Faria *et al.* (1991) performed a study of GH release in normal women during the menstrual cycle. Serum GH was obtained from each of 15 women every 10 minutes for 24 hours during the early follicular, the late follicular and the mid-luteal phase of the menstrual cycle. The mean integrated serum GH concentration in late follicular phase women was higher than that observed in early follicular phase counterparts. The mean integrated serum GH concentration was intermediate in mid-luteal phase but not significantly different from that observed in early and late follicular phase of the cycle. In addition, multiple regressions revealed a positive correlation between maximal GH pulse amplitude and oestrogen and a significant negative correlation between maximal GH pulse amplitude and progesterone (Faria *et al.* 1991). These observations clearly indicate that late follicular phase concentrations of oestrogen may enhance GH pulse amplitude rather than frequency. On the other hand, progesterone may diminish this oestrogen effect. It could also be suggested that pulsatile GH release throughout the menstrual cycle is primarily regulated by way of amplitude modulation.

4.1.6. ENHANCEMENT OF OVULATION BY GROWTH HORMONE

The view of enhancing the ovulation by GH is based on the observations previously performed on experimental animals and these observations indicate;

1- That IGF-I is capable of substantial amplification of gonadotropin hormonal action when examined *in vitro* at the level of either the granulosa or the theca-interstitial cell (Adashi *et al.* 1985).

2- That GH (in concert with oestrogens) may in fact be in position to upregulate the intraovarian generation of IGF-I (Hernandez *et al.* 1989).

It is the combined weight of these two observations that leads to the suggestion that GH may enhance the generation of intraovarian IGF-I and thus amplify gonadotropin hormonal action at the level of the ovary.

Growth hormone has been used in women with hypogonadotropic state and treatment with GH reduced the overall hMG requirement in those women (Homburg *et al.* 1988). In this study, GH significantly augmented the ovarian response to hMG in women undergoing ovulation induction even though all of them were previously hMG resistant. In a following report, again GH administration reduced the duration of treatment and the dose of hMG required (Homburg *et al.* 1990). Furthermore, no significant differences were found in the circulated levels of oestrogen on the day of hCG administration and, more importantly, three of sixteen patients conceived in the first study cycle (Homburg *et al.* 1990). A similar observation was indicated by Bergh *et al.* (1994) that the fertilization rate was increased in infertile patients who had received GH.

4.1.7. ACTIONS OF GROWTH HORMONE AND IGF SYSTEM IN OTHER PARTS OF THE REPRODUCTIVE TRACT

In addition to the ovulation enhancing effects, growth hormone causes a significant increase in circulating serum levels of IGF-I (Bergh *et al.* 1994; Tapanainen *et al.* 1991) and IGFBP-3 (Bergh *et al.* 1994). In trophoblastic cells and on placental membranes, IGFBP-1 inhibits the receptor binding and biological action of IGF-I (Pekonen *et al.* 1988; Ritvos *et al.* 1988). The degree of IGFBP-1 phosphorylation increases from early to late gestation; thus the affinity of IGFBP-1 to bind and inhibit IGF-I action is highest at the end of pregnancy (Koistinen *et al.* 1993). The decidual cells secrete IGFBP-1

(Seppala *et al.* 1994). Because the trophoblast possesses IGF receptors and expresses IGF-II mRNA but not IGFBP-1 mRNA (Pekonen *et al.* 1988), it is possible that IGFBP-1 produced by the stromal cells has an influence on embryonic cells. IGF-I and IGF-II mRNAs have been found in human embryos (Bondy *et al.* 1990). The presence of both the receptor and the binding protein with equal binding affinities for IGFs in adjacent cells indicates a paracrine interaction between trophoblastic and decidual cells (Seppala *et al.* 1994).

The administration of GH may optimize ovulation induction regimens to increase number of oocytes, improve the quality of oocytes, and most importantly it may improve conception rates. However, it is still not clear yet whether GH administration has effects on the other parts of the reproductive tract especially on structure and function of the endometrium which plays a crucial role for implantation and the maintenance of pregnancy. It is the aim the present study to evaluate the effects of GH treatment on the morphology of the endometrium in women with hypopituitary conditions during HRT cycles.

4.2. MATERIALS AND METHODS

4.2.1. SUBJECTS

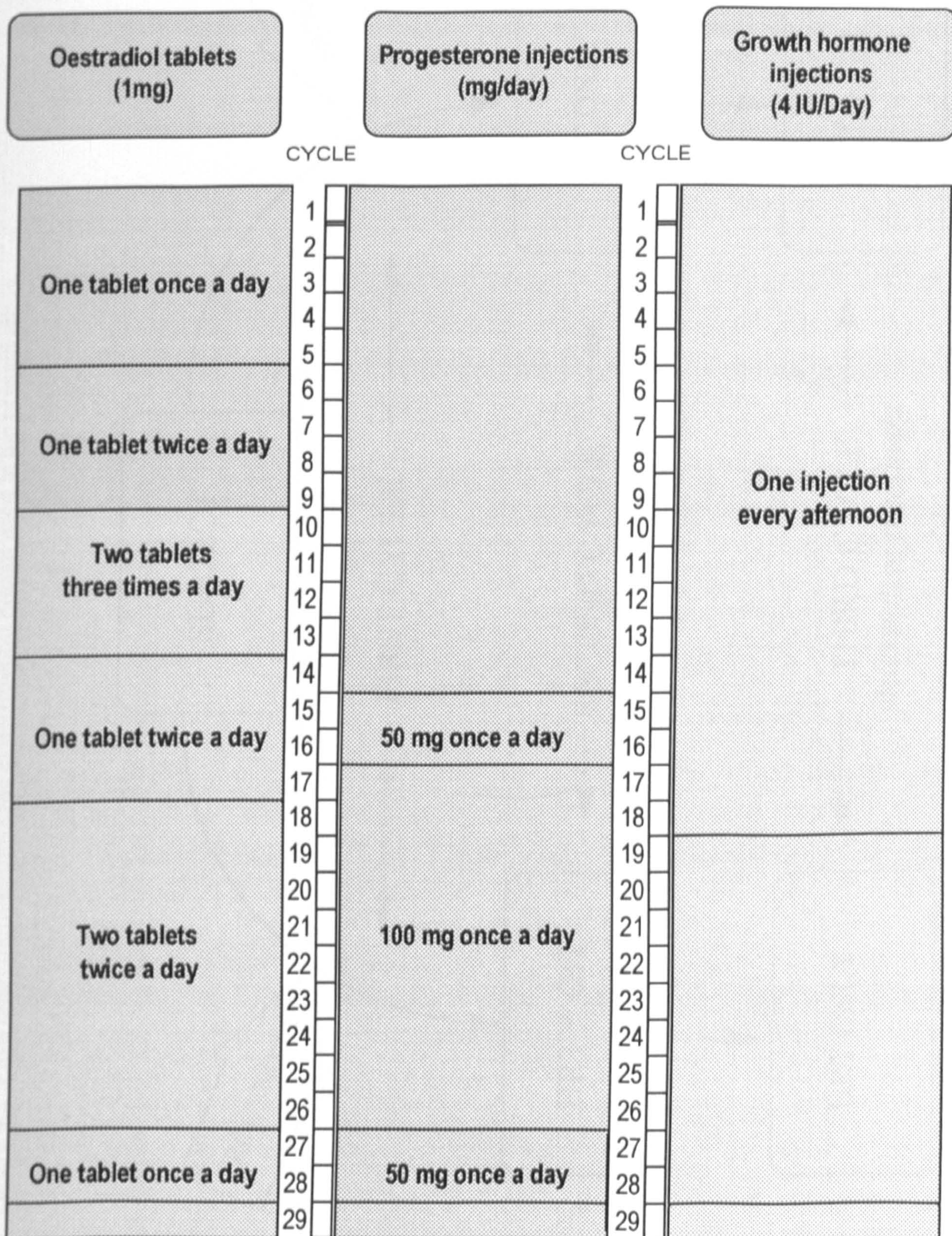
Six infertile subjects were used in the present study; 3 patients with Turner's syndrome and 3 patients with idiopathic premature ovarian failure (POF). All patients were treated on an outpatient basis. Within one month prior to entry into the study, the patients underwent a medical examination to ensure their eligibility for inclusion into the trial. If no contra-indications to participation were found, patients were allocated a study number and commenced a prestudy cycle. Since the patients had no luteinizing hormone production, dating of the endometrial biopsy material in the present study was done from the onset of the next menstrual period (NMP). All experiments performed in the present study had Ethics' Committee approval and informed patient consent. These patients were studied over several years under the treatment of Mr T. C. Li of Jessop Hospital for Women.

4.2.2. EXPERIMENTAL DESIGN

This was an open, random order, controlled, cross - over study designed to determine the effect of growth hormone (Saizen, Serono, U.K.) on the endometrial response to steroids in three patients with idiopathic POF and three patients with Turner's Syndrome.

Following the prestudy medical examination, patients underwent a prestudy cycle of Cyclo-Progynova. Since the patients did not have sex steroid secretion, treatment of Cyclo - Progynova, which is a combination of oestrogen and progesterone, was used to start patients' reproductive response. Seven days after completion of the prestudy cycle, patients began their first study cycle, consisting of a standard hormone replacement cycle (Navot et al. 1986) with or without growth hormone supplementation. All patients underwent two study cycles (each preceded by a cycle of Cyclo-Progynova). After the first treatment cycle, patients crossed - over to receive the alternative treatment (i.e. standard hormone replacement cycle with or without growth hormone supplementation). Figure 4.1 illustrates the treatment process of the present study. Since this study was organised on an out - patient basis, patients received their medication either at the hospital or at home, whatever was most convenient for the patient. If a patient wished to

Figure 4.1: Shows the standard hormone replacement (Navot *et al.* 1986) and growth hormone administration protocols. Each individual had either HRT+GH administration or only HRT during the first treatment cycle, then they were crossed-over and received the alternative treatment (HRT+GH or only HRT) in the second study cycle.



administer the injections herself, she was instructed accordingly and all patients were required to keep a record of medications taken. An endometrial biopsy was taken on day 19 of the each study cycle and was subjected to morphological analysis. The total length of the study for each patient was four months. See Figure 4.2 for the experimental design of the present study.

Biopsy procedure, tissue sampling, histological processing and staining for light and electron microscopy and morphometric methods used in the present study are the same as described in Chapter 2.

4.2.3. STATISTICAL ANALYSIS

Every feature was calculated on a 'per individual' basis. Means and standard errors were calculated for each group and data were analysed using a Student's unpaired *t*-test where the conventional level of 5% was taken as the level of significance. In addition to the comparisons between untreated and GH treated infertile groups, comparisons were also made between fertile day LH+6 and untreated and GH treated infertile groups. Figure 4.3 demonstrates sets of statistical analysis performed in the present study. Since the data from the normal fertile group have already been presented in Chapter 2, they are not repeated in this chapter.

Figure 4.3: Sets of statistical comparisons (Student's *t*-test) between groups performed in the present study.

- a) Untreated Turner's syndrome + idiopathic POF group (n=6) vs GH treated Turner's syndrome + idiopathic POF group (n=6)
- b) Untreated Turner's syndrome group (n=3) vs GH treated Turner's syndrome group (n=3)
- c) Untreated idiopathic POF group (n=3) vs GH treated idiopathic POF group (n=3)
- d) Untreated Turner's syndrome group (n=3) vs untreated idiopathic POF group (n=3)
- e) GH treated Turner's syndrome group (n=3) vs GH treated idiopathic POF group (n=3)
- f) Control group (day LH+6) (n=6) vs untreated Turner's syndrome + idiopathic POF group (n=6)
- g) Control group (n=6) vs GH treated Turner's syndrome + idiopathic POF group (n=6)
- h) Control group (n=6) vs untreated Turner's syndrome group (n=3)
- i) Control group (n=6) vs GH treated Turner's syndrome group (n=3)
- j) Control group (n=6) vs untreated idiopathic POF group (n=3)
- k) Control group (n=6) vs GH treated idiopathic POF group (n=3)

4.3. RESULTS

In the present study, the effects of growth hormone (GH) on human endometrial stromal and vascular morphologies were evaluated morphometrically in two groups of patients; those with Turner's syndrome and those with idiopathic premature ovarian failure (POF) during hormone replacement therapy (HRT) cycles. In addition, these treatment cycles were compared to the results from normal day LH+6 group in order to determine the differences between untreated and GH treated infertility conditions with normal fertile conditions. Day LH+6 is selected since it is the nearest available corresponding time to day 19 of the menstrual cycle. Since the results of day LH+6 group are already presented in Chapter 2, these results are not repeated in the following tables but where necessary, day LH+6 results will be referred to in the corresponding tables of Chapter 2.

4.3.1. GLANDULAR EPITHELIUM

There were no significant differences in any of the glandular features between untreated and GH treated Turner's syndrome and idiopathic POF groups (Tables 4.1; 4.2; 4.3). In addition, Turner's + idiopathic POF groups did not differ significantly before or after the GH treatment (Table 4.1). However, there were significant differences between day LH+6 and infertile groups indicating some abnormalities of the endometrial glandular features in Turner's syndrome and idiopathic POF patients (for comparison see Table 2.1). The volume fraction of gland occupied by gland cell was higher in untreated Turner's syndrome + idiopathic POF group than day LH+6 group, being 0.832 ± 0.021 in day LH+6 group and 0.885 ± 0.010 in Turner's + idiopathic POF group. However, the significance level was marginal in this feature suggesting only a tendency of an increase. On the other hand this increase in gland cell volume density became much higher after treatment with GH being 0.899 ± 0.009 in GH treated Turner's syndrome + idiopathic POF group. This increase was significantly ($p < 0.05$) different between groups. When the day LH+6 group was compared to the untreated Turner's syndrome group, the volume density of gland occupied by gland cell was significantly ($p < 0.02$) higher in untreated Turner's syndrome patients being 0.903 ± 0.012 in this group, suggesting a retardation in endometrial glandular epithelium

Table 4.1: Comparison of the endometrial glandular features between untreated and GH treated patients (both groups consist of Turner's syndrome and idiopathic POF individuals).

Features	Untreated Turner's Syndrome + Idiopathic POF (n=6)	GH Treated Turner's Syndrome + Idiopathic POF (n=6)
Vv of Gland Occupied by Gland Cell	0.885 ± 0.010	0.899 ± 0.009
Vv of Endometrium Occupied by Gland	0.261 ± 0.017	0.309 ± 0.021

Results are means ± SE

n= number of individuals

Table 4.2: Comparison of the glandular features between untreated and GH treated Turner's syndrome groups.

Features	Untreated Turner's Syndrome Group (n=3)	GH Treated Turner's Syndrome Group (n=3)
Vv of Gland Occupied by Gland Cell	0.903 ± 0.012	0.910 ± 0.006
Vv of Endometrium Occupied by Gland	0.268 ± 0.007	0.324 ± 0.032

Results are means ± SE

n= number of individuals

Table 4.3: Comparison of the glandular features between untreated and GH treated idiopathic POF groups.

Features	Untreated Idiopathic POF Group (n=3)	GH treated Idiopathic POF Group (n=3)
Vv of Gland Occupied by Gland Cell	0.866 ± 0.008	0.888 ± 0.017
Vv of Endometrium Occupied by Gland	0.254 ± 0.037	0.293 ± 0.030

Results are means ± SE

n= number of individuals

development. This difference in glandular cell volume density was much higher when treated with GH (0.910 ± 0.006 in GH treated Turner's syndrome group). The difference was highly significant ($p < 0.01$) between these groups, indicating some further effects of GH on endometrial glandular structure and function.

This feature was not significantly different between the day LH+6 group, and untreated idiopathic POF and GH treated idiopathic POF groups suggesting more normal endometrial glandular structure and function in idiopathic POF patients and no effects of GH on this feature. The increase in glandular cell volume density in the Turner's syndrome group may be the result of an increase in glandular cell glycogen content and qualitative observations of the present study showed more glycogen - like material in the subnuclear region of the glandular cells (Figure 4.4a,b).

The volume fraction of endometrium occupied by gland did not significantly differ between the untreated group and the GH treated Turner's syndrome + idiopathic POF group, between untreated Turner's syndrome and GH treated Turner's syndrome groups, nor between untreated idiopathic POF and GH treated idiopathic POF groups (Tables 4.1; 4.2; 4.3). When compared with day LH+6 group, the untreated Turner's + idiopathic POF group again showed no difference. However, this feature significantly ($p < 0.01$) differed between day LH+6 group and the GH treated Turner's syndrome + idiopathic POF group being 0.223 ± 0.017 in day LH+6 group and 0.309 ± 0.021 in GH treated Turner's syndrome + idiopathic POF group (see Table 2.1). This result was consistent with an increase in glandular cell volume density suggesting a possible delay in endometrial glands in the GH treated group.

Similarly, this feature was significantly ($p < 0.05$) different between the day LH+6 group and the untreated Turner's group (0.268 ± 0.007) whereas the difference between day LH+6 and GH treated Turner's was further increased ($p < 0.05$) to 0.324 ± 0.032 in the GH treated Turner's group. On the other hand, there was no significant difference in this feature between day LH+6, and untreated and GH treated idiopathic POF groups suggesting a better response to HRT and GH regimens from POF patients and no evidence for the retarded endometrial glandular epithelium. These results may also indicate that the significant differences found in the glandular epithelial features between

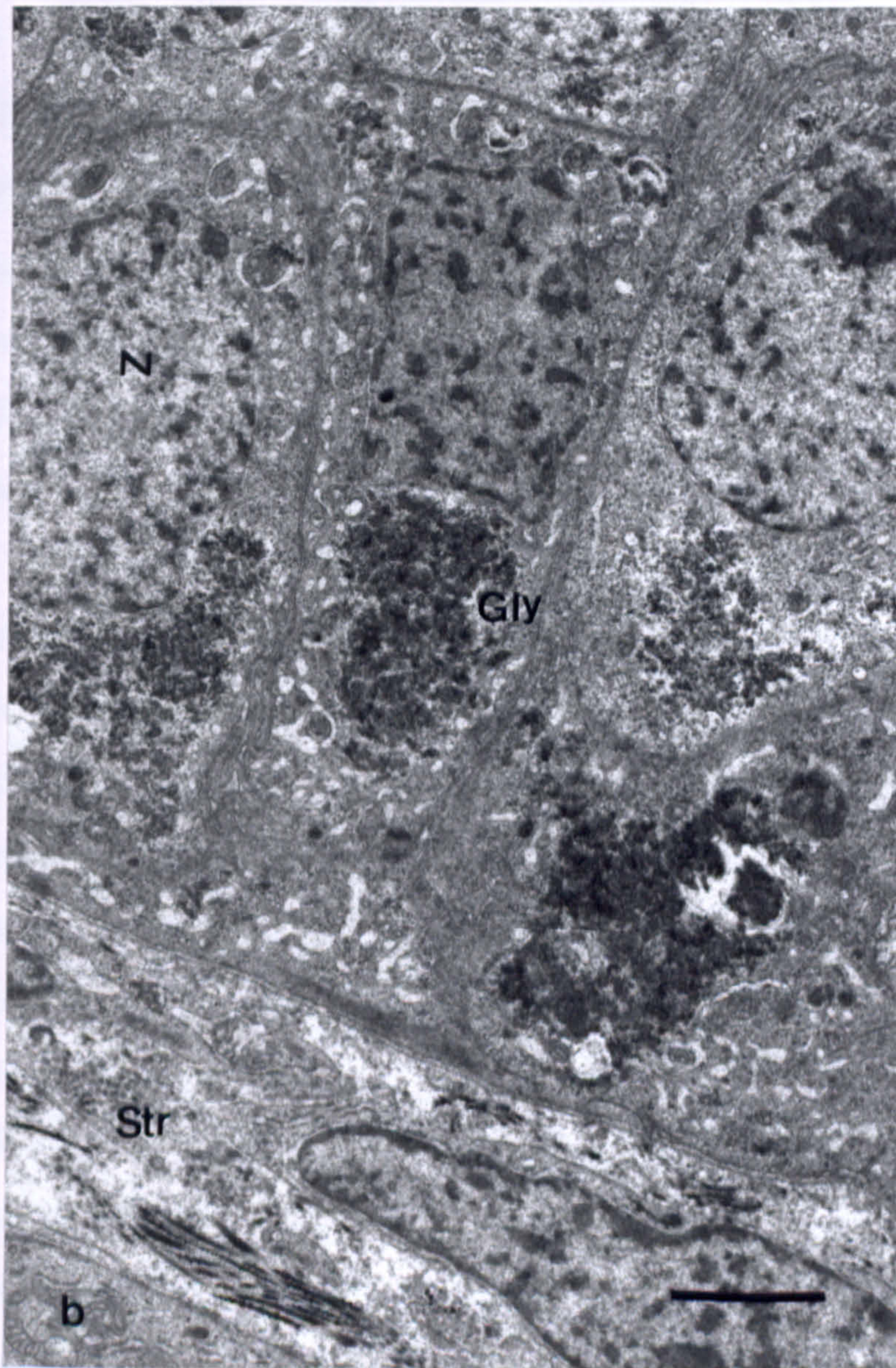
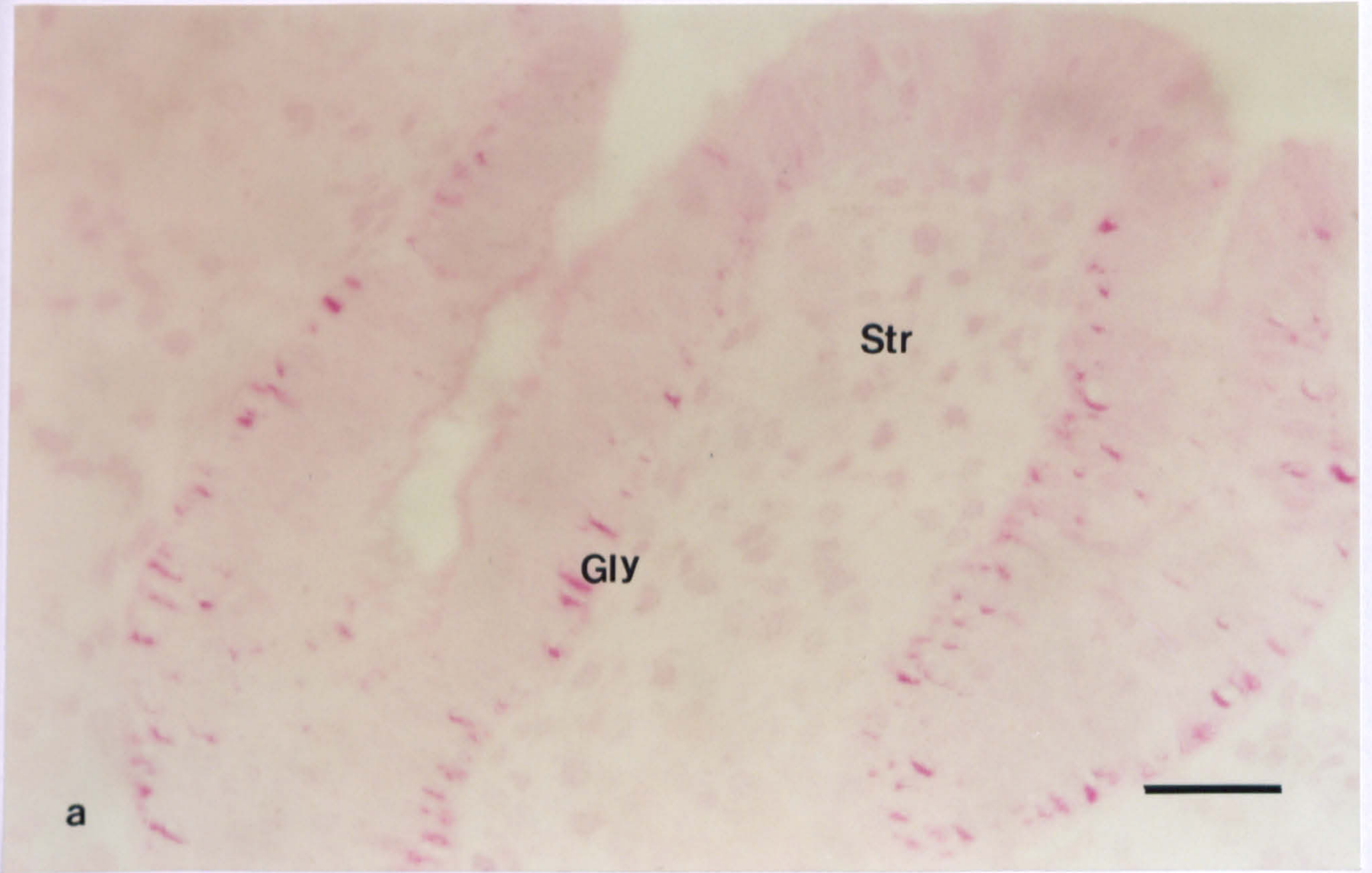
Figures 4.4a,b: Light (a) and electron microscopical (b) demonstration of glycogen (Gly) content in glandular epithelial cells of untreated idiopathic POF group at day 19 of the menstrual cycle. (N) nucleus, (Str) stroma.

Staining: (a) Periodic Acid - Schiff (PAS)

(b) Periodic Acid Thiosemicarbazide - Gelatine Methenamine Silver (PATSC - GMS).

(a) Bar represents 32 μm

(b) Bar represents 2.30 μm



day LH+6 and Turner's syndrome + idiopathic POF groups are likely to be due mainly to the Turner's syndrome group.

4.3.2. STROMA

The volume fraction of endometrium occupied by stroma did not differ between untreated and GH treated Turner's syndrome + idiopathic POF groups, untreated and GH treated Turner's syndrome groups and untreated and GH treated idiopathic POF groups (Tables 4.7; 4.8; 4.9). Moreover, the endometrial stromal volume density was not significantly different between untreated Turner's syndrome and idiopathic POF, and GH treated Turner's syndrome and idiopathic POF groups. While the volume fraction of stroma to endometrium did not differ between day LH+6 and untreated Turner's syndrome + idiopathic POF groups, it was significantly ($p < 0.01$) different between day LH+6 (0.777 ± 0.017) and GH treated Turner's syndrome + idiopathic POF groups (0.691 ± 0.021). In addition, the volume density of endometrial stroma was significantly ($p < 0.05$) different between day LH+6 and untreated Turner's syndrome groups (0.732 ± 0.007) and between day LH+6 and GH treated Turner's syndrome groups (0.676 ± 0.032). There were no significant differences between day LH+6 and untreated idiopathic POF, nor between day LH+6 and GH treated idiopathic POF groups (for comparison see Table 2.2 and Tables 4.7; 4.8; 4.9). These significant differences between the fertile day LH+6 group and infertile groups indicate an abnormal development of the endometrium in these type of the infertility cases. This decreased endometrial stromal volume density was consistent with the increased glandular volume fraction of glands in infertile groups possibly indicating a retarded endometrial development in these groups.

This decreased volume density of stromal compartment might cause an increase in packing density of the endometrial stromal cells. To evaluate this, the volume fraction of endometrium occupied by stromal cell nucleus were measured and there were some significant differences between groups. This feature was not significantly different between untreated Turner's + idiopathics and GH treated Turner's + idiopathics, between untreated Turner's and GH treated Turner's, and between untreated idiopathics and GH treated idiopathics. However, it was significantly ($p < 0.02$) different between

Table 4.4: Comparison of the stromal cell nuclear dimension data between untreated and GH treated groups of both Turner's syndrome and idiopathic POF patients.

Features	Untreated Turner's Syndrome + Idiopathic POF (n=6)	GH Treated Turner's Syndrome + Idiopathic POF (n=6)
Stromal Cell Nuclear Profile Major Axis (µm)	7.01 ± 0.19	6.94 ± 0.18
Stromal Cell Nuclear Profile Minor Axis (µm)	3.92 ± 0.14	3.83 ± 0.10
Stromal Cell Nuclear Profile Mean Diameter (µm)	5.16 ± 0.16	5.09 ± 0.12
Stromal Cell Nuclear Profile Axial Ratio	1.98 ± 0.07	1.99 ± 0.05

Results are means ± SE

n= number of individuals

Table 4.5: Comparison of the stromal cell nuclear dimension data between groups of untreated and GH treated Turner's syndrome patients.

Features	Untreated Turner's Syndrome Group (n=3)	GH Treated Turner's Syndrome Group (n=3)
Stromal Cell Nuclear Profile Major Axis (µm)	6.86 ± 0.35	6.81 ± 0.06
Stromal Cell Nuclear Profile Minor Axis (µm)	4.00 ± 0.28	3.81 ± 0.15
Stromal Cell Nuclear Profile Mean Diameter (µm)	5.17 ± 0.32	5.02 ± 0.11
Stromal Cell Nuclear Axial Ratio	1.87 ± 0.06	1.96 ± 0.10

Results are means ± SE

n= number of individuals

Table 4.6: Comparison of the stromal cell nuclear dimension data between groups of untreated and GH treated idiopathic POF patients.

Features	Untreated Idiopathic POF Group (n=3)	GH treated Idiopathic POF Group (n=3)
Stromal Cell Nuclear Profile Major Axis (µm)	7.16 ± 0.20	7.08 ± 0.38
Stromal Cell Nuclear Profile Minor Axis (µm)	3.83 ± 0.14	3.85 ± 0.15
Stromal Cell Nuclear Profile Mean Diameter (µm)	5.16 ± 0.14	5.16 ± 0.24
Stromal Cell Nuclear Axial Ratio	2.08 ± 0.10	2.02 ± 0.03

Results are means ± SE

n= number of individuals

Table 4.7: Comparison of the volume fraction data of the endometrial stromal features between untreated and GH treated groups of both Turner's syndrome and idiopathic POF patients.

Features	Untreated Turner's Syndrome + Idiopathic POF (n=6)	GH Treated Turner's Syndrome + Idiopathic POF (n=6)
Vv of Endometrium Occupied by Stroma	0.739 ± 0.017	0.691 ± 0.021
Vv of Endometrium Occupied by Stromal Cell Nucleus	0.119 ± 0.007	0.126 ± 0.007
Vv of Stromal Cell Occupied by Nucleus	0.446 ± 0.018	0.442 ± 0.018
Vv of Stromal Cell Nucleus Occupied by Euchromatin	0.811 ± 0.034	0.786 ± 0.027
Vv of Stromal cell Occupied by Mitochondria	0.027 ± 0.003	0.031 ± 0.003
Vv of Stromal Cell Occupied by "Secretory Apparatus"	0.029 ± 0.002	0.035 ± 0.004
Vv of Stromal Cell Occupied by RER	0.033 ± 0.007	0.035 ± 0.005

Results are means ± SE

n= number of individuals

Table 4.8: Comparison of the volume fraction data of the stromal features between groups of untreated and GH treated Turner's syndrome patients.

Features	Untreated Turner's Syndrome Group (n=3)	GH Treated Turner's Syndrome Group (n=3)
Vv of Endometrium Occupied by Stroma	0.732 ± 0.007	0.676 ± 0.032
Vv of Endometrium Occupied by Stromal Cell Nucleus	0.116 ± 0.008	†0.112 ± 0.002
Vv of Stromal Cell Occupied by Nucleus	0.472 ± 0.030	0.435 ± 0.019
Vv of Stromal Cell Nucleus Occupied by Euchromatin	0.828 ± 0.052	0.786 ± 0.049
Vv of Stromal Cell Occupied by Mitochondria	0.024 ± 0.001	0.031 ± 0.005
Vv of Stromal Cell Occupied by "Secretory Apparatus"	0.029 ± 0.003	0.038 ± 0.008
Vv of Stromal Cell Occupied by RER	0.024 ± 0.004	0.031 ± 0.009

Results are means ± SE

n= number of individuals

Table 4.9: Comparison of the volume fraction data of the stromal features between groups of untreated and GH treated idiopathic POF patients.

Features	Untreated Idiopathic POF Group (n=3)	GH Treated Idiopathic POF Group (n=3)
Vv of Endometrium Occupied by Stroma	0.746 ± 0.037	0.707 ± 0.030
Vv of Endometrium Occupied by Stromal Cell Nucleus	0.122 ± 0.013	†0.141 ± 0.007
Vv of Stromal Cell Occupied by Nucleus	0.419 ± 0.007	0.449 ± 0.035
Vv of Stromal Cell Nucleus Occupied by Euchromatin	0.795 ± 0.052	0.787 ± 0.036
Vv of Stromal cell Occupied by Mitochondria	0.029 ± 0.004	0.032 ± 0.004
Vv of Stromal Cell Occupied by “Secretory Apparatus”	0.029 ± 0.004	0.031 ± 0.005
Vv of Stromal Cell Occupied by RER	0.041 ± 0.038	0.038 ± 0.004

Results are means ± SE

n= number of individuals

† Student’s t - Test result (p<0.02) (GH treated Turner’s syndrome vs GH treated idiopathic POF)

GH treated Turner's syndrome (0.112 ± 0.002) and idiopathic POF groups (0.141 ± 0.007) indicating either an increase in packing density of stromal cells or an increase in stromal cell nuclear dimensions after treatment with GH. The volume density of stromal cell nucleus was numerically higher in untreated Turner's syndrome + idiopathic POF group than day LH+6 group, however, the significance level was marginal when the results were compared statistically. On the other hand, this feature was significantly ($p < 0.05$) higher in GH treated Turner's syndrome + idiopathic group being 0.126 ± 0.007 in GH treated Turner's + idiopathics and 0.092 ± 0.010 in day LH+6 group. In addition, the volume fraction of endometrium occupied by stromal cell nucleus was significantly ($p < 0.01$) different between day LH+6 (0.092 ± 0.010) and GH treated idiopathic group (0.141 ± 0.007) whereas day LH+6 and untreated idiopathics did not differ significantly (Tables 4.7; 4.8; 4.9 and Table 2.2).

There were no significant differences between any of the untreated and GH treated Turner's syndrome and idiopathic POF groups in the stromal cell nuclear profile dimension features (Tables 4.4; 4.5; 4.6). Furthermore, there were no significant differences between day LH+6 and any of the untreated and GH treated groups in major profile axis, minor profile axis, profile mean diameter and axial ratio of the stromal cell nucleus (for comparison see Table 2.3 and Tables 4.4; 4.5; 4.6).

The volume fraction of stromal cell occupied by nucleus did not differ significantly between any of the untreated and GH treated infertile groups (Tables 4.7; 4.8; 4.9) (Figures 4.5a,b and Figures 4.6a,b). Further comparisons of this feature between day LH+6 and untreated and GH treated Turner's syndrome + idiopathic POF groups also showed no significant differences. When the volume density of stromal cell nucleus was compared separately between cycle day LH+6 and untreated and GH treated infertile groups, the only significant ($p < 0.05$) difference was found between day LH+6 (0.454 ± 0.013) and untreated idiopathic POF groups (0.419 ± 0.007) indicating either a decreased stromal cell nuclear size or an increased stromal cell cytoplasmic volume in untreated idiopathic POF individuals (Table 2.2 and Table 4.9). The volume density of stromal cell nucleus occupied by euchromatin did not differ significantly between any of the infertile groups, nor between day LH+6 fertile and untreated and GH treated infertile groups (for comparison see Tables 4.7; 4.8; 4.9 and Table 2.2).

Figure 4.5a: Endometrial stromal cells of untreated Turner's syndrome group at day 19 of the cycle. (N) nucleus

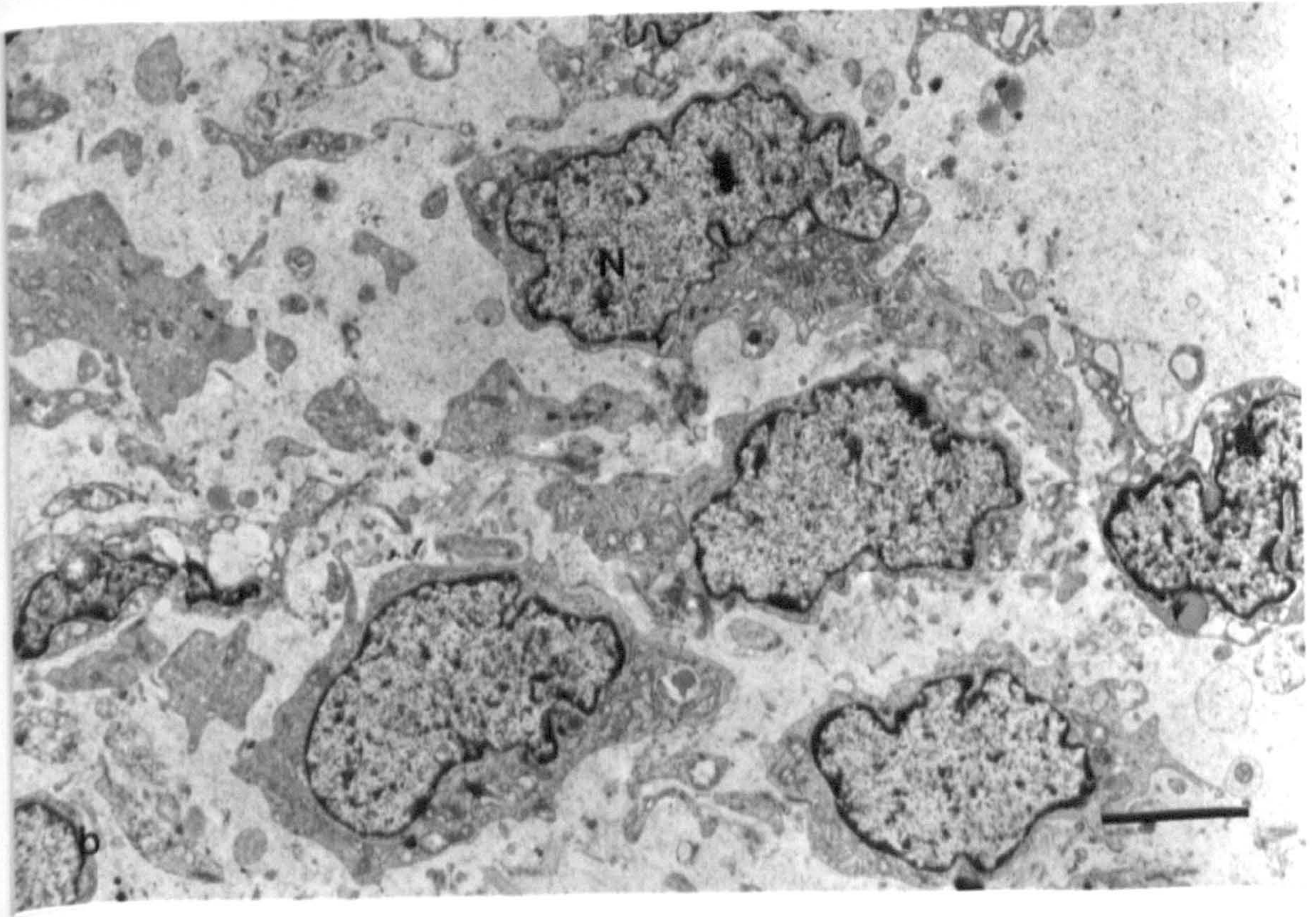
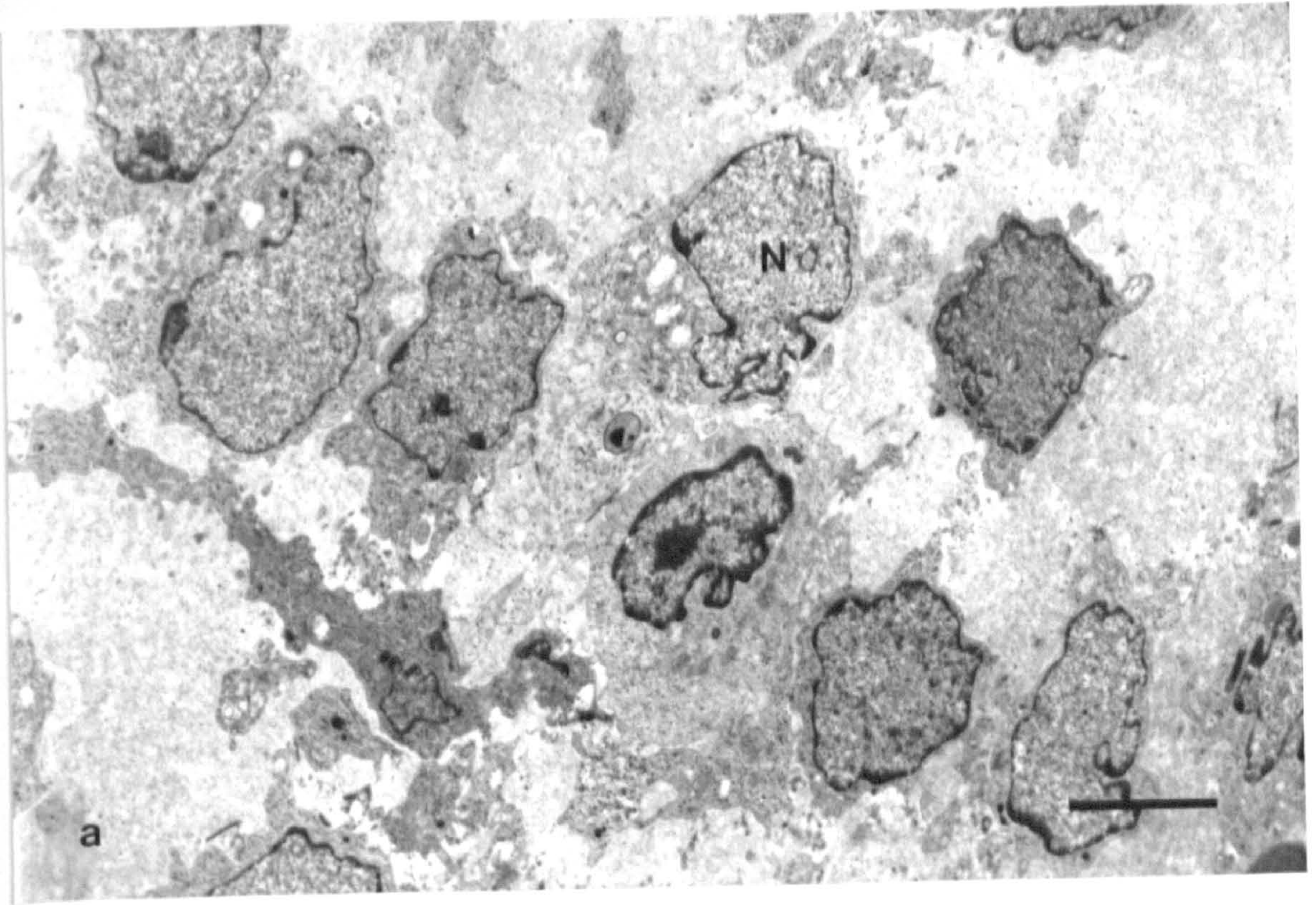
Staining: Uranyl acetate - Lead citrate

Bar represents 3.80 μm

Figure 4.5b: Stromal cells of GH treated Turner's syndrome group at day 19 of the menstrual cycle. (N) nucleus

Staining: Uranyl acetate - Lead citrate

Bar represents 4.60 μm

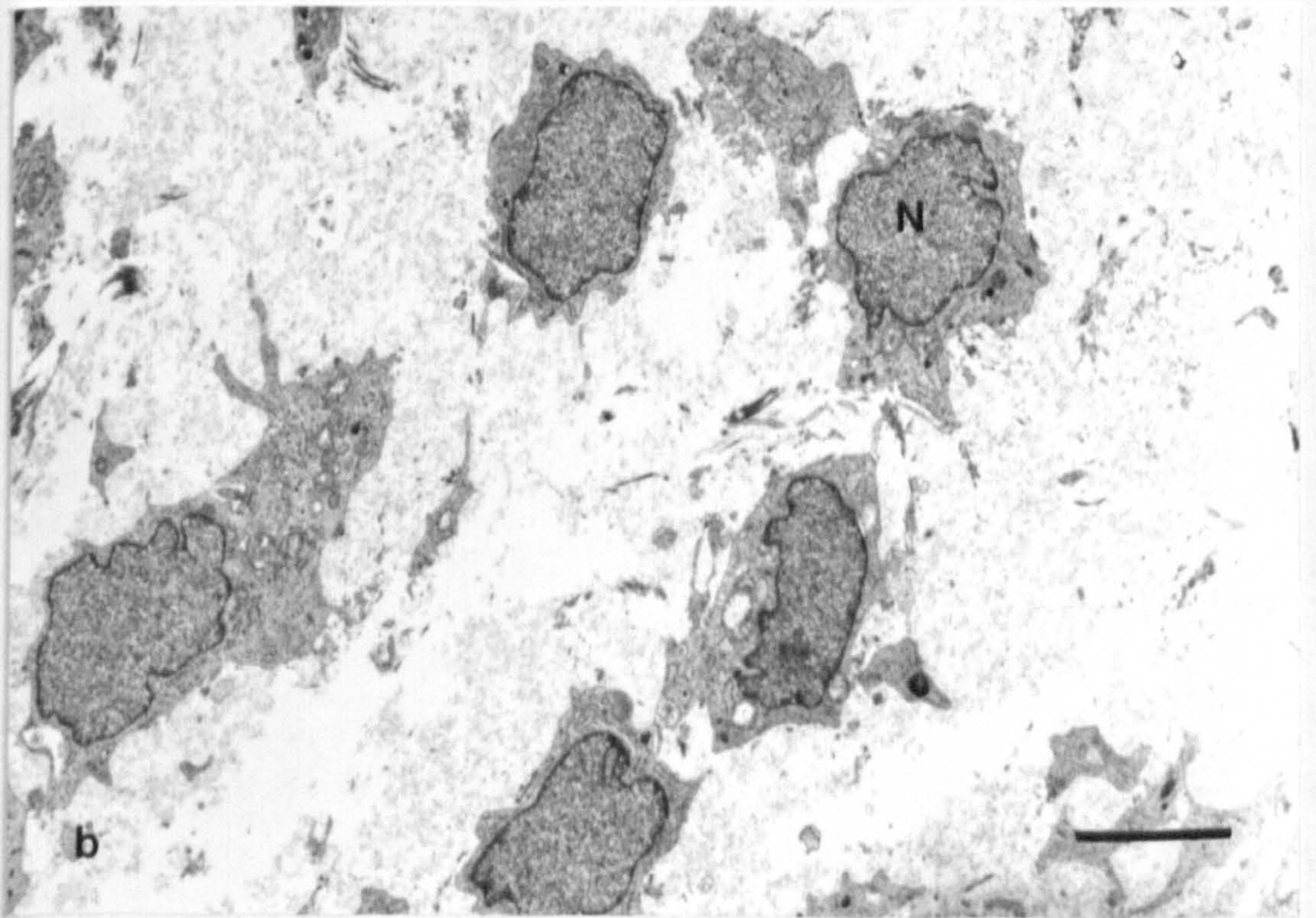
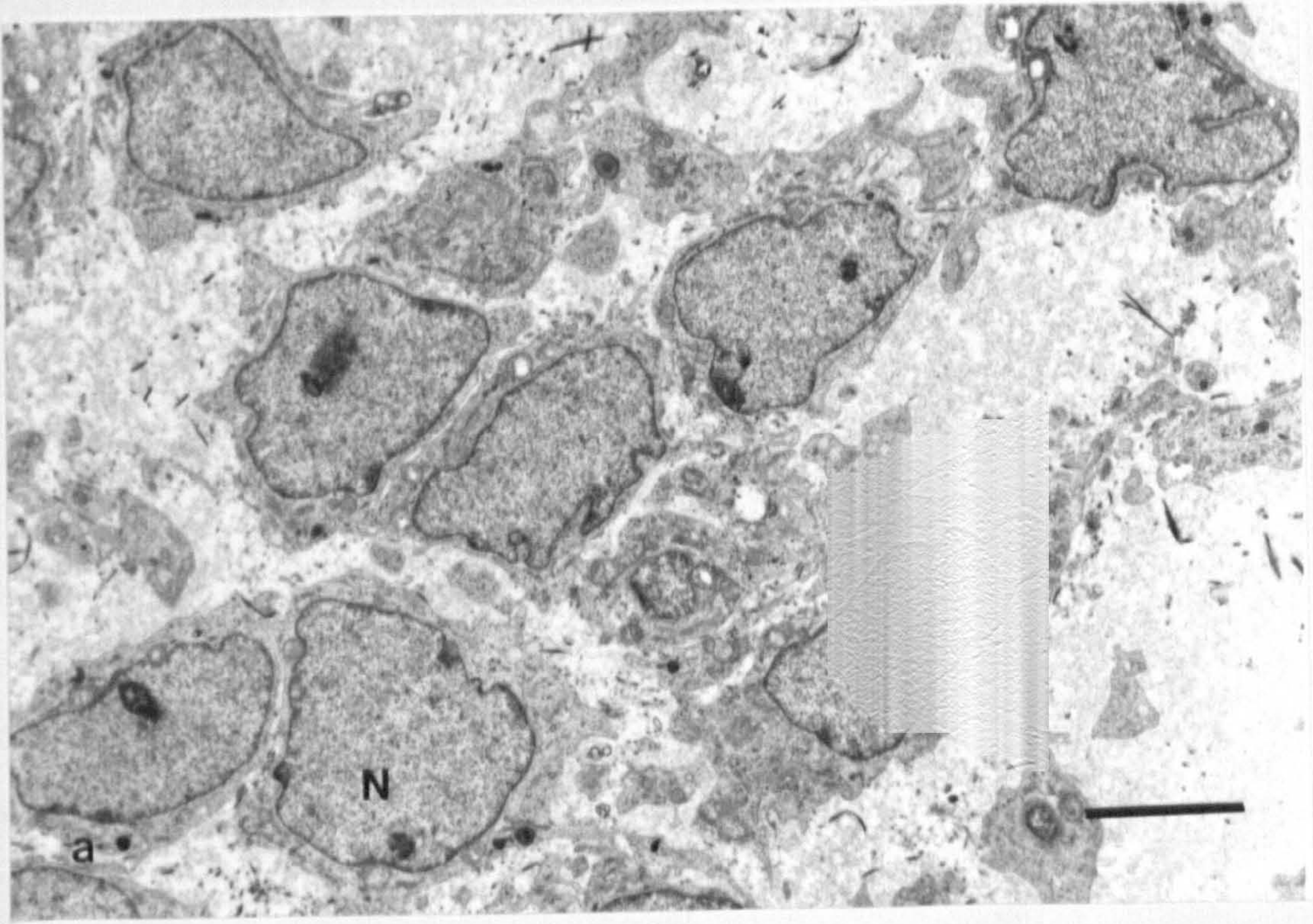


Figures 4.6a,b: Endometrial stromal cells of untreated (a) and GH-treated (b) idiopathic POF group at day 19 of the menstrual cycle. Untreated group cells seem to have larger nuclei (N) than the GH-treated group cells, however quantitative evaluation showed no significant difference between groups.

Staining: Uranyl Acetate - Lead Citrate

(a) Bar represents 4.10 μm

(b) Bar represents 4.00 μm



The volume fraction of stromal cell occupied by mitochondria was not significantly different between any of the infertile groups, nor between day LH+6 and untreated and GH treated Turner's syndrome + idiopathic POF groups. The only significant ($p < 0.05$) difference in the volume density of mitochondria to cell was found between day LH+6 (0.036 ± 0.004) and untreated Turner's syndrome groups (0.024 ± 0.001). The mitochondrial volume density in stromal cells was significantly lower in the untreated Turner's syndrome group suggesting a lower metabolic demand in stromal cells in this particular type of infertility. However, the volume fraction of stromal cell occupied by mitochondria was not significantly different between cycle day LH+6 and untreated and GH treated idiopathic POF groups, possibly indicating a better response from these individuals to HRT and GH treatment (Table 2.2 and Tables 4.7; 4.8; 4.9).

Similar to the mitochondrial volume density data, the volume fraction of "secretory apparatus" to stromal cell was not significantly different between any of the untreated and GH treated infertile groups. However, this feature differed significantly when the cycle day LH+6 group were compared to untreated and GH treated infertile groups (for comparison see Table 2.2 and Tables 4.7; 4.8; 4.9) suggesting large deviations from the normal secretory activity in Turner's syndrome and idiopathic POF individuals. The difference in the volume fraction of stromal cell occupied by "secretory apparatus" was highly significant ($p = 0.0002$) between day LH+6 (0.054 ± 0.004) and untreated Turner's syndrome + idiopathic POF groups (0.029 ± 0.002).

This difference between the same groups in the same feature remained ($p < 0.01$) after treatment with GH, being 0.035 ± 0.004 in the GH treated Turner's syndrome + idiopathic POF group. When the volume density of "secretory apparatus" was compared separately between day LH+6, Turner's syndrome and idiopathic POF groups, they were generally significantly ($p < 0.01$) different with or without growth hormone treatment, except in the comparison of GH treated Turner's syndrome group with cycle day LH+6.

Similar to other organelle features, the volume fraction of stromal cell occupied by RER did not differ between any of the infertile groups. In addition, no significant differences were found between day LH+6 and untreated and GH treated infertile groups, apart from the untreated idiopathics versus day LH+6 comparison. There was a

tendency ($p < 0.1$) of a higher RER to stromal cell volume density in the untreated idiopathic POF group (0.041 ± 0.004) than the cycle day LH+6 group, (0.033 ± 0.004) possibly suggesting a higher synthetic activity in this group.

4.3.3. BLOOD VESSELS

At the light microscope level, the volume fraction of endometrial blood vessel features did not significantly differ between any of the untreated and GH treated infertile groups (Tables 4.10; 4.11; 4.12). While there were no significant differences in the volume fraction of endometrium occupied by blood vessel lumen between cycle day LH+6 and untreated Turner's syndrome + idiopathic POF groups, the volume density of blood vessel tissue significantly ($p < 0.005$) differed between the same groups being 0.028 ± 0.002 in the day LH+6 group and 0.017 ± 0.002 in the untreated Turner's syndrome + idiopathic POF group. Furthermore, the volume fraction of endometrium occupied by total blood vessel (vessels + tissue) significantly ($p < 0.05$) differed between these groups (0.039 ± 0.003 in day LH+6 group and 0.025 ± 0.004 in untreated Turner's + idiopathics). The volume fraction of blood vessel lumen was not significantly different between day LH+6 and GH treated Turner's + idiopathic POF groups. However, the volume density of total blood vessel differed significantly ($p < 0.05$) between the same groups being 0.026 ± 0.005 in GH treated Turner's + idiopathic POF group (for comparison see Table 2.4 and Table 4.10). There was a significant ($p < 0.02$) difference in the volume fraction of endometrium occupied by blood vessel tissue between day LH+6 (0.028 ± 0.002) and untreated Turner's syndrome group (0.017 ± 0.003). The same significant ($p < 0.02$) difference was found in the volume density of total blood vessel when the same groups were compared being 0.039 ± 0.003 in day LH+6 group and $0.021 \pm$ in untreated Turner's syndrome group.

In the Turner's syndrome group after treatment with GH, the significant difference found in the volume density of blood vessel tissue disappeared. However, the significant ($p < 0.05$) difference in the volume fraction of total endometrial blood vessel remained between day LH+6 and GH treated Turner's group. When day LH+6 group were compared to the untreated idiopathic POF group, the only significant ($p < 0.05$) difference found was in the volume density of blood vessel tissue, being 0.018 ± 0.003 in the

Table 4.10: Comparison of endometrial blood vessel features between untreated and GH treated groups of both Turner's syndrome and idiopathic POF patients.

Features	Untreated Turner's Syndrome + Idiopathic POF (n=6)	GH Treated Turner's Syndrome + Idiopathic POF (n=6)
Vv of Endometrium Occupied by Blood Vessel Lumen	0.008 ± 0.003	0.006 ± 0.003
Vv of Endometrium occupied by Blood Vessel Tissue	0.017 ± 0.002	0.020 ± 0.004
Vv of Endometrium Occupied by Blood Vessel Total (Vessels + Lumen)	0.025 ± 0.004	0.026 ± 0.005

Results are means ± SE

n= number of individuals

Table 4.11: Comparison of the volume fraction of endometrial blood vessel features between untreated and GH treated Turner's syndrome groups.

Features	Untreated Turner's Syndrome Group (n=3)	GH Treated Turner's Syndrome Group (n=3)
Vv of Endometrium Occupied by Blood Vessel Lumen	0.004 ± 0.002	0.004 ± 0.003
Vv of Endometrium occupied by Blood Vessel Tissue	0.017 ± 0.003	0.017 ± 0.006
Vv of Endometrium Occupied by Blood Vessel Total (Vessels + Lumen)	0.021 ± 0.005	0.021 ± 0.006

Results are means ± SE

n= number of individuals

Table 4.12: Comparison of the volume fraction of endometrial blood vessel features between untreated and GH treated idiopathic POF groups.

Features	Untreated Idiopathic POF Group (n=3)	GH Treated Idiopathic POF Group (n=3)
Vv of Endometrium Occupied by Blood Vessel Lumen	0.011 ± 0.005	0.008 ± 0.004
Vv of Endometrium occupied by Blood Vessel Tissue	0.018 ± 0.003	0.022 ± 0.004
Vv of Endometrium Occupied by Blood Vessel Total (Vessels + Lumen)	0.029 ± 0.005	0.031 ± 0.008

Results are means ± SE

n= number of individuals

untreated idiopathic POF group. The remaining blood vessel features were not significantly different between the groups indicating a closer pattern of development in the idiopathic POF group to the normal fertile endometrial development. Furthermore, the significant difference seen in idiopathic POF group disappeared after GH treatment, suggesting some unknown effects of GH on endometrial vascularization.

At the electron microscope level, the volume fraction of endothelial cell occupied by nucleus was significantly ($p < 0.02$) different between untreated and GH treated Turner's syndrome + idiopathic POF groups being 0.581 ± 0.018 in untreated group and 0.499 ± 0.012 in GH treated ones (Table 4.13). However, this significant decrease in endothelial cell nuclear volume density disappeared when infertile groups were compared with or without GH treatment (Tables 4.14; 4.15) (Figures 4.7a,b). In addition, there were no significant differences in this feature when untreated and GH treated Turner's syndrome groups were compared to untreated and GH treated idiopathic POF groups separately. While there was no significant difference between day LH+6 and untreated Turner's syndrome + idiopathic POF groups, it was significantly ($p < 0.01$) different between day LH+6 (0.595 ± 0.027) and GH treated Turner's + idiopathic POF groups (0.499 ± 0.012). On the other hand, there was no significant difference between day LH+6 and untreated Turner's syndrome groups, however, these groups were significantly different ($p < 0.05$) when the Turner's syndrome group were treated with GH (0.502 ± 0.022). The same results were observed between fertile day LH+6 and idiopathic POF groups. While there was no difference between day LH+6 and untreated idiopathic POF group, the volume density of endothelial cell nucleus was decreased significantly ($p < 0.02$) after treatment with GH, being 0.495 ± 0.015 (for comparison see Table 2.6 and Tables 4.13; 4.14; 4.15). This decreased endothelial cell nuclear volume density in both groups after GH treatment may suggest either a decrease in nuclear volume or an increase in cytoplasmic volume under the effect of growth hormone or its combination with HRT.

There were no significant differences in the volume fraction of endothelial cell nucleus occupied by euchromatin between any of the untreated and GH treated infertile groups (Tables 4.13; 4.14; 4.15). Therefore, this feature was significantly ($p < 0.05$) different between day LH+6 (0.854 ± 0.019) and untreated Turner's syndrome + idiopathic POF groups (0.770 ± 0.026). Furthermore this significance level ($p < 0.05$) was preserved after

Table 4.13: Comparison of the volume fraction data of endothelial cell features between untreated and GH treated groups of both Turner's syndrome and idiopathic POF patients.

Features	Untreated Turner's Syndrome + Idiopathic POF (n=6)	GH Treated Turner's Syndrome + Idiopathic POF (n=6)
Vv of Endothelial Cell Occupied by Nucleus	0.581 ± 0.018†	0.499 ± 0.012†
Vv of Endothelial Cell Nucleus Occupied by Euchromatin	0.770 ± 0.026	0.752 ± 0.036
Vv of Endothelial Cell Occupied by Mitochondria	0.023 ± 0.002	0.031 ± 0.005
Vv of Endothelial Cell Occupied by "Secretory Apparatus"	0.029 ± 0.003	0.035 ± 0.003
Vv of Endothelial Cell Occupied by RER	0.018 ± 0.003	0.024 ± 0.001

Results are means ± SE

n= number of individuals

† Student's t - Test result (p<0.02) (untreated Turner's + idiopathic POF vs GH treated Turner's + idiopathic POF)

Table 4.14: Comparison of the volume fraction data of endothelial cell features between untreated and GH treated Turner's syndrome groups.

Features	Untreated Turner's Syndrome Group (n=3)	GH Treated Turner's Syndrome Group (n=3)
Vv of Endothelial Cell Occupied by Nucleus	0.606 ± 0.016	0.502 ± 0.022
Vv of Endothelial Cell Nucleus Occupied by Euchromatin	0.782 ± 0.026	0.743 ± 0.063
Vv of Endothelial Cell Occupied by Mitochondria	0.022 ± 0.003	0.029 ± 0.008
Vv of Endothelial Cell Occupied by "Secretory Apparatus"	0.023 ± 0.004	0.032 ± 0.003
Vv of Endothelial Cell Occupied by RER	0.017 ± 0.005	0.023 ± 0.001

Results are means ±SE

n= number of individuals

Table 4.15: Comparison of the volume fraction data of endothelial cell features between untreated and GH treated idiopathic POF groups.

Features	Untreated Idiopathic POF Group (n=3)	GH Treated Idiopathic POF Group (n=3)
Vv of Endothelial Cell Occupied by Nucleus	0.556 ± 0.028	0.495 ± 0.015
Vv of Endothelial Cell Nucleus Occupied by Euchromatin	0.758 ± 0.050	0.761 ± 0.048
Vv of Endothelial Cell Occupied by Mitochondria	0.024 ± 0.003	0.035 ± 0.009
Vv of Endothelial Cell Occupied by "Secretory Apparatus"	0.034 ± 0.003	0.037 ± 0.007
Vv of Endothelial Cell Occupied by RER	0.018 ± 0.004	0.024 ± 0.003

Results are means ± SE

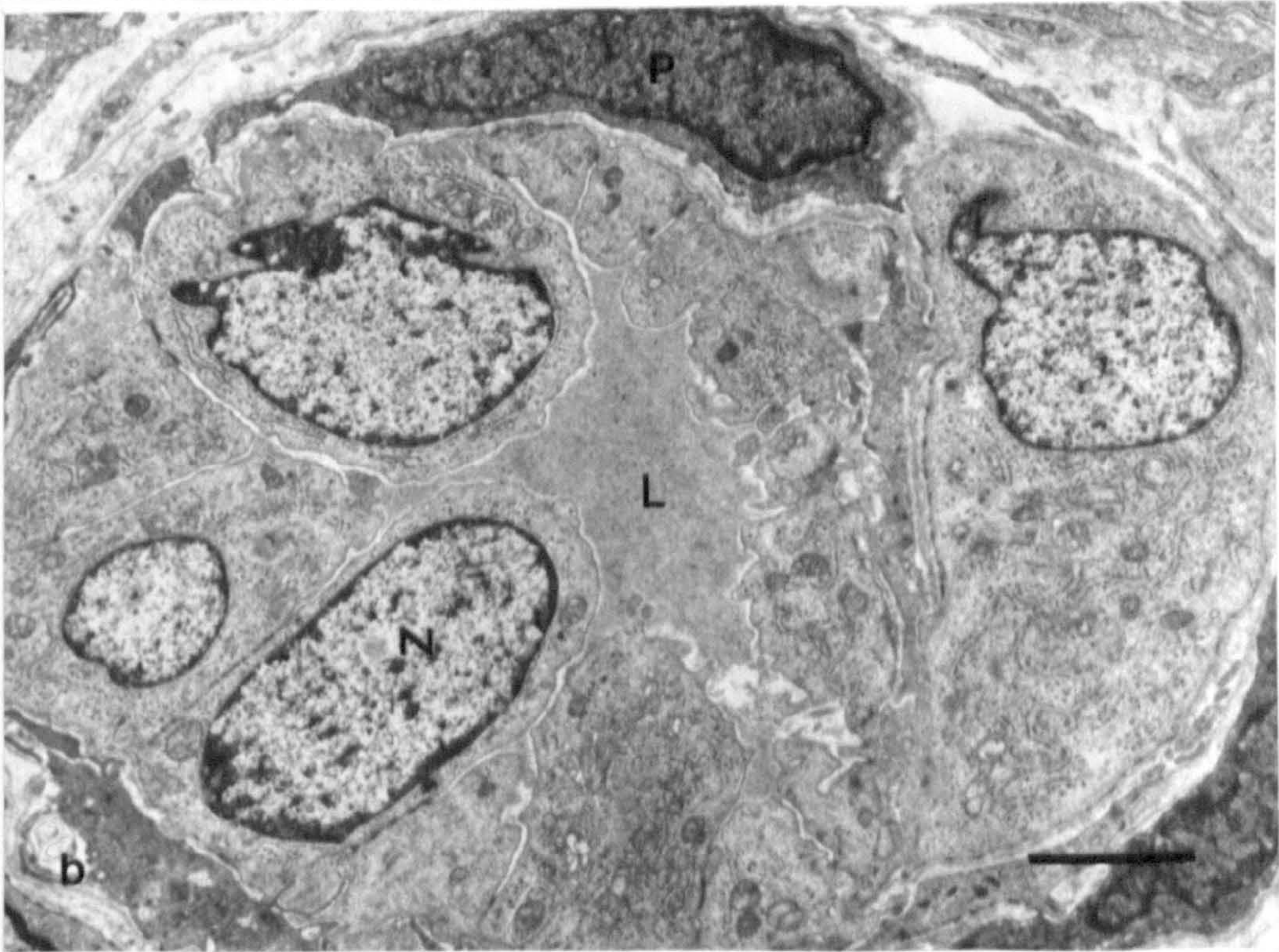
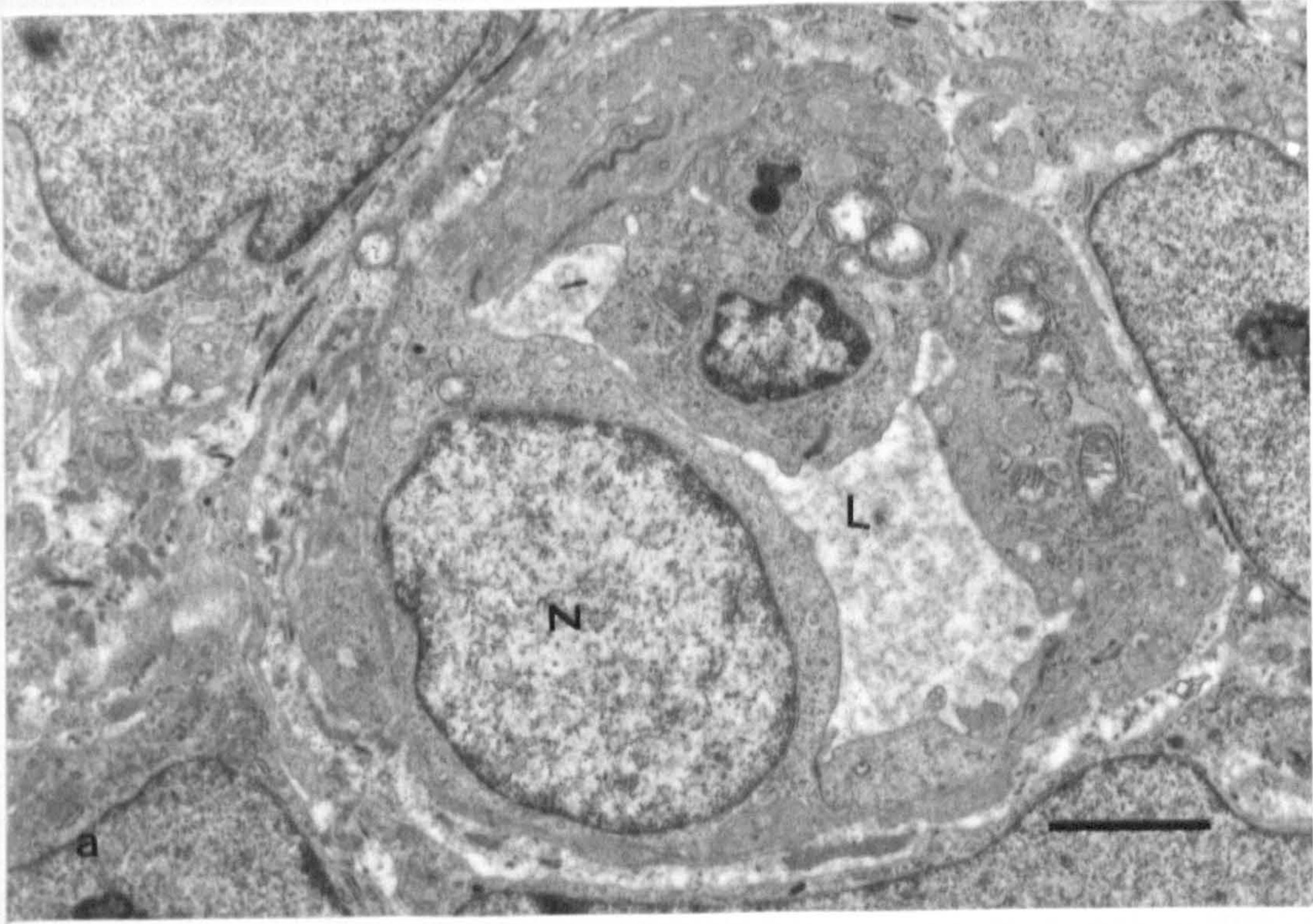
n= number of individuals

Figures 4.7a,b: Untreated idiopathic POF group (a) and GH-treated Turner's syndrome group (b) blood vessels. Since there were no significant differences in endothelial cells between untreated and GH-treated groups, GH-treated idiopathic POF group and untreated Turner's syndrome group blood vessel electron micrographs are not presented here. (N) nucleus, (L) lumen, (P) pericyte.

Staining: Uranyl Acetate - Lead Citrate

(a) Bar represents 1.95 μm

(b) Bar represents 1.85 μm



treatment with GH, being 0.752 ± 0.036 in GH treated Turner's syndrome + idiopathic POF group (for comparison see Table 2.6 and Table 4.13). However, there was only a tendency of a decrease in the volume density of endothelial cell nuclear euchromatin between day LH+6 (0.854 ± 0.019) and GH treated Turner's syndrome groups (0.743 ± 0.063). There were no differences in this feature between day LH+6 and untreated and GH treated idiopathic POF groups, possibly because of the relatively high coefficients of variation within the infertile groups.

No significant differences were found in the volume fraction of endothelial cell occupied by mitochondria between any of the untreated and GH treated infertile groups nor between day LH+6 and infertile groups, suggesting no change in the metabolic demand of the endothelial cells after treatment with GH and no differences in this feature between fertile (day LH+6) and infertile groups (Tables 4.13; 4.14; 4.15 and Table 2.6 in Chapter 2). The volume density of "secretory apparatus" to endothelial cell was also not significantly different between infertile groups. However, it differed significantly ($p < 0.05$) between day LH+6 (0.039 ± 0.003) and untreated Turner's syndrome + idiopathic POF groups (0.029 ± 0.003) suggesting either a decreased secretory activity of endothelial cells or a decreased transport between stroma and blood in the infertile groups. This significant difference disappeared after GH treatment of the infertile individuals. When day LH+6 was compared to the infertile groups separately, the volume fraction of "secretory apparatus" was significantly ($p < 0.02$) different between day LH+6 (0.039 ± 0.003) and untreated Turner's syndrome groups (0.023 ± 0.004). However, there was no difference between day LH+6 and GH treated Turner's syndrome group, nor between day LH+6 and untreated and GH treated idiopathic POF groups indicating either a more normal secretory activity of endothelial cells in idiopathic POF individuals or a better response to HRT from them.

Similar to the other endothelial cell features, the volume density of RER to endothelial cell was not significantly different between any of the infertile groups (Tables 4.13; 4.14; 4.15). On the other hand, it was significantly ($p < 0.05$) different between the day LH+6 and untreated Turner's syndrome + idiopathic POF groups being 0.031 ± 0.004 in day LH+6 group and 0.018 ± 0.003 in untreated Turner's + idiopathic POF group. When the infertile groups received GH treatment, this significant difference

between fertile and infertile groups disappeared (Tables 4.13; 4.14; 4.15). Interestingly, the separate comparisons in this feature between day LH+6 and untreated and GH treated infertile groups did not generally show significant differences, apart from tendencies to decrease in untreated Turner's syndrome and untreated idiopathic POF groups.

There were no significant differences between any of the untreated and GH treated infertile groups in the blood vessel arithmetic and harmonic mean basement membrane thicknesses (Tables 4.16; 4.17; 4.18). The arithmetic mean basement membrane thickness was significantly ($p < 0.01$) different between day LH+6 (81.17 ± 1.92 nm) and untreated Turner's syndrome + idiopathic POF groups (101.41 ± 5.41 nm). It was again significantly ($p < 0.002$) different between day LH+6 and GH treated Turner's syndrome + idiopathic POF groups (105.19 ± 4.93 nm) (for comparison see Table 2.7 and Table 4.16). When infertile groups were compared to the fertile group separately, the arithmetic mean thickness was significantly ($p < 0.02$) different between day LH+6 and untreated Turner's syndrome group (103.92 ± 9.36 nm). The significance level ($p < 0.001$) between day LH+6 and Turner's syndrome group increased when the Turner's syndrome group was treated with GH (101.88 ± 1.82 nm). The arithmetic mean basement membrane thickness was significantly ($p < 0.05$) different between day LH+6 and untreated idiopathic POF groups (97.90 ± 7.40 nm) and the significance level ($p < 0.01$) was preserved when the idiopathic POF group was treated with GH (108.49 ± 10.35 nm) (Tables 4.17; 4.18 and Table 2.7). While there were marginally significant differences in the blood vessel harmonic mean basement membrane thickness between day LH+6 and untreated Turner's syndrome groups, and between day LH+6 and untreated idiopathic POF groups, this feature was significantly ($p < 0.01$) different between day LH+6 (82.74 ± 1.87 nm) and GH treated Turner's syndrome groups (93.32 ± 0.69 nm), and between day LH+6 and GH treated idiopathic POF groups (95.86 ± 3.07 nm) (Table 2.7 and Tables 4.17; 4.18). These significant differences between fertile group and infertile groups indicate an abnormal blood vessel structure and function in these types of infertility cases. In addition, it is noteworthy that the differences were more significant after treatment of infertile groups with growth hormone, indicating some unknown effects of GH on endometrial blood vessel structure.

Table 4.16: Comparison of endometrial blood vessel basement membrane thicknesses between untreated and GH treated groups of both Turner's syndrome and idiopathic POF patients.

Features	Untreated Turner's Syndrome + Idiopathic POF (n=6)	GH Treated Turner's Syndrome + Idiopathic POF (n=6)
Endometrial Blood Vessel Arithmetic Mean Basement Membrane Thickness (nanometers)	101.41 ± 5.41	105.19 ± 4.93
Endometrial Blood Vessel Harmonic Mean Basement Membrane Thickness (nanometers)	92.82 ± 2.98	94.59 ± 1.52

Results are means ± SE

n= number of individuals

Table 4.17: Comparison of endometrial blood vessel basement membrane thicknesses between untreated and GH treated Turner's syndrome groups.

Features	Untreated Turner's Syndrome Group (n=3)	GH Treated Turner's Syndrome Group (n=3)
Endometrial Blood Vessel Arithmetic Mean Basement Membrane Thickness (nanometers)	103.92 ± 9.36	101.88 ± 1.82
Endometrial Blood Vessel Harmonic Mean Basement Membrane Thickness (nanometers)	94.16 ± 4.74	93.32 ± 0.69

Results are means ± SE

n= number of individuals

Table 4.18: Comparison of endometrial blood vessel basement membrane thicknesses between untreated and GH treated idiopathic POF groups.

Features	Untreated Idiopathic POF Group (n=3)	GH Treated Idiopathic POF Group (n=3)
Endometrial Blood Vessel Arithmetic Mean Basement Membrane Thickness (nanometers)	97.90 ± 7.40	108.49 ± 10.35
Endometrial Blood Vessel Harmonic Mean Basement Membrane Thickness (nanometers)	91.47 ± 4.50	95.86 ± 3.07

Results are means ± SE

n= number of individuals

4.4. DISCUSSION

The present study has dealt with the effects of growth hormone (GH) on the morphology of endometrial stromal and vascular structures in patients with Turner's syndrome and patients with idiopathic POF during standard hormone replacement therapy (HRT). Endometrial biopsies were obtained at day 19 of the cycle. LH dating of the endometrial biopsies was not performed since the subjects had no endogenous gonadotrophin secretion due to their infertility. Both untreated and GH treated infertile groups' data were compared in order to determine the effects of GH on endometrial structure and function. The data from infertile subjects were also compared with data from normally ovulating fertile subjects (at day LH+6) for a more complete comparison of endometrial structure between these types of infertility conditions and normal fertile conditions. Day LH+6 has been selected for the fertile group since it was the nearest available corresponding time to day 19 of the menstrual cycle.

As it can be seen in Figure 4.3, the experimental design of the present study is a very complicated one and the statistical comparisons involve the combined infertile groups either with or without GH treatment, and their comparisons with the day LH+6 fertile controls. Furthermore, the comparisons were also made between GH treated and untreated groups of Turner's syndrome and idiopathic POFs separately and also between these infertile groups and the day LH+6 controls.

In the present study, the small sample size for the statistical comparisons was an unavoidable disadvantage, due to the difficulty of obtaining the endometrial tissue for this type of study. These blocks represent the total tissue available for GH treated POF and Turner's syndrome subjects over almost 3 years. Untreated and GH treated infertile Turner's syndrome and idiopathic POF groups were combined together since they both lacked endogenous ovarian hormone secretion (although their aetiologies are different - see Introduction). These two infertile groups were also compared separately to the other groups, however, their small sample size may be one reason for the relatively low significance levels in separate statistical comparisons between Turner's syndrome and idiopathic POF groups.

While there were no major changes in overall endometrial morphology between any of the untreated and GH treated infertile groups, they were significantly different

between fertile LH+6 group and infertile groups (either with or without GH), suggesting an impairment in the development of endometrium in infertile groups. In addition, the significance levels in some endometrial features between the day LH+6 fertile group and GH treated infertile groups were higher than between the day LH+6 fertile group and untreated infertile groups, suggesting that the treatment with GH may have some unknown effects on endometrial development. Since there are very few publications dealing with the effects of GH on the endometrial morphology (especially on the stromal and vascular compartments), findings of the present study will be discussed in separate subheadings and then comparisons with the existing available literature will be made at the end of this section.

4.4.1. GLANDULAR EPITHELIUM

No major changes were found in glandular epithelial morphology between any of the untreated and GH treated infertile groups. However, both untreated and GH treated infertile groups differed significantly from the normal fertile group in a way which suggested a retardation in glandular epithelium of the infertiles. The volume density of gland cell to gland was significantly higher (6%) in untreated Turner's + idiopathic POF than the control group and the level of significance became larger when the infertile group was treated with GH (8% higher in the GH treated Turner's syndrome + idiopathic POF group). This high gland cell to gland volume fraction may be due to the high glycogen content present in the glandular epithelial cells of the infertile groups. Glycogen-like material in glandular epithelium is normally at its peak at day LH+4 of the menstrual cycle (corresponding to day 17 of the traditional dating according to Noyes *et al.* 1950) and a substantial decrease occurs after this time, eventually resulting the disappearance of glycogen-like material from gland cells to the gland lumen later in the menstrual cycle (see Discussion in Chapter 2). In the present study, from qualitative observations both at light and electron microscope levels using glycogen specific staining procedures, more glycogen-like material was present in the glandular epithelium of infertile groups than in the LH+6 fertile group, possibly indicating a delay of secretion from glands in these individuals. The significant difference between both untreated and GH treated infertile groups (when Turner's syndrome and idiopathic POF groups were

compared) was seen to be mainly due to the changes that occurred in Turner's syndrome group when these 2 groups were compared separately.

While there were no significant differences in the volume density of gland cell to gland between day LH+6 controls and either untreated or GH treated idiopathic POF groups, this feature differed significantly between day LH+6 control group and Turner's syndrome group with or without GH. It was 8% higher in the untreated Turner's syndrome group than the day LH+6 fertile group and became 9% higher when the Turner's syndrome subjects were treated with GH. In addition, the difference between these groups was more significant after GH treatment indicating some extra effects of GH on endometrial development in patients with Turner's syndrome.

Li *et al.* (1991b) reported that the patients with premature ovarian failure had better response to HRT than the Turner's syndrome patients. In their ultrasonographic and histological study, 18 women with ovarian failures (10 idiopathic ovarian failure, 4 Turner's syndrome and 4 POF patients) were examined during hormone replacement therapy and demonstrated that the women with Turner's syndrome had the smallest uterine thickness, endometrial thickness and the least advanced histological dating (both traditional and by morphometry) whereas POF patients had more normal endometrial thickness and a closer histological appearance to the normal endometrium. In their study, the results of histological dating of the endometrial biopsy were found to be positively correlated with the endometrial thickness on day 19 of the menstrual cycle. Although simple morphometry was used in their study at the light microscope level, their findings give certain implications on the effects of HRT on endometrial morphology in women with premature ovarian failure. Their findings agreed with the data from the present study that the idiopathic POF subjects, receiving HRT, had a better response to sex steroids whether or not they also had growth hormone supplementation.

On average, the volume fraction of endometrium occupied by gland did not differ significantly between the untreated and the GH treated Turner's + idiopathic groups, between untreated Turner's syndrome and GH treated Turner's syndrome groups, nor between untreated and GH treated idiopathic POF groups. However, this feature again differed significantly between day LH+6 fertile group and both the untreated and the GH treated infertile groups. The reason for this difference in volume density of gland to

endometrium between infertile groups and the fertile group may well be the delayed glycogen accumulation in the gland cells and thus the abnormality in development of the endometrium in these type of infertility conditions. Yet again the significant difference between the day LH+6 fertile group and Turner's syndrome + idiopathic POF group in this feature was due mainly to the Turner's syndrome group indicating a great deviation of the endometrial development in Turner's syndrome individuals from the fertile ones and even from the idiopathic POF group.

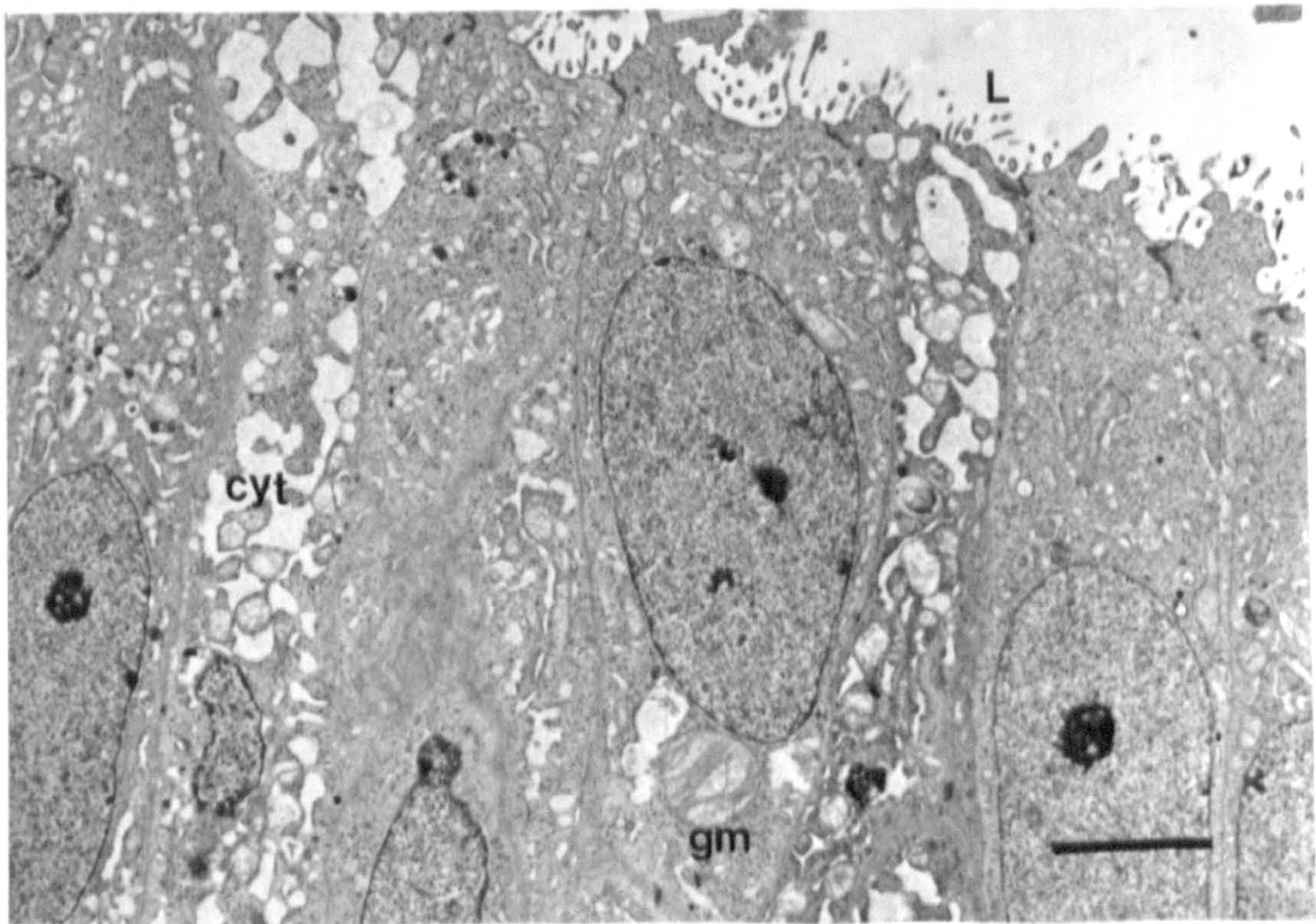
In the present study, qualitative observations of the glandular epithelium in infertile groups demonstrated some atrophic cells as well as the accumulation of glycogen-like material within these cells (Figure 4.4a and Figure 4.8). Although these glandular epithelial cells were present in both infertile groups, they were more abundant in the Turner's syndrome group regardless of treatment with GH. The question arises as to whether these cells were atrophic or apoptotic. However, in terms of structural patterns that apoptotic cells demonstrate (Piyawinijwong 1993), the cells with abnormal appearance observed in the present study appeared atrophic or degenerative cells not exhibiting features of apoptosis. For example, in the central nervous system, some nerve cells in the inferior olivary nucleus of the brain show apoptosis which is characterised by an apparent space between neighbouring cells, the presence of extremely dense nuclei, and smaller cells than the normal ones in appearance (Piyawinijwong 1993). In the present study, abnormal cells that were observed in the Turner's syndrome group had small nuclei with an appearance closer to that of euchromatic nuclei, and a cytoplasmic degeneration at different degrees in the neighbouring cells which did not loose contact with each other, unlike the cells described as undergoing apoptotic in brain (Figure 4.8). The underlying reason for the presence of these degenerated cells in the Turner's syndrome endometria could be of genetic origin or due to secondary endocrinological factors, since these individuals are genetically abnormal (lacking the second sex chromosome - 45, X0). The presence of these atrophic cells in the Turner's syndrome endometrial glandular epithelia may possibly suggest an abnormal endometrial structure and development in the Turner's syndrome individuals, either due to direct genetic causes or as an altered response to HRT.

Figure 4.8: Glandular epithelial cells of GH-treated Turner's syndrome group. Cells demonstrate atrophic cytoplasm (cyt) with increased amounts of vacuolation. (gm) giant mitochondria, (L) lumen.

Staining: Uranyl Acetate - Lead Citrate

Bar represents 3.85 μm

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The reason for the use of women with Turner's syndrome in the present study was mostly to obtain a group of women without endogenous ovarian hormones, but for a different reason than the idiopathic POF group. As can be seen in the experimental design of the present study, the sample size of both POF group and Turner's syndrome group was relatively small. As well as lack of ovarian steroids, Turner's syndrome patients have several abnormalities, such as short stature, webbing of the neck and lacking of the gonadal functions (see Introduction). Although women with Turner's syndrome may have gonads, the number of follicles present in ovaries is reduced to 20% of the normal quantity after puberty. This reduced quantity of the ovarian follicles is accompanied by the lack of steroid hormone secretion, resulting in obvious reproductive tract abnormalities. For example Rogers *et al.* (1992) reported no visible tight junctional complexes between uterine epithelial cells in 9 out of 11 Turner's syndrome patients receiving hormone replacement therapy. Their observations agree with the data from the present study that the Turner's syndrome subjects may have abnormal endometrial structure and development causing infertility in this type of condition. The opinion that the Turner's syndrome patients might be poor responders to the sex steroid treatment was also agreed by Rudolf *et al.* (1989). In their study, Turner's syndrome patients were treated with synthetic sex steroids and the endometria from those patients showed no proliferation or secretion after hormone treatment indicating no response of the endometrium.

4.4.2. STROMA

Similar to the glandular features, there were few significant differences between any of the infertile groups, either with or without GH treatment, suggesting that both of the infertile groups may display a similar type of endometrial structure and a similar response (or lack of response) to GH treatment. However, these features in infertile groups did differ significantly from the day LH+6 fertile group, indicating a great diversity from the normal endometrial development and function in these types of infertility cases.

While there was no significant difference in the volume density of endometrium occupied by stroma between day LH+6 group and untreated Turner's + idiopathic POF group, it was significantly higher in the day LH+6 fertile group than the GH treated

Turner's syndrome + idiopathic POF group indicating a decrease in stromal volume density and a significant effect of GH on this feature. This feature did not differ between the day LH+6 group and either untreated or GH treated idiopathic POF group. However, the volume density of stroma to endometrium was significantly lower in untreated Turner's syndrome group than the fertile group and this significant decrease became larger after treatment with GH hormone. This decreased endometrial stromal volume density was consistent with the increased glandular volume density in infertile groups, especially the Turner's syndrome group, possibly indicating a retarded endometrial development in infertile groups. The significant decrease of stromal volume density in the overall infertile group (Turner's + idiopathic POF) was mainly due to the Turner's group, since the idiopathic POF group had similar volume fraction values to the fertile group before and after GH treatment.

The volume fraction of stromal cell nucleus to endometrium was examined in order to help evaluate, when combined with other measurements, indirectly whether or not the packing density of the cells in endometrial stroma was affected by GH treatment. This feature was significantly different between GH treated Turner's syndrome group and GH treated idiopathic POF group, being greater in the idiopathic POF group. This result may indicate a different response in GH treatment due to the different type of infertility. The higher volume density of stromal cell nucleus to endometrium in GH treated idiopathics suggests there is relatively more stromal cell nucleus than endometrium, possibly due to either an increase in the stromal cell packing density or that the stromal cell nuclear dimensions are larger in this group. However, the stromal cell nuclear profile dimensions did not differ between any of the infertile groups nor between the day LH+6 fertile and any of the infertile groups, either with or without GH supplementation. This result suggests that the reason for the increased stromal cell nuclear volume density in GH treated idiopathic POF group is due to the increased packing density of the stromal cells.

The volume fraction of stromal cell nucleus to endometrium was significantly higher in GH treated Turner's syndrome + idiopathic POF group than the day LH+6 fertile group. This feature was significantly higher in GH treated idiopathic POF group than the

day LH+6 group whereas the fertile group and untreated idiopathics did not differ significantly.

The volume fraction of nucleus to stromal cell differed only between the day LH+6 group and untreated idiopathic POF group, being 8% lower in the latter. This result may indicate an increased stromal cell cytoplasmic volume since it was already confirmed that there were no significant differences in the stromal cell nuclear dimensions (shape and size) between the day LH+6 group and untreated idiopathic POF group. In addition, there were no significant changes in the volume fraction of stromal cell nucleus occupied by euchromatin. These unchanged stromal cell nuclear features contrast with the findings of the stromal cell nucleus volume fraction to cell results which was significantly different between the GH treated combined group and the day LH+6 control group and suggest that, at the functional level, stromal cell nuclei did not change either with or without GH supplementation and they were not significantly different from those day LH+6 controls.

The volume density of stromal cell mitochondria to cell did not differ between untreated and GH treated infertile groups and between day LH+6 and infertile groups, with only one exception; the volume density of stromal cell occupied by mitochondria which was significantly higher in the day LH+6 fertile group than the untreated Turner's syndrome group. These results suggest that the untreated Turner's syndrome group stromal cells demand a lower metabolic activity, than in the controls. These results may further indicate a better response from idiopathic POF individuals to HRT by not being significantly different from day LH+6 fertile group.

A similar result to the stromal cell mitochondrial volume density has been observed for the volume fraction of "secretory apparatus" to stromal cell feature in that there were no significant differences between any of the untreated and GH treated infertile groups. However, there were significant differences when the day LH+6 control group were compared separately to the infertile groups, suggesting an abnormal endometrial development and function in these infertile subjects. This feature was significantly different between the day LH+6 group and the Turner's syndrome + idiopathic POF group both before and after treatment with GH. Further comparisons revealed that both infertile groups, with or without growth hormone administration, significantly differed

from those fertile individuals with the exception of the GH treated Turner's syndrome group. However, a high coefficient of variation within the GH treated Turner's syndrome group was observed possibly accounting for its lack of statistical significance. These generally decreased mitochondrial volume densities in stromal cells suggest that at the endometrial stromal cell level both Turner's syndrome and idiopathic POF individuals required less metabolic activity than did the fertile group, however whether this is the cause of the infertility or a consequence of it remains unknown.

The volume fraction of stromal cell occupied by rough endoplasmic reticulum (RER) was not significantly different between any of the untreated and GH treated infertile groups, nor between day LH+6 fertile group and untreated and GH treated infertile groups, with the exception that there was a tendency of an increase in this feature in the untreated idiopathic POF group when compared to the day LH+6 fertile group. The overall conclusion for stromal cell features could be that the idiopathic POF individuals had a better response to HRT than Turner's syndrome subjects. However, GH treatment might cause some unknown effects on the stromal compartment of both infertile groups, since they had larger significant differences when compared to the day LH+6 fertile group following GH treatment.

4.4.3. BLOOD VESSELS

Human endometrium is a very dynamic tissue which renews itself every 28 day, and a precise sequence of events in the endometrial compartments are required for the development of a receptive endometrium. Vascularization of the endometrium after menses is one of these crucial events that prepares the endometrium for a successful implantation and gestation. Any interruption and abnormality in the vascularization of the endometrium could cause infertility in women. For the above reasons, the effects of growth hormone on endometrial blood vessel structure, as well as on the glandular and stromal morphologies, have been examined at the light and electron microscope levels for a more precise evaluation of the endometrial structure in the Turner's syndrome and idiopathic POF infertility conditions.

At the light microscope level, the volume density of endometrial blood vessel features were not significantly different between any of the untreated and GH treated infertile

groups. In addition, the volume fraction of endometrium occupied by blood vessel lumen did not differ significantly between the day LH+6 fertile group and any of the infertile groups. However, the volume density of blood vessel tissue was significantly higher in the day LH+6 group than the untreated Turner's syndrome + idiopathic POF group, possibly suggesting a poor endometrial vascularization in these types of infertility cases. The volume fraction of endometrium occupied by total blood vessel, which includes both lumen and the tissue, was again significantly higher in the day LH+6 group than the untreated Turner's syndrome + idiopathic POF group. Furthermore, the volume density of total blood vessel to endometrium was significantly higher in the fertile group than the GH treated Turner's + idiopathic POF group.

However, these significant differences between the normal fertile group and the combined infertile groups (Turner's + idiopathics) is most likely due to the Turner's syndrome group, since there were more significant differences between the fertile group and the Turner's syndrome group (with or without GH supplementation). For example, the volume fractions of blood vessel tissue and the total blood vessel were significantly lower (39% and 46% respectively) in the untreated Turner's syndrome group than the fertile group, indicating an abnormal endometrial vascularization and development in this group. However, after treatment with growth hormone, the difference in the volume density of blood vessel tissue between Turner's syndrome group and the fertile group was not significant. Although the total blood vessel volume density remained significantly lower in this infertile group, the significance level was reduced suggesting an unknown positive effect of GH on the endometrial vascular structure and function.

When the day LH+6 control group were compared to the idiopathic POF group before and after GH treatment, the only significant difference found in the endometrial blood vessel features was between the fertiles and the untreated POF group. This significant difference disappeared after treatment with GH, again indicating a positive effect of GH treatment on the vascularization and development of endometrium. These results suggest that the women with Turner's syndrome respond suboptimally to steroid hormones and have an abnormal endometrial structure and development whereas the women with POF have a more normal endometrial vascularization and development and have a better response to HRT and that both groups respond positively to GH treatment.

At the electron microscope level, the volume density of nucleus to endothelial cell was significantly higher in the untreated Turner's syndrome + idiopathic POF group than the GH treated one, indicating either an increase in endothelial cell nuclear volume or a decrease in endothelial cell cytoplasmic volume in the untreated group. However, this significant difference disappeared when infertile groups were compared separately before and after GH treatment, probably because of the reduced sample size of infertile groups after separating the combined infertile group into two different infertile groups (Turner's syndrome and idiopathic POF groups). Although the volume density of the endothelial cell nucleus to cell in the infertile group (Turner's + POFs) was not different from the fertile group without GH treatment, a significant decrease occurred in the infertile group after treatment with GH. Similarly, while the volume density of endothelial cell nucleus was not significantly different between day LH+6 group and the untreated Turner's syndrome group, a significant decrease occurred in the Turner's syndrome group after treatment with growth hormone. Yet again the same feature did not differ between the fertile group and untreated idiopathic POF group. However, it decreased significantly when the idiopathic POF group was treated with GH. This decreased endothelial cell nuclear volume density in both infertile groups after GH treatment may suggest either a decrease in nuclear volume or an increase in cytoplasmic volume as a result of the effects of the GH treatment.

Similar to the other endothelial cell features, the euchromatin distribution in the endothelial cell nucleus did not differ between any of the infertile groups. However, the volume fraction of endothelial cell nucleus occupied by euchromatin differed significantly between the fertile group and the Turner's syndrome + idiopathic POF group, either with or without GH, being lower in the latter and possibly indicating a lower transcriptional rate of the endothelial cells in this group. However, there was only a tendency of a decrease in this feature when the day LH+6 group was compared to the untreated Turner's syndrome group. In addition, there were no significant differences between the day LH+6 group and untreated and GH treated idiopathic POF groups possibly because of the high coefficients of variation and small sample size of these fertile groups. On the other hand, this unchanged euchromatin distribution in the idiopathic POF group may

also suggest a closer resemblance of endometrial development to the controls in this group than in the Turner's syndrome group.

While the volume fraction of endothelial cell occupied by mitochondria did not differ either between any of the infertile groups or between day LH+6 fertile group and infertile groups before and after GH treatment, there were significant differences in the volume density of "secretory apparatus" to endothelial cell between groups. The unchanged mitochondrial volume density may indicate an unchanged metabolic demand in these cells with or without GH treatment. It may also suggest that the mitochondrial pattern of the endothelial cells in the infertile groups seem to have a close resemblance to the normal fertile mitochondrial structure and function. The volume density of endothelial cell occupied by "secretory apparatus" did not differ significantly between untreated and GH treated infertile groups. However, it was significantly lower in the untreated Turner's syndrome + idiopathic POF group than day LH+6 control group, suggesting either a decreased secretory activity of the endothelial cells or a decreased transport between the stroma and the blood in infertile groups. After treatment of infertile groups with growth hormone, the significant difference between fertile and infertile groups in the volume density of endothelial cell "secretory apparatus" disappeared suggesting an unknown effect of GH on the blood vessel structure and function. When the day LH+6 group was compared separately to infertile groups, the volume fraction of "secretory apparatus" to endothelial cell was significantly lower in the untreated Turner's syndrome group. However, there was no significant difference between GH treated Turner's syndrome group and the fertile group, nor between the fertile group and either untreated or GH treated idiopathic POF groups. These results may indicate an effect of GH on the secretory activity of the blood vessel endothelial cells in the Turner's syndrome group, and may possibly suggest either a more normal secretory activity of the endothelial cells in idiopathic POF individuals or a better response to HRT from idiopathic individuals than the Turner's syndrome patients.

Similar to the other endothelial cell features, the volume fraction of endothelial cell occupied by RER did not differ significantly between any of the infertile groups with or without GH treatment. On the other hand, it was significantly lower in untreated Turner's syndrome + idiopathic POF group when compared to the control group,

indicating a lower protein synthetic activity in this particular group. However, this significant difference disappeared between the same groups after treatment with GH (as in most of the other stromal and endothelial cell features), again suggesting an effect of growth hormone on endothelial structure and function. Interestingly the separate comparisons in this feature between fertile group and untreated and GH treated infertile groups did not show significant differences apart from tendencies to decrease in untreated Turner's syndrome and untreated idiopathic POF groups were seen. This may be the result of the small sample size and thus high coefficients of variation in the infertile groups each of which consists of 3 subjects whereas the combined Turner's syndrome + POF group consists of 6 subjects.

For a complete evaluation of the effect of GH on the endometrial blood vessel morphology, arithmetic and harmonic mean basement membrane thicknesses were determined for Turner's syndrome and POF patients and these results were also compared to the fertile control group. Although there were no significant differences between any of the untreated and GH treated infertile groups, the infertile groups mostly differed from the fertile group. The arithmetic and harmonic mean basement membrane thicknesses were both significantly different between the day LH+6 fertile group and untreated and GH treated Turner's syndrome + idiopathic POF groups, being higher (around 20% in arithmetic mean thickness and 10% in the harmonic mean thickness) in the infertile groups. Separate basement membrane thickness comparisons between the day LH+6 fertile group and the Turner's syndrome and idiopathic POF groups demonstrated that the harmonic and arithmetic mean basement membrane thickness values of the Turner's syndrome and POF subjects were significantly higher (again, around 20% higher in arithmetic mean thickness and 10% in harmonic thickness) than the control group values, both before and after treatment with GH. These basement membrane evaluations suggest that the infertile subjects had thicker blood vessel basement membrane than the day LH+6 controls, and that the significance levels further increased when the infertile subjects received the GH treatment indicating an effect of GH on the endometrial blood vessel structure and function.

Basement membranes have been demonstrated in the attachment, migration, growth, repair and differentiation of the overlying cell populations as well as in the fluid filtration

and gas exchange (Wracko 1982). A thick endometrial blood vessel basement membrane may cause alterations of these functions in infertile groups. It could also indicate a lower fluid filtration and gas exchange between blood and endometrium in these groups, and this decreased transport rate may lead to a less normal endometrial development and function eventually causing infertility. However, it is likely that the increased basement membrane thickness could be only one of several underlying pathologies which all together may cause the infertility in these subfertile individuals.

Young women, who lack ovaries or whose ovaries are not functional, make up a small but relatively important group of sterile patients. Hormone replacement therapy (HRT) has been an interest of several investigators in the establishment of successful pregnancies in women with primary ovarian failure (Lutjen *et al.* 1984; Navot *et al.* 1986). There are different types of primary ovarian failure such as gonadal dysgenesis (either in a pure form with 46,XX chromosomal complement or in conjunction with Turner's syndrome - 45,XO) and premature ovarian failure (premature menopause). Patients with gonadal dysgenesis have primary amenorrhea and sexual infantilism whereas patients with premature ovarian failure usually present with permanent ovarian failure occurring after menarche but before the normal menopausal age (generally before the age of 35) (Coulam and Ryan 1979; Friedman *et al.* 1983).

Two factors are probably crucial in successful embryo implantation: receptivity of the endometrium and synchronisation between endometrial and embryonic development. Both these factors depend on the cyclic hormonal activity of the ovary and timely release of a fertilizable oocyte. In a fertile cycle, the ovaries produce adequate quantities of sex steroids in a certain temporal pattern and ratio to promote the necessary growth of the endometrium and to regulate its timely differentiation throughout the early stages of gestation (Maslar 1988). However, in the case of primary ovarian failure the development of a receptive endometrium, capable of supporting implantation and maintaining pregnancy, is achieved when the uterus is exposed to an appropriate sequence and ratio of oestrogen and progesterone (Navot *et al.* 1986; Younis *et al.* 1994). Endometrial stimulation is performed sequentially by oestrogen in the follicular phase and by oestrogen and progesterone in the luteal phase. A precise co-ordination of these events are thought to maintain effective reproduction (Younis and Laufer 1992). It

is well established that oestrogen in the follicular phase is indispensable for endometrial priming whereas the luteal phase progesterone is essential to allow implantation and to maintain pregnancy (Lenton *et al.* 1982; Lenton and Woodward 1988). In the case of patients with primary ovarian failure, as in the present study, a certain sequence and ratio of these sex steroids are required for the normal ovulation and the development of a receptive endometrium. Results of the present study indicated that the endometrial morphology of the infertile patients, who received standard hormone replacement therapy, significantly differed from the normal endometrial structure of the fertile subjects. These results may suggest that the abnormal endometrial development in patients with primary ovarian failure, especially in those with Turner's syndrome, is permanent and thus incurable. However, previous work has shown that such conditions can be treated to result in some successful pregnancies, despite the significant differences in endometrial morphology reported in the present study.

While there appear to be no reports on the effects of GH on endometrial structure, there have been several reports on the effects of GH on other tissues (Beer *et al.* 1994; Doi *et al.* 1988, 1990). In genetically dwarf (GH deficient) rats, the crypt volume and epithelial cell height of the small intestine were significantly lower than the controls, however, these reduced epithelial features were reversed to the control values when rats were treated with exogenous GH (Beer *et al.* 1994). They suggested that GH deficiency has subtle effects on intestinal epithelial morphology and reconstitution with GH is capable of reversing many of these changes. In addition, mesangial cell proliferation followed by glomerulosclerosis was observed in the kidney of transgenic mice chronically expressing growth hormone and growth hormone releasing factor (Doi *et al.* 1988, 1990). In the present study, women with Turner's syndrome had abnormal endometrial glandular epithelia when compared to controls, but this abnormality did not disappear when subjects were treated with GH, suggesting that there might be differences in the physiology of different epithelial types, and each of these tissues may have their own different factors, hormonal actions and metabolic activities which influence their responsiveness to GH.

In the human reproductive tract, the actions of GH, gonadotrophic hormones, sex steroids and the insulin-like growth factor (IGF) system are closely related to each other

(see Introduction). Growth hormone enhances ovulation via regulating the gonadotrophic hormone, sex steroid and IGF system actions which include; IGF-I action that is capable of substantial amplification of gonadotrophin hormonal action (Adashi *et al.* 1985). Alternatively, GH, in concert with oestrogens, may upregulate the intraovarian generation of IGF-I (Hernandez *et al.* 1989). Because IGF-I mRNA is stimulated by oestrogen (Murphy and Ghahary 1990) and IGFBP-I secretion by progesterone (Rutanen *et al.* 1986) and IGFBP-I inhibits binding of IGF-I to its endometrial IGF receptors (Rutanen *et al.* 1988), the IGF system is likely to be an important mechanism through which the biological actions of oestrogen and the anti-oestrogenic effects of progesterone are mediated.

It has been suggested that progesterone-induced stromal IGFBP-I inhibits epithelial growth by inhibiting the action of IGFs (Seppala *et al.* 1994). Indeed, when the endometrium comes under the sustained influence of progesterone, i.e. during pregnancy or systemic progesterone treatment, the endometrium undergoes stromal hyperplasia and epithelial atrophy (Dallenbach-Helweg 1981). This is consistent with the data from the present study that there were atrophic glandular epithelial cells when the morphology of the endometria of infertile groups were examined qualitatively after hormone replacement therapy consisting of oestrogen and progesterone (Figure 4.8).

Growth hormone and steroid hormone interaction has been a new field of interest in reproductive biology. Bezcny *et al.* (1992) reported a significant increase in oestrogen receptor concentration in the guinea-pig uterus after treatment with bovine growth hormone. In their study, the amount of cytosolic oestradiol receptor per unit of uterine weight was increased 7-14 fold in GH treated animals, and the nuclear oestradiol receptor concentration was 3-7 fold higher than in control animals. The present data strongly suggest that GH administration may have substantial effects on the uterus, especially on endometrium, as well as having influences on the ovulation enhancement in ovaries.

It appears that growth hormone, gonadotrophins, IGF system (with its ligands, receptors and binding proteins) and sex steroids (oestrogen and progesterone) have very complicated interactions and thus effects on the reproductive tract through the pituitary - ovary - endometrial axis (see earlier). However, despite its ovulation enhancing effect,

the exact role and mechanism of GH in the reproductive biological use still remains unclear.

The main conclusion to be drawn from the present study is that while untreated and GH treated infertile groups did not differ significantly at the endometrial stromal and vascular levels, these infertile groups (Turner's syndrome and idiopathic POF) differed significantly from the day LH+6 fertile group either with or without GH administration. In addition, the significance levels between the fertile group and infertile groups were further increased in some of the stromal and vascular features when the infertile groups were treated with GH, suggesting that the GH treatment may have subsequent effects on the endometrial development of these infertile subjects. However, further studies are required for the understanding of GH action in the reproductive tract and especially in the endometrium for a better use of GH in this field.

CHAPTER 5

HUMAN ENDOMETRIAL STROMAL MORPHOLOGY IN UNEXPLAINED RECURRENT MISCARRIAGE

5.1. INTRODUCTION

The World Health Organisation's (WHO) definition for miscarriage is "the expulsion or extraction from its mother of an embryo or fetus weighing 500g or less" (WHO 1977). In repeated abortions, women manage to achieve pregnancy but on several occasions lose the pregnancy before it is viable or before baby has a reasonable chance of survival (Stirrat 1990a). The definition of recurrent miscarriage, agreed following an European Society of Human Reproduction and Embryology (ESHRE) workshop, is two or more consecutive pregnancy losses (Crosignani and Rubin 1991). It has been reported that recurrent miscarriage occurs in 1% of the total female population (Coulam 1986). The aetiology of recurrent miscarriage varies according to many factors, but is often classified on the basis of whether the abortion occurs in the first trimester or in the second trimester of the pregnancy.

It has been estimated that up to 65% of all first trimester abortions are due to the fertilized oocyte being chromosomally faulty (Warburton and Strobino 1987; Olson and Magenis 1988). The remaining 35% of spontaneous abortions are due to undiscovered causes but there are several implicated factors including uterine causes, hormonal deficiencies, faults in implantation, infections and others. It is the aim of the present study to investigate the endometrial morphology of women who have had recurrent miscarriages followed by successful pregnancies compared to subjects with persisting miscarriage. In both groups other common causes of miscarriage (such as chromosomal abnormalities, see later) can be discounted leaving the endometrium as a likely factor in the miscarriage. In order to give a full account of this topic, major causes for recurrent pregnancy losses are described below.

5.1.1. ANATOMICAL DISORDERS

The reported incidence of uterine abnormalities associated with recurrent miscarriage is between 15% and 30% (Harger *et al.* 1983; Stray-Pedersen and Stray-Pedersen 1984). The true incidence of congenital uterine anomaly in recurrent miscarriage is unknown (Stirrat 1990b), but congenital uterine anomalies appear to be present in about 12% of recurrent miscarriage patients (Bennett 1987). However, it is well established that successful pregnancy can occur in the presence of some uterine anomaly, and the

percentage of uterine anomalies remaining undiagnosed in the general female population is unknown. Due to this lack of information, it is not possible to conclude about the relative importance of uterine anomaly in recurrent miscarriage.

Another anatomical disorder which may play an important role in recurrent miscarriage is cervical incompetence. In a study of 66 patients with 3 or more consecutive miscarriages, where no apparent cause could be found for their repeated loss, Ayers *et al.* (1982) found an 89% incidence of cervical incompetence in the first trimester of the pregnancy. The incidence of cervical incompetence in recurrent miscarriage has been reported to vary from 13% to 20% (Stray-Pedersen and Stray-Pedersen 1984; McDonald 1987).

5.1.2. GENETIC CAUSES

It is thought that at least 50% of first trimester pregnancy loss and 20% of second trimester pregnancy loss exhibit a fetal chromosomal abnormality (Olson and Magenis 1988). However, the accuracy of these figures is speculative, because of the difficulties in culturing chromosomal material from abortus specimens and the unnoticed loss of very early fetuses. Repetitive aneuploidies, the most common of which is trisomy, appear to be significantly higher among recurrent miscarriers (de Braekeleer and Dao 1990).

5.1.3. ENDOCRINE FACTORS

Insufficient luteal function associated with recurrent miscarriage has been reported to occur in 20-60% of cases, but there is no reliable way to recognise inadequate luteal function in the already pregnant patient (Fritz 1988). Endometrial biopsy examination of the non-pregnant uterus is the best way of determining the luteal phase defect in women, however, it requires careful sampling and a time consuming effort. Although it is the commonest endocrinopathy diagnosis in association with recurrent pregnancy loss, there is no consensus of luteal phase inadequacy, the method of diagnosis, or its proper treatment (Fritz 1988). The hypothesis is that the defective corpus luteum produces low levels of progesterone which are insufficient to allow proper endometrial development, implantation, and placentation. Studies have shown that peripheral oestrogen (McClure *et al.* 1993), and progesterone levels in some women suffering from recurrent

miscarriage appear to be lower than normal women (Fritz 1988). However, other studies have found no evidence of a relationship between luteal phase defect and miscarriage (Baird *et al.* 1991). There has also been a lack of correlation between plasma progesterone levels and endometrial histology (Rosenfield *et al.* 1980) and it has recently been recognised that endometrial development is influenced not only by peripheral steroid hormone levels, but also by the intrinsic response of the endometrium to those hormones (Li *et al.* 1989).

It has been suggested that polycystic ovary syndrome (PCOS) might be associated with an increased risk of recurrent miscarriage (Sagle *et al.* 1988). In their study 46 out of 56 (82%) women with recurrent miscarriage had PCOS compared with 2 out of 11 (18%) parous controls, and they postulated a hostile endometrial environment or abnormal production of immunoglobulins as possible causes for miscarriage.

Thyroid dysfunction is another commonly quoted cause of recurrent pregnancy loss (Harger *et al.* 1983; Stray-Pedersen and Stray-Pedersen 1984). Thyroid function tests are a traditional part of routine evaluation of the recurrent miscarriage cases. Diabetes mellitus is another traditionally quoted cause. In a study of spontaneous abortion among insulin-dependent-diabetic women the incidence of miscarriage was 15% in women with good diabetic control in early pregnancy and 45% in those with poor control (Miodovnik *et al.* 1985). It has been reported that diabetic women with good metabolic control are probably no more likely to miscarry than non-diabetic women (Mills *et al.* 1988).

Other chronic medical disorders such as epilepsy have been implicated in recurrent pregnancy loss but there is scant evidence for such speculations.

5.1.4. IMMUNOLOGICAL CAUSES

Immunological causes of recurrent miscarriage are probably the least well understood group, because the immunology of the normal pregnancy is yet to be fully described. Both autoimmune and alloimmune immunological mechanisms have been implicated in the aetiology of unexplained recurrent pregnancy loss. The most common autoimmune connective tissue disorder that affects women is systemic lupus erythematosus (SLE) or lupus anticoagulant (anticardiolipin) (Conley and Hartman 1952; Lubbe and Liggins 1988). The presence of serum auto antibodies has been shown to correlate with adverse

pregnancy outcome, and fetal loss may complicate up to 70% of pregnancies in women with SLE (Scott and Bird 1990). 10 to 30% of SLE patients possess antiphospholipid antibodies (APA), and they are at increased risk of fetal loss, especially second and third trimester loss (Branch *et al.* 1985).

Pregnancy immunology is a relatively new science and the mechanisms that an immunocompetent or immunoincompetent fetus surviving in a potentially hostile maternal immunological environment are poorly understood. There are two hypothesised mechanisms whereby the fetus is protected from maternal rejection: either that placental antigens may stimulate the production of blocking factors, or secondly they may alter the maternal immune cell function (Stirrat 1990b). The full story is likely to be a combination of factors.

5.1.5. REPRODUCTIVE TRACT INFECTIONS

The possibility that a bacterial or viral infective organism cause repeated pregnancy loss is very small, and it would have to persist for long periods of time, produce maternal symptoms so slight as to escape diagnosis and treatment, and gain access to the fetal compartment to cause loss either by infection of fetal tissue or by stimulation of an inflammatory response. Previous data suggest that microorganisms can cause individual pregnancy losses, but the role of maternal and fetal infections in recurrent first-trimester loss remains controversial (Watts and Eschenbach 1980).

The other factors that can cause recurrent miscarriage are drug exposure, environmental factors, such as smoking, and family history. For example, it appears that smokers are not only at increased risk of miscarriage, but the risk is directly proportional to the number of cigarettes smoked (Parazzini *et al.* 1991).

Recurrent pregnancy loss appears to be a highly complicated phenomenon that occurs as result of several of the factors described above. It is the aim of the present study to determine the endometrial morphology in patients having persisting recurrent miscarriages in comparison with women that, previously having a recurrent miscarriage problem, recently had a successful pregnancy.

5.2. MATERIALS AND METHODS

5.2.1. SUBJECTS AND EXPERIMENTAL DESIGN

Twelve subjects were used in the present study, 6 individuals in the successful pregnancy group and 6 for unsuccessful pregnancy group. Subjects were recruited from the Recurrent Miscarriage clinic at the Jessop Hospital for Women, Sheffield. The inclusion criterion of subjects into the study was to have a history of 3 or more consecutive miscarriages prior to 28 weeks gestation. There was no apparent cause for repeated miscarriage, since no abnormality was detected after peripheral leukocyte karyotyping, antiphospholipid antibodies, protein S and C concentrations, endocervical culture for chlamydia and mycoplasma and hysterosalpingography (Serle *et al.* 1994). Unsuccessful pregnancy group subjects had 3 or more consecutive pregnancy losses and were currently miscarriers. Successful pregnancy group individuals also previously had 3 or more consecutive miscarriages but then had successful pregnancy and delivery. In the present study, endometrial biopsies from those two groups were compared morphometrically in order to determine the differences in endometrial morphology between previously miscarrying and persistently miscarrying individuals. Endometrial biopsies were carried out at day LH+7 of the menstrual cycle in both groups.

The endometrial biopsy procedure, tissue processing, sectioning and staining procedures for light and electron microscopy were the same as described in the Materials and Methods section of Chapter 2.

5.2.2. STATISTICAL ANALYSIS

In the present study, every endometrial feature was calculated on a 'per individual' basis. Means and standard errors were calculated for each group and data were analysed using Student's unpaired *t*-test where the conventional level of $p < 0.05$ was taken as the level of significance. Morphometric results were compared statistically between recurrent miscarriage groups; the first group (successful pregnancy group) consisted of 6 individuals with a history of 3 or more previous repeated pregnancy losses but having successful pregnancies thereafter and the second group (unsuccessful pregnancy group) ($n=6$) consisted of previously and currently miscarrying individuals.

5.3. RESULTS

In the present study, endometrial stromal and vascular morphologies were examined in two groups of patients; subjects with a persistent recurrent miscarriage problem and subjects who previously had recurrent pregnancy losses but had successful pregnancies thereafter. The quantitative morphological data were compared statistically in order to determine the differences between the unsuccessful pregnancy group and the successful pregnancy group. There were no significant differences in glandular, stromal and vascular features between successful and unsuccessful pregnancy groups, indicating no structural change in human endometrium after successful pregnancy.

5.3.1. GLANDULAR EPITHELIUM

The volume fraction of gland occupied by gland cell was not significantly different between unsuccessful and successful pregnancy groups, nor the volume density of gland to endometrium (Table 5.1). The coefficients of variation in both features were consistently similar within the groups, being around 7% in the volume density of gland cell to gland feature and 30-35% in the volume fraction of endometrium occupied by gland, indicating a higher intersubject variation of the latter feature in both groups than the former feature. In addition, the qualitative investigation of the endometrial glandular epithelium demonstrated nuclear channel systems (NCS) (Figure 5.1) in the unsuccessful pregnancy group at day LH+7, a feature typical of human endometrial glandular epithelial cells between days LH+4 and LH+7 of the menstrual cycle (Dockery *et al.* 1988b).

5.3.2. STROMA

Similar to the volume density of glandular epithelium to stroma, the volume fraction of endometrium occupied by stroma did not differ significantly between groups, nor did the volume density of stromal cell nucleus to endometrium (Table 5.3). The coefficients of variation in the volume density of stromal cell nucleus to endometrium were around 20% in both successful and unsuccessful pregnancy groups, suggesting similar and relatively high intersubject variations in both groups.

Table 5.1: Comparison of glandular features between successful pregnancy group and unsuccessful pregnancy group.

Feature	Successful Pregnancy Group (n=6)	Unsuccessful Pregnancy Group (n=6)
Vv of Gland Occupied by Gland Cell	0.855 ± 0.024	0.861 ± 0.023
Vv of Endometrium Occupied by Gland	0.272 ± 0.040	0.230 ± 0.029

Results are means ± SE

n = number of individuals

Table 5.2: Represents the stromal cell nuclear diameter data compared between successful pregnancy group and unsuccessful pregnancy group.

Feature	Successful Pregnancy Group (n=6)	Unsuccessful Pregnancy Group (n=6)
Stromal Cell Nucleus Profile Major Axis (μm)	7.34 ± 0.28	6.90 ± 0.16
Stromal Cell Nucleus Profile Minor Axis (μm)	3.91 ± 0.09	3.73 ± 0.10
Stromal Cell Nucleus Profile Mean Diameter (μm)	5.28 ± 0.08	5.01 ± 0.10
Stromal Cell Nucleus Profile Axial Ratio	2.06 ± 0.14	1.99 ± 0.06

Results are means \pm SE

n = number of individuals

Table 5.3: Comparison of endometrial stromal volume fraction data between successful and unsuccessful pregnancy groups

Features	Successful Pregnancy Group (n=6)	Unsuccessful Pregnancy Group (n=6)
Vv of Endometrium Occupied by Stroma	0.728 ± 0.040	0.770 ± 0.029
Vv of Endometrium Occupied by Stromal Cell Nucleus	0.132 ± 0.010	0.120 ± 0.011
Vv of Stromal Cell Occupied by Nucleus	0.503 ± 0.010	0.489 ± 0.014
Vv of Stromal Cell Nucleus Occupied by Euchromatin	0.733 ± 0.021	0.732 ± 0.023
Vv of Stromal Cell Occupied by Mitochondria	0.022 ± 0.002	0.025 ± 0.002
Vv of Stromal Cell Occupied by "Secretory Apparatus"	0.026 ± 0.005	0.038 ± 0.003
Vv of Stromal Cell Occupied by RER	0.034 ± 0.005	0.030 ± 0.002

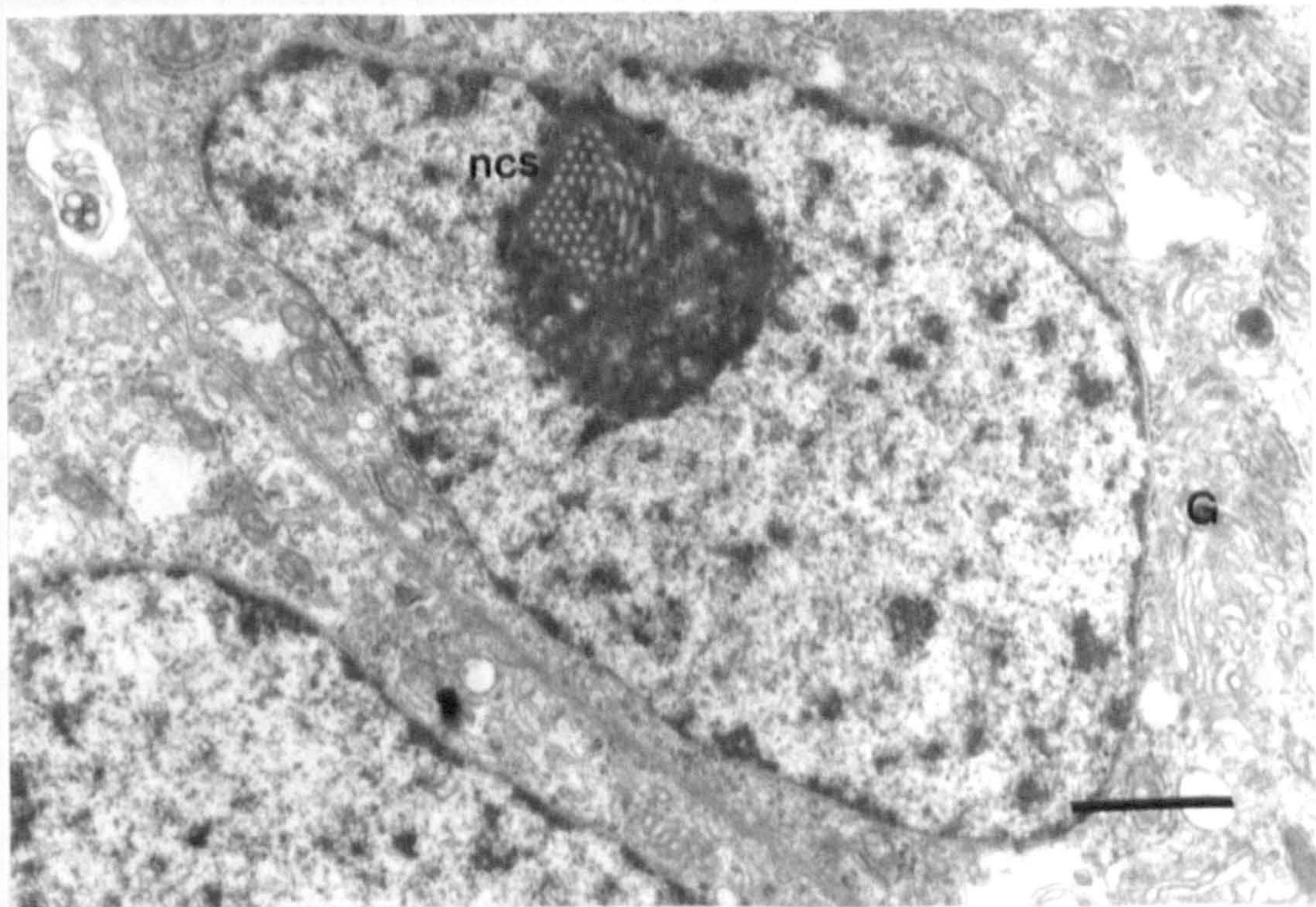
Results are means ± SE

n = number of individuals

Figure 5.1: Unsuccessful pregnancy unexplained recurrent miscarriage group glandular cell which demonstrates a nuclear channel system (ncs). (G) Golgi apparatus.

Staining: Uranyl Acetate - Lead Citrate

Bar represents 1.10 μm



Although the stromal cell nuclear profile dimensions were not significantly different between groups (Table 5.2), the mean profile diameter tended ($p < 0.10$) to be higher in the successful pregnancy group. The mean stromal cell nuclear profile diameter was $5.01 \mu\text{m}$ in the unsuccessful pregnancy group and $5.28 \mu\text{m}$ in the successful pregnancy group (Table 5.2), possibly indicating an increased stromal cellular size and activity. However, it was only a tendency of an increase and such implications are speculative. The coefficient of variation in the stromal cell nuclear profile axial ratio was almost twice as much (17%) in the successful pregnancy group than the unsuccessful pregnancy group (7%) indicating a greater intersubject variation in the successful pregnancy individuals.

There was no significant difference in the volume fraction of stromal cell occupied by nucleus. In addition, the volume density of euchromatin to stromal cell nucleus did not differ between groups nor did the volume density of stromal cell occupied by mitochondria (Table 5.3) (Figures 5.2a,b). On the other hand, there was a tendency of an increase in the volume density of "secretory apparatus" to stromal cell in the unsuccessful pregnancy group possibly indicating an increased secretory activity of the stromal cell in this group. It was 0.026 ± 0.005 in the successful group and 0.038 ± 0.003 in the unsuccessful pregnancy group (Table 5.3). The coefficient of variation of this feature in the successful pregnancy group was about 50% which was almost 3 times greater than the unsuccessful group (20%). Yet again the volume fraction of rough endoplasmic reticulum to stromal cell was not significantly different between groups with a higher coefficient of variation in the successful pregnancy individuals (37%) than the unsuccessful ones (19%).

5.3.3. BLOOD VESSELS

None of the volume fraction of endometrial blood vessel features [which were blood vessel lumen, blood vessel tissue and total blood vessel (vessel + lumen)] significantly differed between groups (Table 5.4), however the coefficient of variation in the unsuccessful pregnancy group was dramatically higher than the successful ones. For example, the coefficient of variation in blood vessel tissue was 8% in the successful group and 47% in the unsuccessful pregnancy group. Similarly, it was 14% and 50% respectively in the total blood vessel volume density, indicating that there was a greater

Table 5.4: Comparison of endometrial vascular volume fraction data between successful and unsuccessful pregnancy groups.

Features	Successful Pregnancy Group (n=6)	Unsuccessful Pregnancy Group (n=6)
Vv of Endometrium Occupied by Blood Vessel Lumen	0.009 ± 0.002	0.013 ± 0.005
Vv of Endometrium Occupied by Blood Vessel Tissue	0.028 ± 0.009	0.030 ± 0.006
VV of Endometrium Occupied by Blood Vessel Total (Vessels + Lumen)	0.037 ± 0.002	0.043 ± 0.009

Results are means ± SE

n = number of individuals

Table 5.5: Comparison of endothelial cell volume fraction data between successful and unsuccessful pregnancy groups.

Features	Successful Pregnancy Group (n=6)	Unsuccessful Pregnancy Group (n=6)
Vv of Endothelial Cell Occupied by Nucleus	0.561 ± 0.015	0.557 ± 0.025
Vv of Endothelial Cell Nucleus Occupied by Euchromatin	0.750 ± 0.026	0.706 ± 0.014
Vv of Endothelial Cell Occupied by Mitochondria	0.019 ± 0.002	0.023 ± 0.003
Vv of Endothelial Cell Occupied by “Secretory Apparatus”	0.034 ± 0.005	0.039 ± 0.004
Vv of Endothelial Cell Occupied by RER	0.017 ± 0.002	0.015 ± 0.002

Results are means ± SE

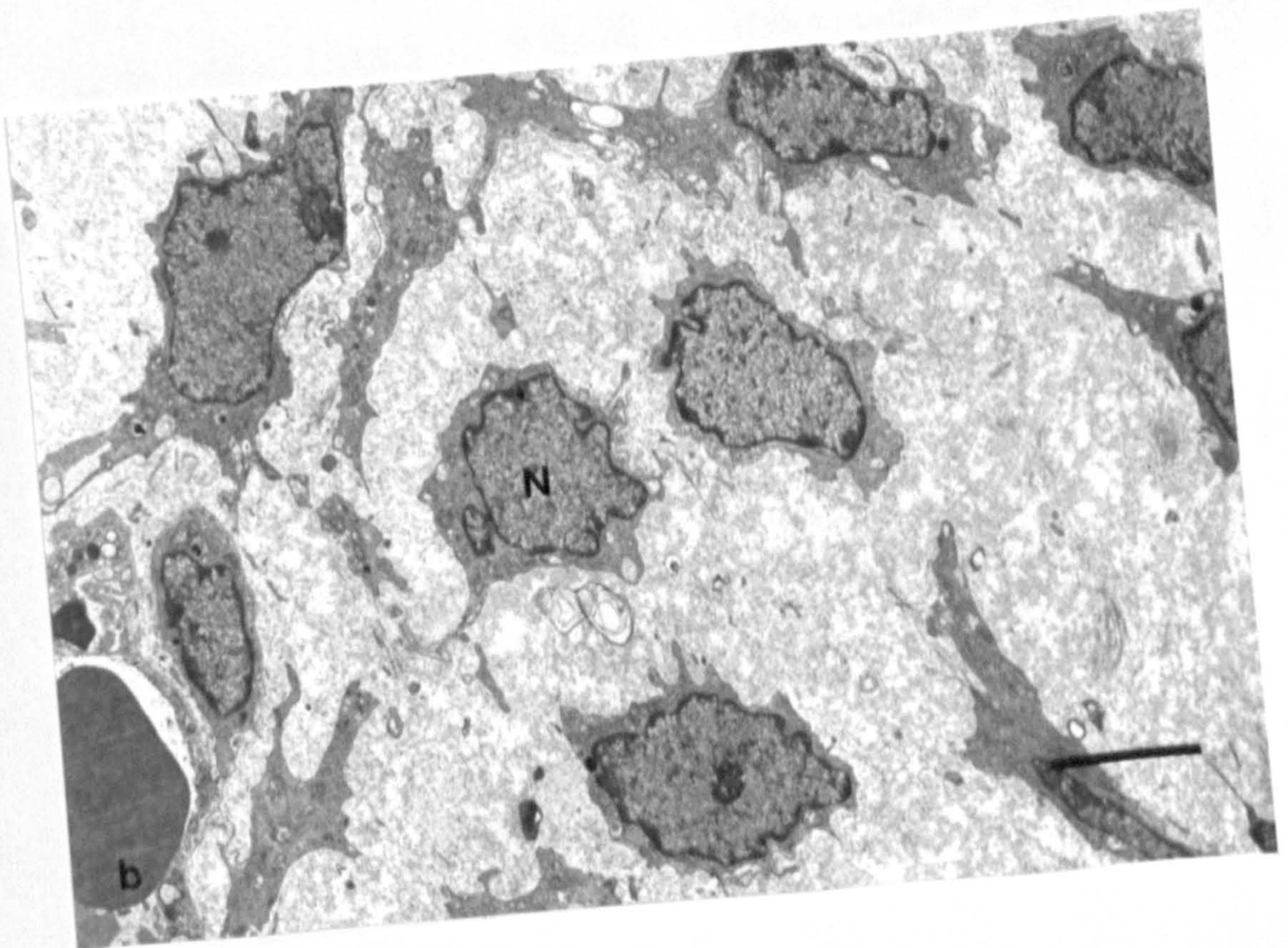
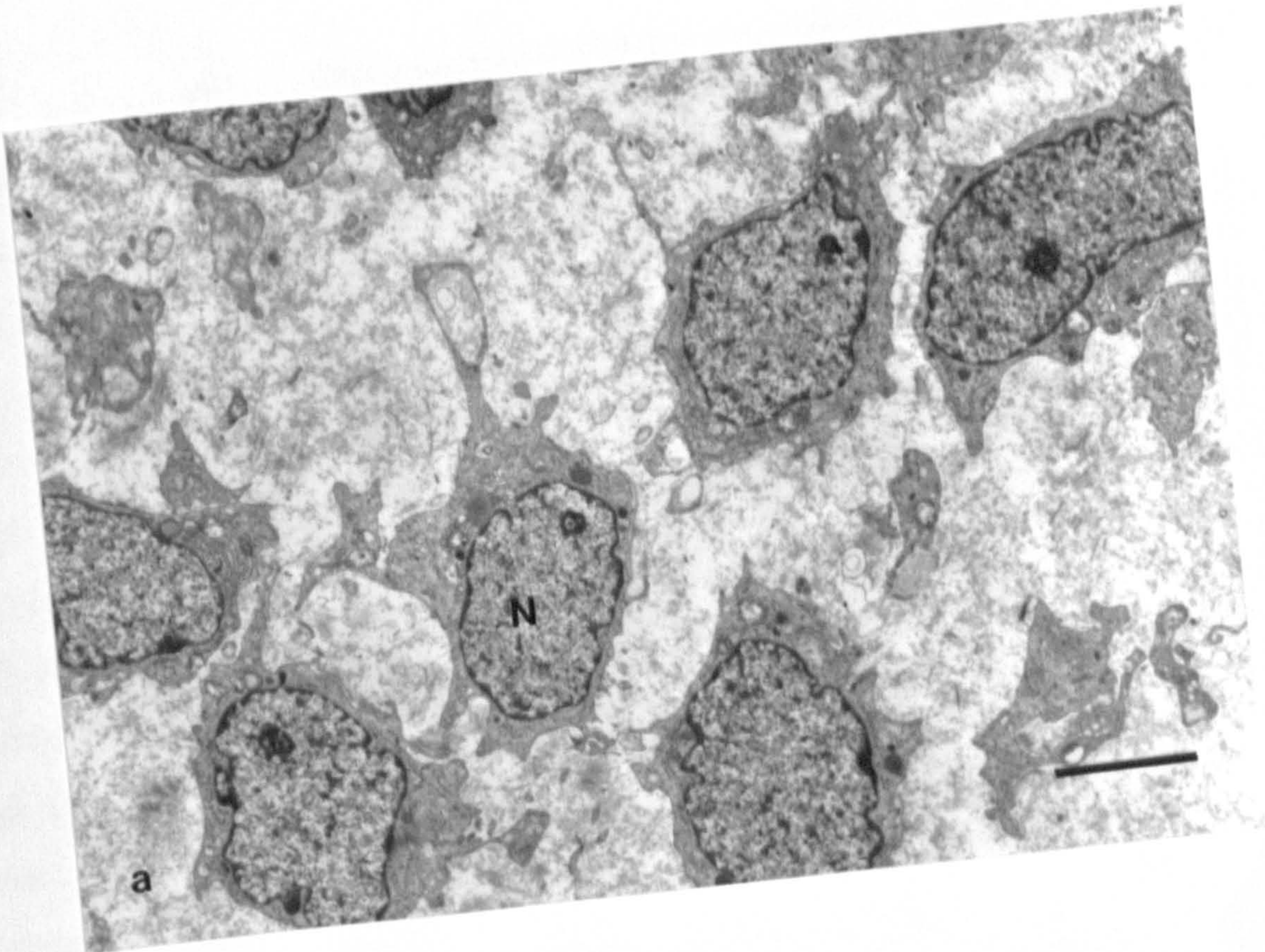
n = number of individuals

Figures 5.2a,b: Unexplained recurrent miscarriage stromal cells from unsuccessful (a) and successful (b) pregnancy groups at day LH+7 of the menstrual cycle. Both groups show euchromatic nuclei (N) and cytoplasmic projections.

Staining: Uranyl Acetate - Lead Citrate

(a) Bar represents 3.95 μm

(b) Bar represents 3.95 μm



intersubject variation in the unsuccessful pregnancy group in this feature. However, the implication of this remain unknown.

The morphometric evaluation of the endothelial cell features did not show any significant differences, similar to stromal cells (Table 5.5). The volume densities of endothelial cell occupied by nucleus and the euchromatin to endothelial cell were not significantly different between groups, indicating no change in the endothelial cell nuclear shape and function. The endothelial cell organelle volume densities again did not show significant differences (Table 5.5). However, while the coefficient of variation in the mitochondrial feature was consistently similar in both groups, the coefficient of variation in the volume density of RER was greater in the unsuccessful pregnancy group (35%) than the successful pregnancy group (23%). In contrast, the coefficient of variation in the volume fraction of “secretory apparatus” to endothelial cell was greater in the successful group (33%) than the unsuccessful one (23%).

5.4. DISCUSSION

The present study has examined the endometrial stromal and vascular morphologies in subjects with recurrent unexplained miscarriage. Two groups of individuals were used; members of the first group previously had 3 or more consecutive pregnancy losses, but then had a successful pregnancy and the other group included patients with a history of 3 or more previous consecutive miscarriages and persistent unexplained miscarriages. These particular infertile individuals were selected for the present study in order to determine whether or not there were any endometrial morphological differences between the 2 groups. To make subjects (unexplained recurrent miscarriers) eligible for the inclusion into the study, common causes for miscarriage [chromosomal anomalies, immunological and bacteriological factors (see earlier)] were excluded. The general hypothesis being that exclusion of other common, identifiable causes from the present study left the endometrial factor as the most likely cause for the miscarriage. However, this of course does not exclude other unknown causes for the persistent miscarriage which would influence the interpretation of the present study.

Results of these two groups were also compared to day LH+6 group results, in order to determine whether or not recurrent miscarriage patients differed from those normal fertile individuals (for comparison Table 2.1 and Table 5.1). The results of the day LH+6 fertile group were not repeated in the result section of this chapter since they were presented in Chapter 2. However, differences between recurrent miscarriage groups and the day LH+6 fertile group will be mentioned in this section in order to give a full profile of the endometrial morphology in persistent recurrent miscarriage and previous miscarriage cases.

Previously miscarrying subjects in the "successful pregnancy group" had had at least one pregnancy with a single birth. However, data on the final outcome of pregnancies from previously miscarrying women remain controversial. Reginald *et al.* (1987) reported that 30% of such babies were small for gestational age, 28% were born preterm, and the perinatal mortality rate was 161/1000 births, indicating a greater risk of these complications in women with the history of recurrent miscarriage. On the other hand, Hughes *et al.* (1991) found no significant differences in the incidence of small-for-gestational age infants, prematurity, low-birth-weight infants and toxemia between a

group consisting of women with a previous history of recurrent miscarriage and the control group. These data could suggest that women with previous miscarriages may still have some abnormalities related to their previous sub-fertility, however such suggestions remain speculative since an abnormal pregnancy outcome could be due to other reasons unconnected with any previous fertility problems.

In the present study, the endometrial stromal and vascular features did not show any significant differences between the 2 groups. However, there were high coefficients of variation in some features, indicating their high intersubject variation within the group. In addition, stromal and vascular features of the two recurrent miscarriage groups differed from those of day LH+6 fertile groups indicating an abnormal endometrial structure in recurrent miscarriage patients, and perhaps more interestingly, it may indicate an abnormal endometrial development in those patients with previous miscarriage history who eventually had at least one successful pregnancy.

5.4.1. GLANDULAR EPITHELIUM

The volume densities of endometrium occupied by gland, and gland cell to gland did not differ between successful and unsuccessful pregnancy groups. However, the coefficient of variation in the gland to endometrium volume fraction was high in both groups indicating a large intersubject variation of this feature in both groups.

In addition to the quantitative examination, qualitative observations of the endometrial gland cells in the unsuccessful pregnancy group demonstrated pre-implantation period characteristics such as the nuclear channel system (NCS). These are spiral channels within the nucleus which are continuous with the inner leaflet of the nuclear membrane. Although their function is still unclear, they consistently appear around day LH+4 and disappear soon after day LH+7 in normally cycling fertile women, under the influence of progesterone (Dockery and Rogers 1989). The presence of nuclear channel systems is crucial for development of normal endometrium, and a recent report by Birkenfeld *et al.* (1991) has suggested that the presence of nuclear channels 'may serve as a fine marker of adequate secretory transformation'. It has also been reported that endometrial NCS formation was delayed in women with unexplained infertility (Dockery *et al.* 1994). In the present study the presence of nuclear channels in

glandular epithelial cells of recurrent miscarriage individuals at day LH+7 may indicate a normal endometrial development, and thus eliminates the possibility any gross distortion in endometrial development causing infertility in these individuals, although it does not rule out some minor delay. In addition there were no significant differences between the day LH+6 fertile group and unsuccessful and successful pregnancy groups, indicating that the glandular compartment of the human endometrium was not a major cause of infertility in patients with recurrent miscarriage.

Serle *et al.* (1994) indicated a retarded endometrial development in recurrent miscarriage patients during the peri-implantation period in a morphological and immunohistochemical study. In their study, 15 out of 25 (60%) recurrent miscarriers had retarded endometrial development accompanied by reduced levels of four mucin-related secretory epitopes, determined by immunohistochemistry. In addition, when the histological dating of the recurrent miscarriage group was compared to the chronological dating (LH dating), there was a 2 day delay on average in the recurrent miscarriage group (Serle *et al.* 1994). In their study, the volume fraction of endometrium occupied by gland was significantly lower in the recurrent miscarriage group with retarded endometrium (22%) than the fertile control group (44%). However, the volume fraction of endometrium occupied by gland was not significantly different between the recurrent miscarriage group with unretarded endometrium and the control group. The volume density of gland occupied by gland cell was significantly higher in the recurrent miscarriage group with retarded endometrium (88%) than in the control group (72%). On the other hand, this feature was not significantly different between recurrent miscarriage group with normal endometrium and the control group. In addition, the number of subnuclear vacuoles per 100 gland cells was dramatically higher in the infertile group with retarded endometrium (4.70 per 100 gland cells) than the controls (0.70 per 100 gland cells) and the recurrent miscarriage group with normal endometrium (also 0.70 per gland 100 cells).

In the present study comparison of endometrial glandular features between the recurrent miscarriage group and the previous recurrent miscarriage group were made. There were no significant differences between these 2 groups. It was not possible to make comparisons between day LH+7 fertile individuals and these recurrent miscarriage

groups, since control biopsies at day LH+7 were not available. However, there were no significant differences in the glandular features between the nearest available biopsies (day LH+6 fertile group) and any of the recurrent miscarriage groups (for comparison see Table 2.1 and Table 5.1). Recurrent miscarriage group biopsies were obtained at day LH+7 and while it may not be entirely appropriate to compare the data between the day LH+6 and the day LH+7 groups, these were the best controls available at the time. Also there were no significant differences between day LH+6 and day LH+8 fertile groups (see Table 2.1), suggesting that it is unlikely that major differences in endometrial glandular values occurred between the day LH+7 and the day LH+6 endometria. This unchanged endometrial glandular morphology may indicate that the reason for repeated pregnancy losses may not lie with endometrial glandular development, but could be due to other unknown pathologies in the stromal and vascular compartments.

Saleh *et al.* (1995) investigated the peri-implantation period luminal epithelial differences between fertile women and women with several types of infertility including recurrent miscarriages using morphometric methods at the light microscopical level. In their study, most of the luminal epithelial features did not differ between the fertile group and the recurrent miscarriage group, apart from the luminal epithelial cell height which was significantly reduced in the recurrent miscarriage group. In addition they found numerically higher coefficients of variation within the recurrent miscarriage group when compared to the fertile group. However, their study was only at the light microscopical level and compared only basic luminal epithelial cell features. In addition, recurrent miscarriage is not a failure of implantation (which may be due to luminal epithelium) but rejection of the embryo some time after implantation and therefore Saleh's report may not provide much useful information to the present study. Endometrial factors in rejection of the implanted embryo are more likely to be due to either blood vessels or stroma.

In the present study, there were no significant differences in glandular features between the persistent recurrent miscarriage group and the group with previous recurrent miscarriages but recent successful pregnancies. Thus, it may be that such high intersubject variations (CVs) could suggest sub-groups within the population studied.

Better diagnosis of these potential sub-groups would be likely to substantially improve our quantitative assessment of these samples.

Although in most recurrent miscarriage cases no apparent reason for pregnancy loss can be found, recent evidence indicates that endometrial function plays an important role for continuation of pregnancy (Stirrat 1990b; Tulppala *et al.* 1993; Li *et al.* 1994). It has been reported that luteal phase defect (LPD) of endometrium was present in 17% of habitual aborters (Tulppala *et al.* 1991). Determination of serum placental protein 14 (PP14) levels is reported to be an accurate and reliable indicator for the diagnosis of LPD (Klentzeris *et al.* 1994; Tulppala *et al.* 1995; Dalton *et al.* 1995). It has been suggested that in the luteal phase of the menstrual cycle, serum PP14 levels were lower in patients with habitual abortion than in fertile control women, but the differences in PP14 levels between habitual aborters with or without LPD were not significant (Tulppala *et al.* 1995). They speculated that “deficient production of endometrial PP14 may result in an altered immunological environment during the early phases of pregnancy”. Dalton *et al.* (1995) also suggested a correlation between retarded endometrium and lower PP14 levels in patients with recurrent miscarriage. However, the explanation for these phenomena in human fertility still remains unknown, and more effort is needed for a fully understanding on this field of human reproductive biology.

5.4.2. STROMA

The volume fraction of endometrium occupied by stroma was not significantly different between successful and unsuccessful pregnancy groups and this result was consistent with the unchanged glandular volume density. The volume fraction of stromal cell nucleus to endometrium did not change when the successful and unsuccessful pregnancy groups were compared statistically, and when considered with the data above, this could suggest an unchanged packing density of stromal cells in recurrent miscarriage individuals. The coefficients of variation in both successful and unsuccessful pregnancy groups were around 20% which was relatively high.

The stromal cell nuclear profile dimensions showed no significant differences between the 2 groups, although there was a tendency of an increase in the stromal cell mean profile diameter in the unsuccessful pregnancy group. This numerically higher (5%)

stromal cell nucleus mean profile diameter in the successful pregnancy group could suggest an increased nuclear size and activity in this group. However, on average the volume densities of stromal cell occupied by nucleus and euchromatin to stromal cell nucleus did not differ significantly between groups, indicating no change in the stromal nuclear volume and transcriptional rate. In addition, the stromal cell profile axial ratio did not differ between groups indicating no change in the stromal cell nuclear shape. The coefficient of variation in the stromal cell nuclear axial ratio was more than two fold that of the successful pregnancy group (17%) when compared to the unsuccessful group (7%), indicating a greater intersubject variation in the former group.

The volume fraction of stromal cell occupied by mitochondria did not differ between groups suggesting that there was no change of metabolic demand between persistently miscarrying and previously miscarrying individuals. Similarly, the volume density of RER to stromal cell was not different between groups, again indicating no change of synthetic activity in stromal cells. On the other hand, the volume density of "secretory apparatus" to stromal cell tended to be higher in the unsuccessful pregnancy group, suggesting an increased secretory activity or membrane turnover between the two groups. Although the numerical difference between mean values was relatively high (31%) it was not significant, probably due to the high coefficients of variation of this feature in the successful pregnancy group (50%) and unsuccessful ones (20%). In addition, the coefficient of variation in the volume fraction of stromal cell occupied by RER in the successful pregnancy group (37%) was double that of the unsuccessful one (19%).

In contrast to the comparison between two recurrent miscarriage groups, there were significant differences between the day LH+6 fertile group and both recurrent miscarriage groups (see Tables 2.2 - 2.3 and Tables 5.2 - 5.3 for details). For example the mean profile diameter of the stromal cell nucleus was significantly higher in the successful pregnancy group than the day LH+6 group. This feature was not significantly different between the fertile group and the unsuccessful pregnancy group. Since the results were already presented in previous sections, they have not been repeated here. In addition, while some stromal features were significantly different between the day LH+6 group and the unsuccessful pregnancy group, some differed significantly between fertile

group and successful pregnancy group, indicating an unstable pattern of results between groups. These differences may also suggest an abnormal endometrial stromal development in the recurrent miscarriage patients, which could affect the later stromal cell differentiation (decidualization).

5.4.3. BLOOD VESSELS

At the light microscope level, none of the endometrial blood vessel features differed significantly between groups, however, the coefficients of variation in these features (blood vessel lumen, tissue and total blood vessel volume densities) were consistently higher in the unsuccessful pregnancy group than the successful ones, indicating a greater range of endometrial vascularization in the persistently miscarrying individuals. This high intersubject variation in the unsuccessful pregnancy group could be due to different underlying pathologies in individuals allocated to the same group.

At the electron microscope level, the morphometric evaluation of the endothelial cell features showed no significant differences between recurrent miscarriage groups. However, the coefficients of variation in some features were relatively high in both groups, indicating a high intersubject variation within groups. Although there were no significant differences between the 2 recurrent miscarriage groups in blood vessel endothelial cell features, some features in both groups differed significantly when compared to the day LH+6 fertile group. For example, the volume fraction of endothelial cell nucleus occupied by euchromatin was significantly lower in recurrent miscarriage groups than the fertile group, indicating a lower level of transcription in endothelial cell nucleus in both recurrent miscarriage groups. This low transcriptional rate in endothelial cell was consistent with a decreased volume fraction of endothelial cell occupied by RER in both recurrent miscarriage groups. These differences in endothelial cell synthetic activity may indicate an involvement of blood vessels in the failure of the pregnancy and finally rejection of the embryo by endometrium.

Rejection of the embryo occurs after implantation and placentation has begun; it is convenient to now review the events that take place during these stages of a conception cycle. The depth of trophoblastic invasion is influenced by the degree of decidual response. Where the decidual response is inadequate, invasion is much more aggressive

and penetrating (Johnson and Everitt 1988). The maternal blood vessels in the uterus are eroded to nurture the trophoblastic cells in maternal blood. With the formation and invasion of the decidua, implantation is completed, a physical hold and a nutritional source of decidua is established, and the basis of placental development (leading to adjacent blood circulations and exchange of nutrients) is initiated (Guillomot *et al.* 1993).

As the *mesoderm* of the conceptus becomes more extensive, it develops blood vessels and forms an extensive vascular network. Blood formation occurs in the yolk sac *mesoderm* and a primitive heart forms in the cardiac *mesoderm* within the developing embryo itself. With further development, the vascularity in the conceptus becomes particularly marked in the yolk sac mesoderm and where the yolk sac and chorionic mesoderm fuse together. A corresponding vascularity develops within the endometrium adjacent to this site of fusion, and together the two adjacent, highly vascular sites form the placenta (Johnson and Everitt 1988), with the decidua on the maternal site of the placenta. The functional chorio-allantoic placenta is characterized by 1) extensive proliferation of the chorionic tissue to form villi that penetrate into, and interdigitate with, the endometrial tissue, and 2) by highly developed vascularity of both fetal and maternal components (Johnson and Everitt 1988). These developments in fetal and maternal sites are important to mention in this section, since the loss of pregnancy occurs during this time of the pregnancy, and any abnormality in these processes could lead to the miscarriage of the embryo.

Miscarriage is not a failure of implantation, but it is the rejection of already implanted embryo. Therefore stromal and vascular compartments, rather than glandular and luminal epithelia, are likely to be major endometrial contributors for the rejection of embryo, and thus failure of the pregnancy. Results of the present study generally agree with this hypothesis that recurrent miscarriage individuals differed from the fertile group at the stromal cell and blood vessel levels, indicating the importance of these compartments throughout the pregnancy process.

Although there are a small number of reports dealing with the endometrial morphology in women with unexplained recurrent miscarriage, these reports mainly involve the morphology of glandular and luminal epithelial compartments of the human

endometrium at the light microscopical level. The present study appears to be the first one dealing with the morphological changes that occur in the endometrial stromal and vascular components. In addition, it is the first one to examine endometrial glands using objective stereological methods at the light and electron microscope levels in cases of unexplained recurrent pregnancy losses.

Endometrial secretions may also play a big role in the recurrent miscarriage. The precise role of the secreted factors is unknown, but they are postulated to have a role in embryo nourishment and implantation, and therefore may be essential for successful pregnancy (Dalton *et al.* 1995). Endometrial secretory activity may be important in conditions such as recurrent miscarriage where the problem may be due to endometrial dysfunction. Proteins such as PP 14 (Tulppala *et al.* 1995) and CA 125 (Klentzeris *et al.* 1994) are secreted from endometrial glands, and are believed to play crucial role in the continuation of a conception cycle. These endometrial secretions must be considered as potential factors of repeated pregnancy losses. Several sub-groups were also indicated by Serle *et al.* (1994) within the recurrent miscarriage groups consisting of patients with retarded endometrium and patients with unretarded endometrium. In the present study, high coefficients of variation in some endometrial features support this hypothesis, and inclusion criteria into the recurrent miscarriage groups should be precisely chosen in such studies.

In summary, there were no significant differences in the stromal and vascular parts of the endometrium between successful and unsuccessful pregnancy groups. However, the coefficients of variation of most of the endometrial features were higher in the unsuccessful pregnancy group than the successful pregnancy group whereas in some features, it was higher in the successful pregnancy group. It can be concluded that the stromal and vascular morphologies did not differ significantly around the time of implantation in women with persistent pregnancy losses from those who had previous recurrent miscarriages but had successful pregnancy. On the other hand, these recurrent miscarriage groups differed significantly from the day LH+6 fertile group, suggesting an abnormal endometrial development at the stromal and endothelial cell levels in recurrent miscarriage individuals. However, examination of these endometrial compartments at the later stages of the menstrual cycle may be more appropriate since crucial events

(such as decidualization) take place towards the end of a conception cycle. In addition, more effort is needed for a complete understanding of mechanisms that cause the recurrent pregnancy loss and complicated interactions between steroid hormone levels, endometrial structure and function (especially endometrial secretions), and the development of maternal and fetal tissues such as decidua formation and placentation in the case of recurrent miscarriage.

CHAPTER 6

ESTABLISHMENT OF A 3-DIMENSIONAL TISSUE CULTURE

MODEL FOR HUMAN ENDOMETRIAL STROMAL CELLS

6.1. INTRODUCTION

The mechanisms of recognition and adhesion of the blastocyst to the endometrial epithelium and embedding of the embryo into the endometrial stroma are poorly understood phenomena in Man. The precise cytological events which occur during interaction between embryo and endometrium are not known, and direct *in vivo* microscopic studies of the structural changes that occur in the endometrial epithelium and stroma during implantation and gestation of a conception cycle are virtually impossible in humans for both ethical and practical reasons. *In vivo* animal experiments may be of some use in these studies, but remain limited since the processes of implantation in animals is very different in detail from those in Man. *In vitro* endometrial cell culture models using human tissue have been reported by several investigators (e.g. Luginbuhl 1968; Bongso *et al.* 1988; Hill *et al.* 1993 and 1994; Bentin-Ley *et al.* 1994) in an attempt to overcome the existing difficulties in the understanding of the cellular events that take place in the human endometrium around the time of implantation, and also during the later stages of the pregnancy involving gestation and placentation.

Previous studies on *in vitro* human endometrial morphology have concentrated on epithelial cells and were performed using organ culture systems (Luginbuhl 1968), traditional monolayer cell culture techniques (Liszczyk *et al.* 1977; Bongso *et al.* 1988) and three-dimensional cell culture methods (Bentin-Ley *et al.* 1994; Hill *et al.* 1994) that allow cells to grow in polarized conditions so they appear similar to those seen *in vivo*. It is well established that the morphology of epithelial cells in monolayer culture does not resemble to those seen *in vivo* and they do not demonstrate a full complement of cellular structures such as apical surface structures and cellular polarization whereas those in three-dimensional culture systems closely resemble cells *in vivo* (Hill *et al.* 1994). However, there is less published information on stromal cells *in vitro*.

The present thesis has examined the effects of various hormonal influences on the stromal compartment of endometrium. However, it is ethically and practically impossible to examine detailed effects of repeated hormone treatment on the same women during the same cycle *in vivo*. Therefore in this chapter an experiment is reported that was designed to produce a novel *in vitro* model system of human stromal cells and to use it to

examine the effects of hormonal administration on cultured cells and where possible to relate it to *in vivo* conditions reported previously.

6.1.1. ENDOMETRIAL TISSUE AND CELL CULTURE MODELS

The first endometrial *in vitro* model was described by Ehrmann *et al.* (1961) using endometrial tissue explants . In this method, endometrial curettings or strips from hysterectomy specimens were cut into 1 or 2 mm fragments and placed in Petri dishes covered with gel surfaces and then a nutrient agar (medium) was added to the culture dishes. These so-called tissue explants were cultured in this media for up to 10 days and were used for specific purposes such as morphological investigation (Luginbuhl 1968). However, endometrial tissues cultured with this method had a poor survival rate and the cells in the stromal compartment of human endometrium had particularly poor survival rates in this culture system (Luginbuhl 1968).

Traditional monolayer cell culture has also been used for morphological and especially for physiological studies. In this technique, cells were grown in culture dishes containing nutrient medium and additional substances such as steroid hormones (Liszcak *et al.* 1977; Bongso *et al.* 1988). It has been suggested that the morphology of cells grown in monolayer culture were similar to those seen *in vivo* (Liszcak *et al.* 1977). However, polarization of the epithelial cells (markers of the cell polarization include apical surface specializations such as cilia and microvilli) is not achieved in monolayer culture systems as indicated by microscopic studies (Hill *et al.* 1994).

In recent years, three-dimensional cell culture models have been developed (Hill *et al.* 1994; Bentin-Ley *et al.* 1994). In these culture systems, epithelial cells are grown on or in an artificial ECM in a multiwell culture plate using a millicell insert that contains a filter at the bottom allowing the cells to be nourished from their bases. It has been shown that epithelial cells grown with this technique are morphologically much closer to those *in vivo* than the cells in monolayer culture (Hill *et al.* 1994). Bentin-Ley *et al.* (1994) used a slightly different endometrial cell culture model for a three-dimensional growth of epithelial cells. In their model, stromal cells were seeded onto a collagen gel and epithelial cells were cultured on top of this stromal cell layer. Microscopical

observations showed a polarized growth of epithelial cells with abundant apical surface specializations.

In recent years human endometrial stromal cell differentiation (including decidualization) and the production of some cytokines and growth factors (such as interleukins) by decidual cells has become an important area of interest in human reproductive biology. For the reasons mentioned earlier, human *in vivo* studies on this subject can provide little information. Therefore new *in vitro* stromal cell culture models are needed for a better understanding of the events that take place in the human endometrial stromal compartment under the influence of sex steroids in the menstrual cycle and during pregnancy. One aim of the present study was to grow stromal enriched human endometrium in three-dimensional culture as a model for *in vivo* conditions. The second aim was to use this model to study the effects of oestrogen and progesterone on stromal cell morphology at light and electron microscope levels. This should be regarded as a preliminary study on human endometrial stromal cell morphology in a novel three-dimensional culture model and so a large part of the study deals with the technical aspects of the culture system such as the stromal and epithelial cell separation techniques. However, preliminary reports on the qualitative morphological appearance of the stromal cells obtained with this culture model both with and without hormonal supplementation are also included.

6.2. MATERIALS AND METHODS

6.2.1. SUBJECTS AND EXPERIMENTAL DESIGN

Endometrial biopsy tissue from 5 individuals were used in the present study; 2 were taken in the proliferative and 3 in the secretory phase of the menstrual cycle. Biopsies were obtained with informed consent and Ethic's Committee approval from subjects attending the Jessop Hospital for Women, Sheffield (see Table 6.1 for details of the subjects used). From each biopsy a piece of tissue was fixed in 3% glutaraldehyde in 0.1M phosphate buffer, and processed for standard electron microscopical observations (see earlier). This specimen provided a morphological reference, and was designated the "original biopsy". It was used to make comparisons, where possible, between endometrial stromal cell structure *in vitro* and normal human stromal cell morphology *in vivo*. The remaining tissue was processed for *in vitro* tissue culture experiments (see later). The first aim of the present study was to produce a good *in vitro* model of stromal cells *in vivo*. In addition, the morphological differences between proliferative phase and secretory phase endometrial stromal cell primary cultures were evaluated. The effects of ovarian sex steroids on the stromal cells *in vitro* were also examined at each stage of the menstrual cycle.

Table 6.1: Shows the age, cycle length and biopsy date of subjects used in the present study.

Subjects	Age (years)	Cycle Length (days)	Biopsy Date (in cycle)
1	33	28	15 (Secretory)
2	39	28	21 (Secretory)
3	48	28	18 (Secretory)
4	38	28	2 (Proliferative)
5	47	26	8 (Proliferative)

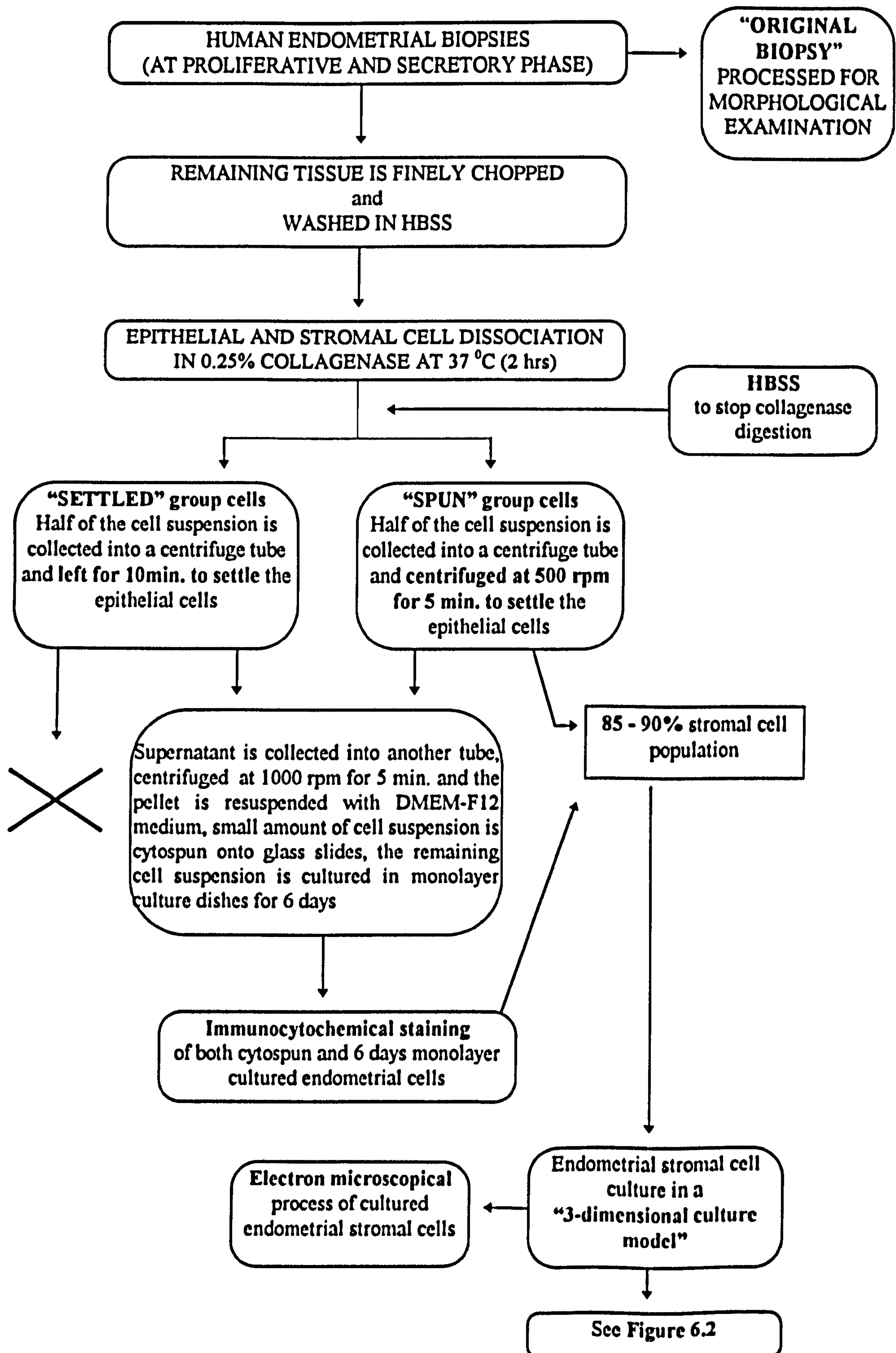
6.2.2. TISSUE CULTURE

6.2.2.1. PILOT STUDY: SELECTION OF THE BEST METHOD FOR STROMAL CELL SEPARATION

At the beginning of the present study, two separation techniques were performed to decide which gave the best cell separation method for obtaining the stromal cell rich cultures. These are described below (also see Figure 6.1).

Endometrial biopsies were removed using a Sharmann's curette (Downs Surgical, Sheffield, UK) by an experienced gynaecologist, and following this procedure endometrial tissues were washed with several changes of 10% Hank's balanced salt solution (HBSS, Life Technologies Ltd., Paisley, Scotland) in distilled water to remove any blood, non-endometrial tissue and cellular debris. The tissue was then chopped finely with a sharp surgical blade and washed further in 10% HBSS. The finely chopped tissue was placed in dissociation media consisting of 0.25% collagenase (Sigma Chemicals Co., UK) in HBSS to remove connective tissue. The mixture was incubated in a water bath at 37 °C for 2 hours with mechanical disruption intermittently by a vortex. After 2 hours of dissociation, the supernatant was removed into two centrifuge tubes to which 10% HBSS was added to stop further collagenase digestion. The first tube ("settled" tube) was left for 10 minutes to settle down the epithelial cells which are larger and heavier than the stromal cells, whereas the second tube ("spun" tube) was centrifuged at 500 rpm for 5 minutes. Then supernatants from both tubes were collected separately into 2 other tubes, and further centrifuged at 1000 rpm for 5 minutes. One ml of Dulbecco's Hams F12 (DMEM-F12, Life Technologies Ltd., Paisley, Scotland) media was added into each tube and 0.5 ml of the resulting cell suspensions were seeded onto coverslips in monolayer culture dishes. The remaining 0.5 ml of cell suspensions were cytopspin (Cytospin, Shandon Southern, UK) onto glass slides (at 400-500 rpm speed for 2 minutes) and stained with cytokeratin antibody for epithelial cell demonstration and vimentin antibody for stromal cell demonstration (see next section for details of the immunocytochemical staining protocol). Cells in monolayer culture dishes were also processed for immunocytochemistry following 6 days of incubation. This last procedure was performed to examine if the proportion of epithelial and stromal cells

Figure 6.1: Flow chart summarises the human endometrial stromal cell separation and culture procedures.



changed after 6 days of culture and to determine the final outcome of the cell types. Results of both cell separation methods were evaluated, and it was decided to use the second (“spun”) method for the rest of experiments.

6.2.2.2. PREPARATION OF CELLS IN 3-DIMENSIONAL CULTURES

The pellet containing stromal cells (after centrifugation at 1000 rpm for 5 minutes) was collected and resuspended in DMEM-F12 culturing media (amount of media used depended on the volume of the cells and ranged from 2 ml to 5 ml). Half millilitre of cell suspension was seeded into each Matrigel (Collaborative Biomedical Products, Bedford, USA) coated millicell insert (Millipore, U.K.) (see Appendix 2) and 0.5 ml of DMEM-F12 media was placed around the millicell insert (Figure 6.2a). The multiwell culture plate (Costar, USA) was then placed in an incubator with an atmosphere of 5% CO₂ and 95% air at 37 °C. Medium was changed every 2 days and the culturing time for these stromal cells was approximately 6-7 days (see Figure 6.1). Following 6 days in culture, cells were washed with pre-warmed 0.1 M phosphate buffer and pre-warmed 3% glutaraldehyde was added into culture plates (the remaining procedures such as tissue fixation, dehydration, embedding, sectioning and staining protocols for electron microscopy was the same as described in Chapter 2 Materials and Methods section. Except that the insert was cut up into smaller pieces before being embedded along with the cells it contained). The separation procedure of the stromal cells from epithelial cells was slightly modified from the report by Bongso *et al.* (1988).

6.2.3. CYTOKERATIN AND VIMENTIN IMMUNOSTAINING METHOD (ALKALINE PHOSPHATASE - ANTI-ALKALINE PHOSPHATASE : APAAP TECHNIQUE)

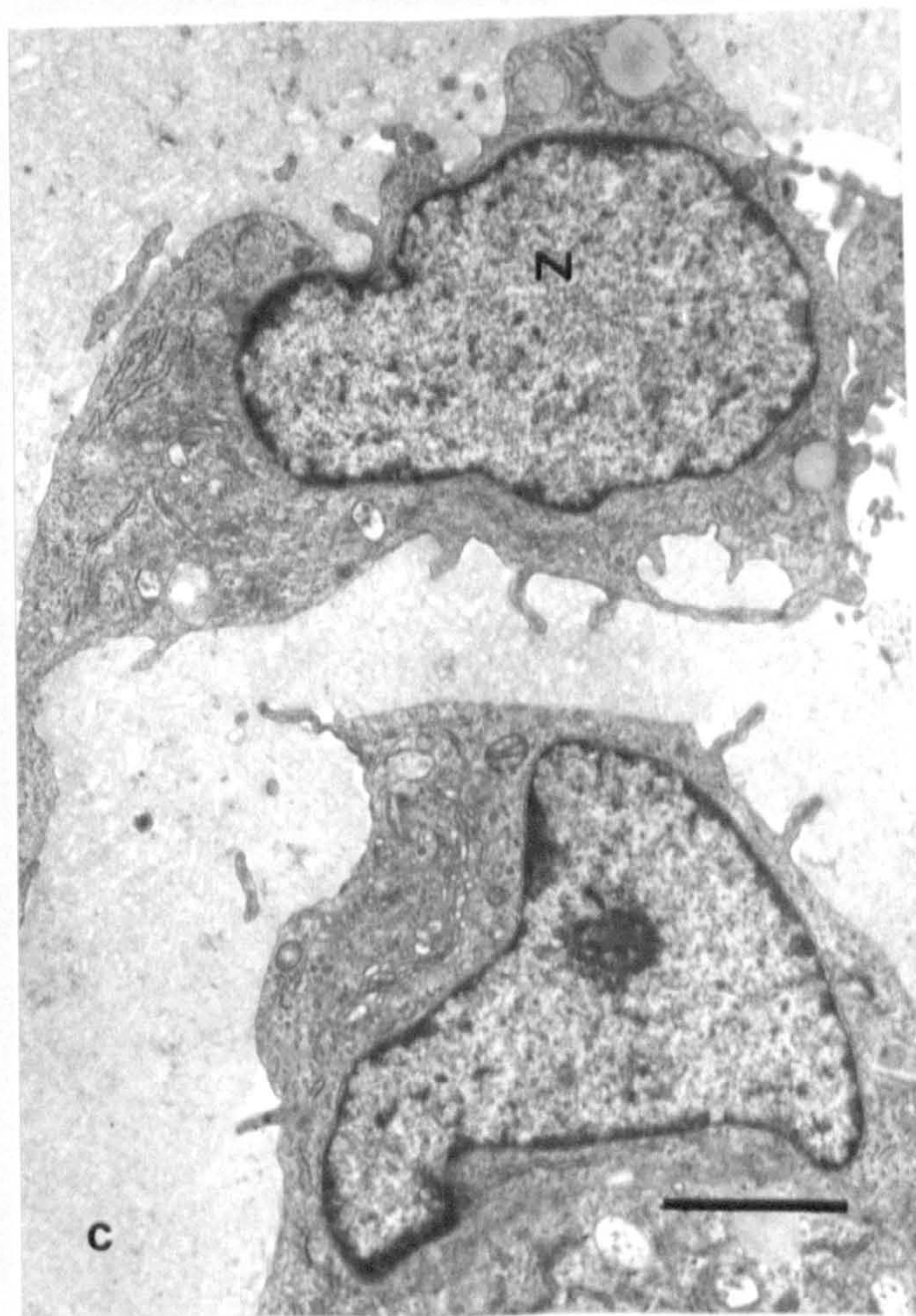
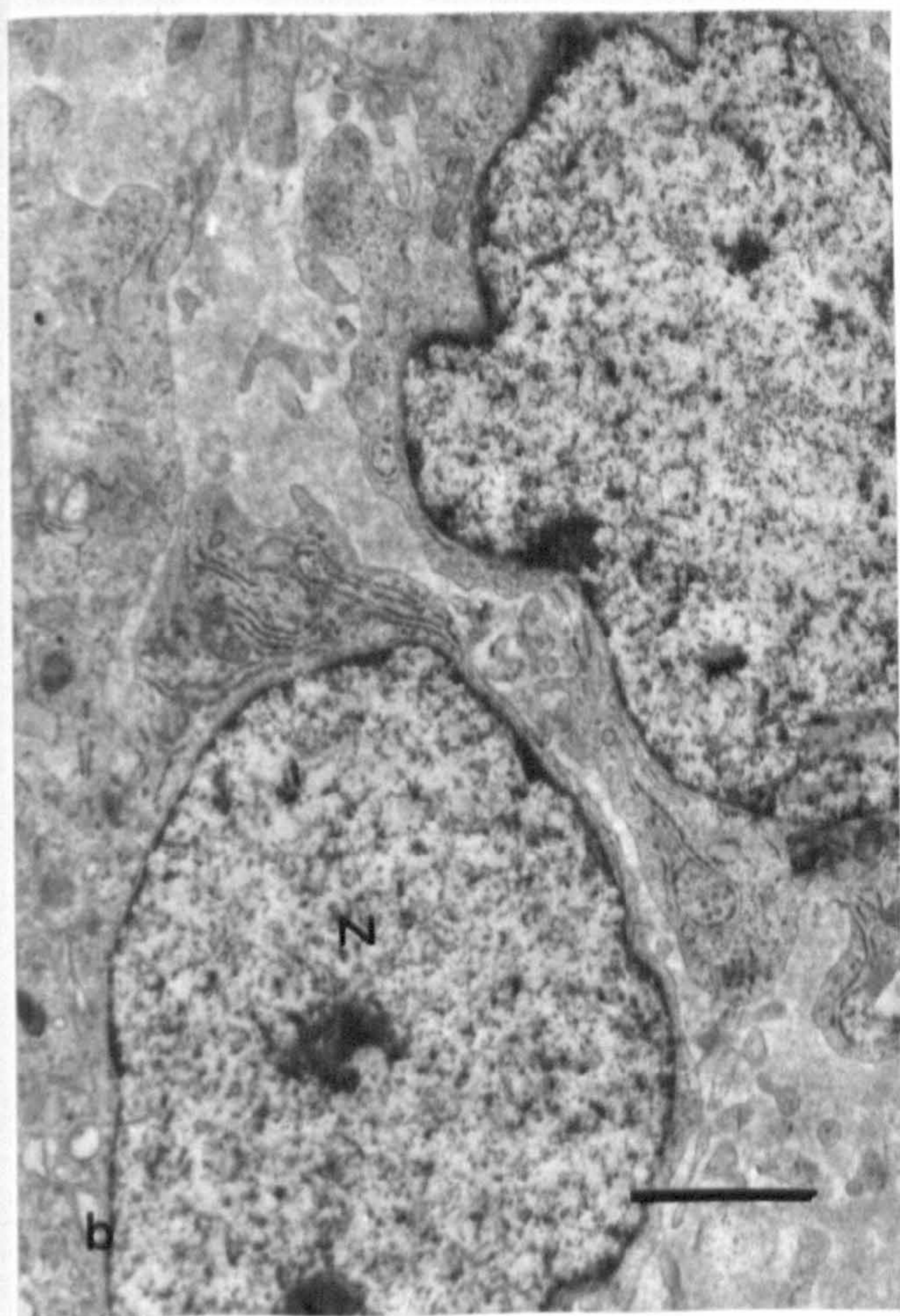
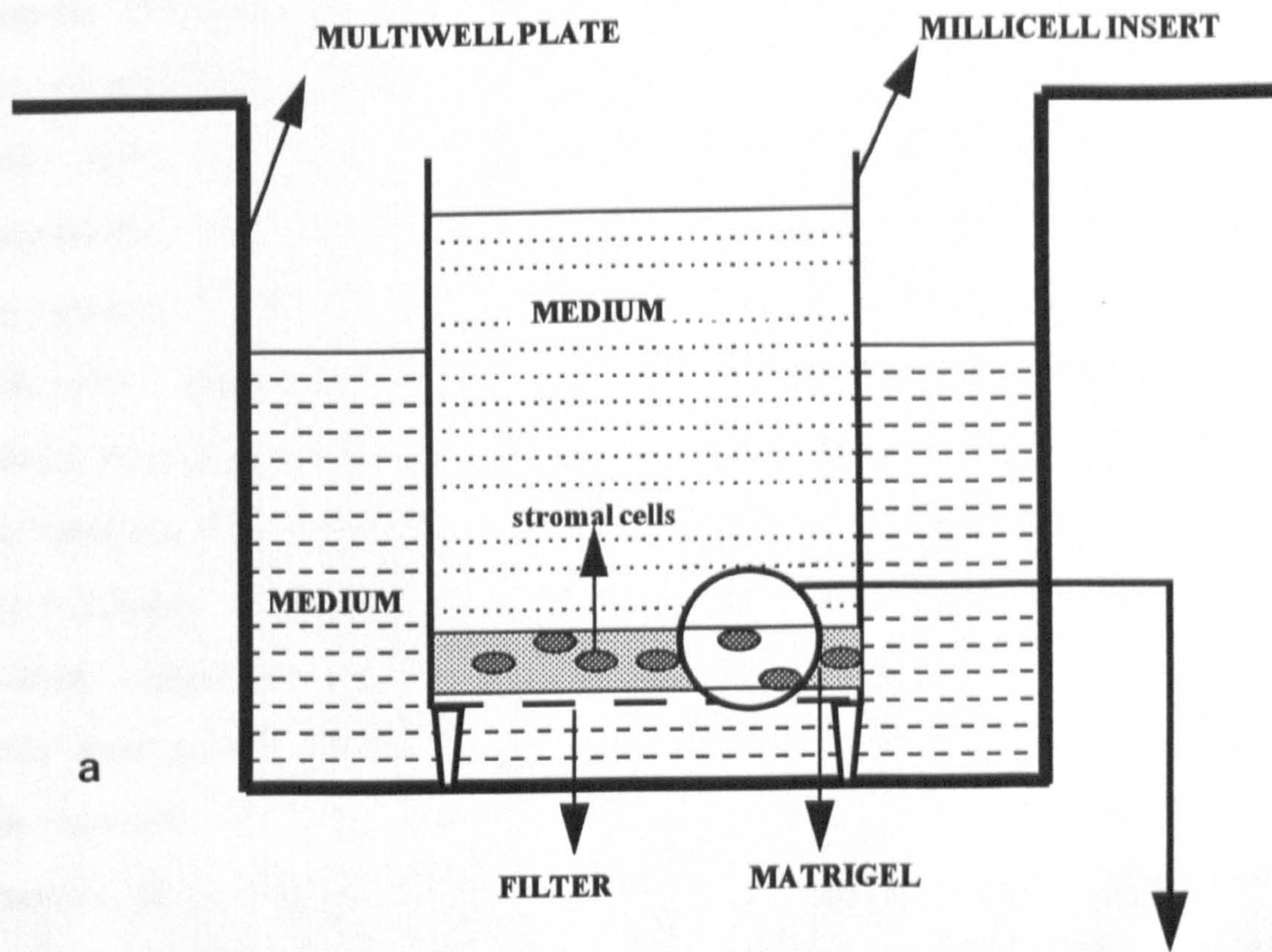
Immunocytochemical staining (APAAP) procedure of human endometrial cells was modified from the report by Cordell *et al.* (1984). Cytospun cells and cultured cells in monolayer culture dishes were fixed in pre-cooled 50:50 methanol/acetone fixative for 1 minute 30 seconds. Cells were then washed in TBS (tris buffered saline) (pH 7.6) for 5 minutes. Approximately 30 µl of monoclonal antibody [either anti-cytokeratin (Monoclonal Anti-Pan Cytokeratin: Sigma Immunochemicals, UK)- at 1:100 dilution or

Figures 6.2a,b,c: (a) demonstrates the diagram of the 3-dimensional culture system used in the present study. Figures 6.2b and (c) show electron micrographs of stromal cells taken from *in vivo* and *in vitro* samples respectively. The appearance of these cells is generally similar. (N) nucleus.

Staining: Uranyl Acetate - Lead Citrate

(b) Bar represents 1.75 μm

(c) Bar represents 1.80 μm

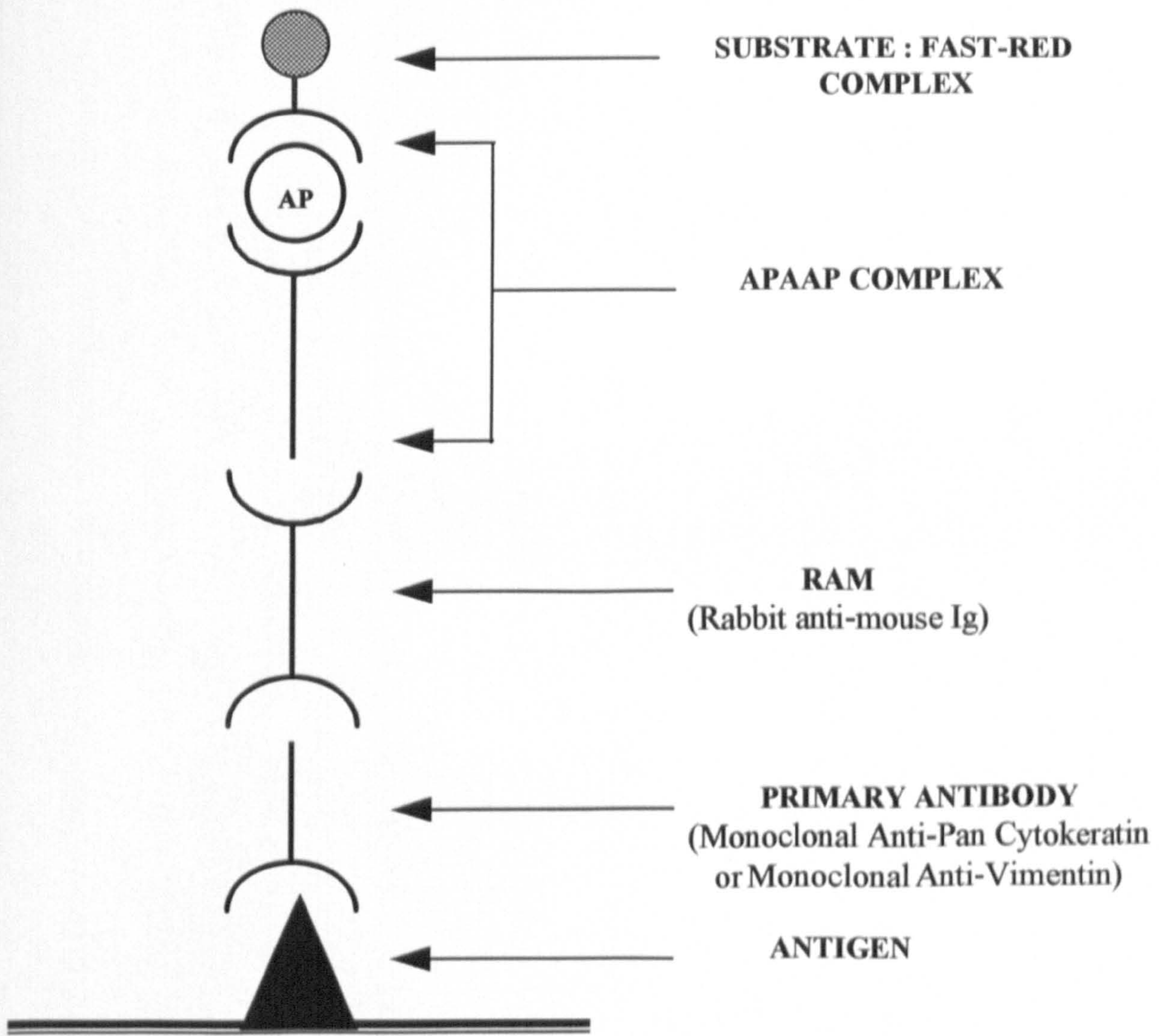


anti-vimentin (Monoclonal Anti-Vimentin: Sigma Immunochemicals, UK)- at 1:50 dilution] was applied for 2 hours at room temperature then washed in TBS for 5 minutes. The cells were incubated in rabbit anti-mouse (RAM - Rabbit Anti-Mouse Immunoglobulins, Dako A/S, Denmark) polyclonal antibody (preparation of RAM is given in Appendix 2) at a dilution of 1:20 (30 μ l) for 30 minutes at room temperature, and cells were washed in TBS for 5 minutes. Cells were then incubated in mouse monoclonal alkaline phosphatase - anti-alkaline phosphatase complex (APAAP: Sigma Immunochemicals, U.K.) (Figure 6.3) for 30 minutes at room temperature and washed in TBS for 5 minutes. RAM and APAAP steps of the procedure were repeated to amplify the reaction. Substrate-Fast Red solution (10 mg of Fast Red TR-Salt in 10 ml of substrate) were applied for approximately 15 minutes (preparation of the substrate is given in Appendix 2). The cells were washed in running water, counter stained with haematoxylen for a few minutes and then mounted with glycerine. Cytokeratin or vimentin positive cell cytoplasm were stained in orange or red colour (Figures 6.5a,b).

6.2.4. OESTROGEN AND PROGESTERONE SUPPLEMENTATION OF HUMAN ENDOMETRIAL STROMAL CELLS IN CULTURE

Steroid hormone administration of the stromal cell culture was performed in order to determine the effects of oestrogen and progesterone on stromal cell ultrastructure *in vitro*. To do this, 0.5 ml of DMEM-F12 media containing oestrogen and/or progesterone hormones (in 10^{-6} M dilution) was added into the culture plates. This procedure was repeated in every change of media, which occurred every 2 days. Oestrogen used in the present study was β -estradiol (Sigma Chemicals, UK), and a synthetic progesterone (Sigma Chemicals, UK) was used. Both hormones were dissolved in absolute ethanol (up to the 10^{-2} M dilution), and these 10^{-2} M solutions were diluted to a final concentration of 10^{-6} M using DMEM-F12 medium. For each biopsy, one set of hormone supplementation protocol was performed in a multiwell culture plate; e.g. 1 well for oestrogen, 1 for progesterone, 1 for oestrogen + progesterone combination and one for unsupplemented cell growth.

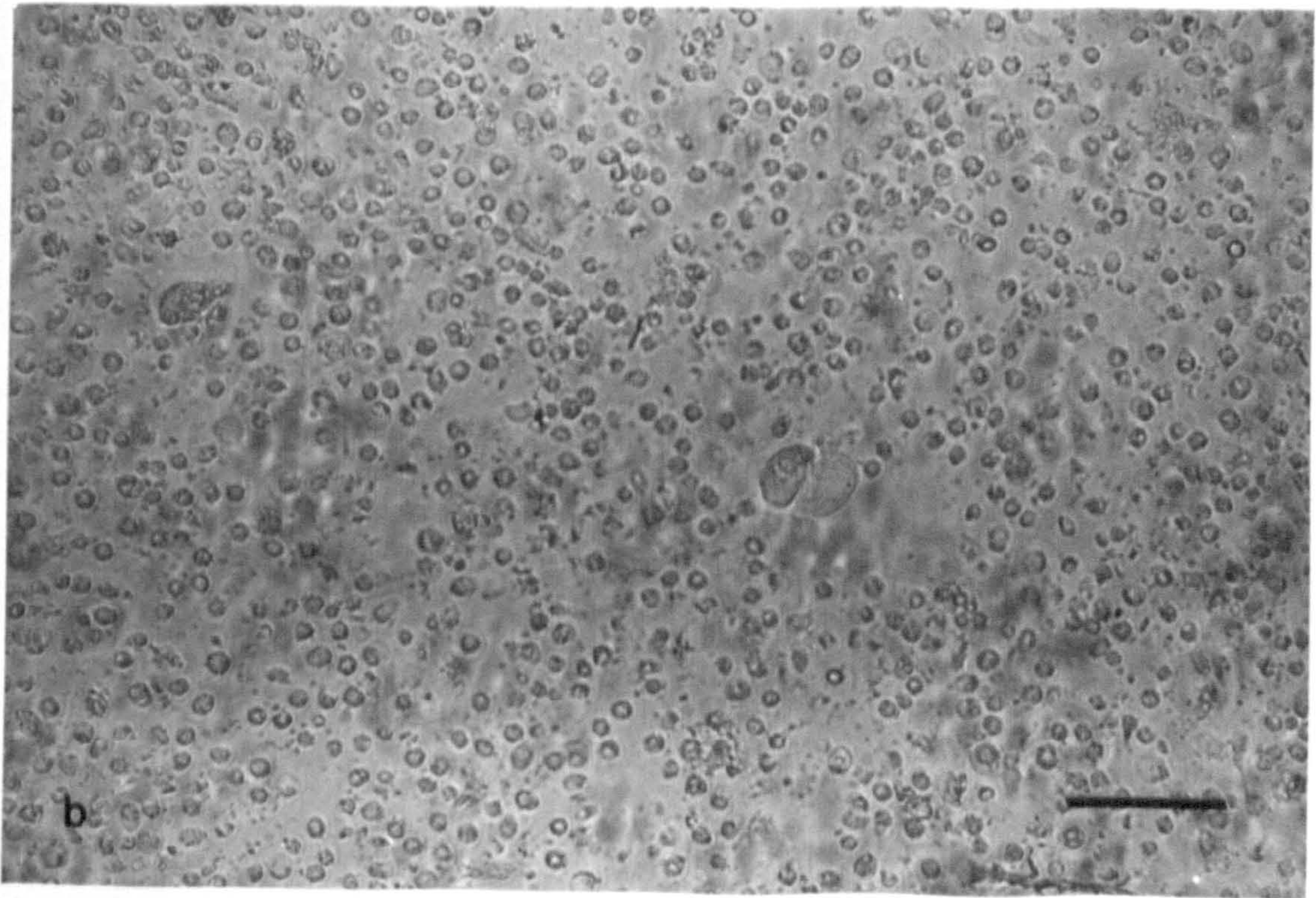
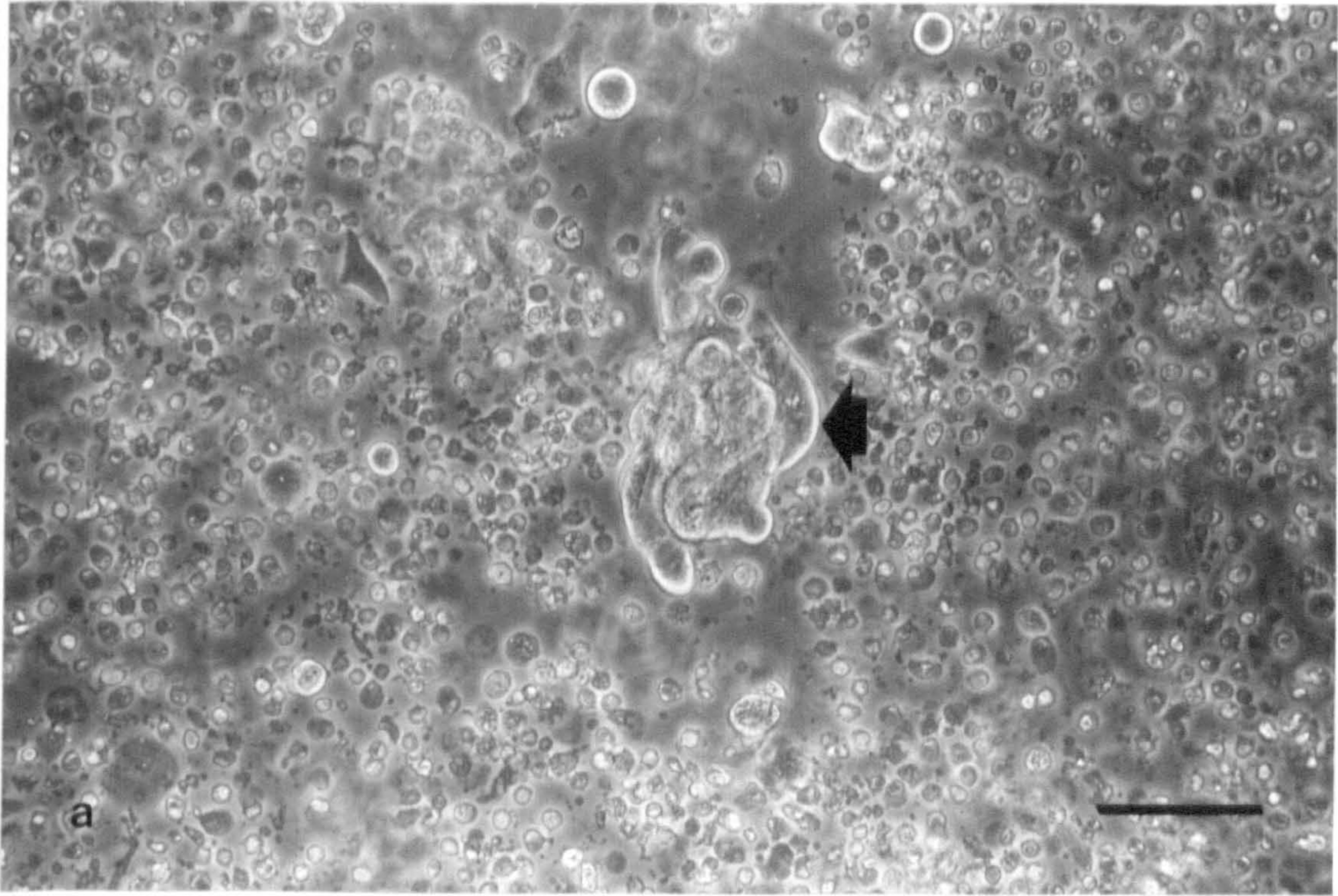
Figure 6.3: Schematic illustration of the monoclonal APAAP procedure (modified from Cordell *et al.* 1984).



Figures 6.4a,b: Phase-contrast microscopical appearance of “settled” cells (a) and “spun” cells (b) after 6 days in monolayer culture. While “settled” group culture contains large cells (arrow) (presumed epithelial; see Figure 6.5a), “spun” group culture consists of mostly smaller cells (presumed stromal; see Figure 6.5b).

(a) Bar represents 13 μm

(b) Bar represents 11.5 μm



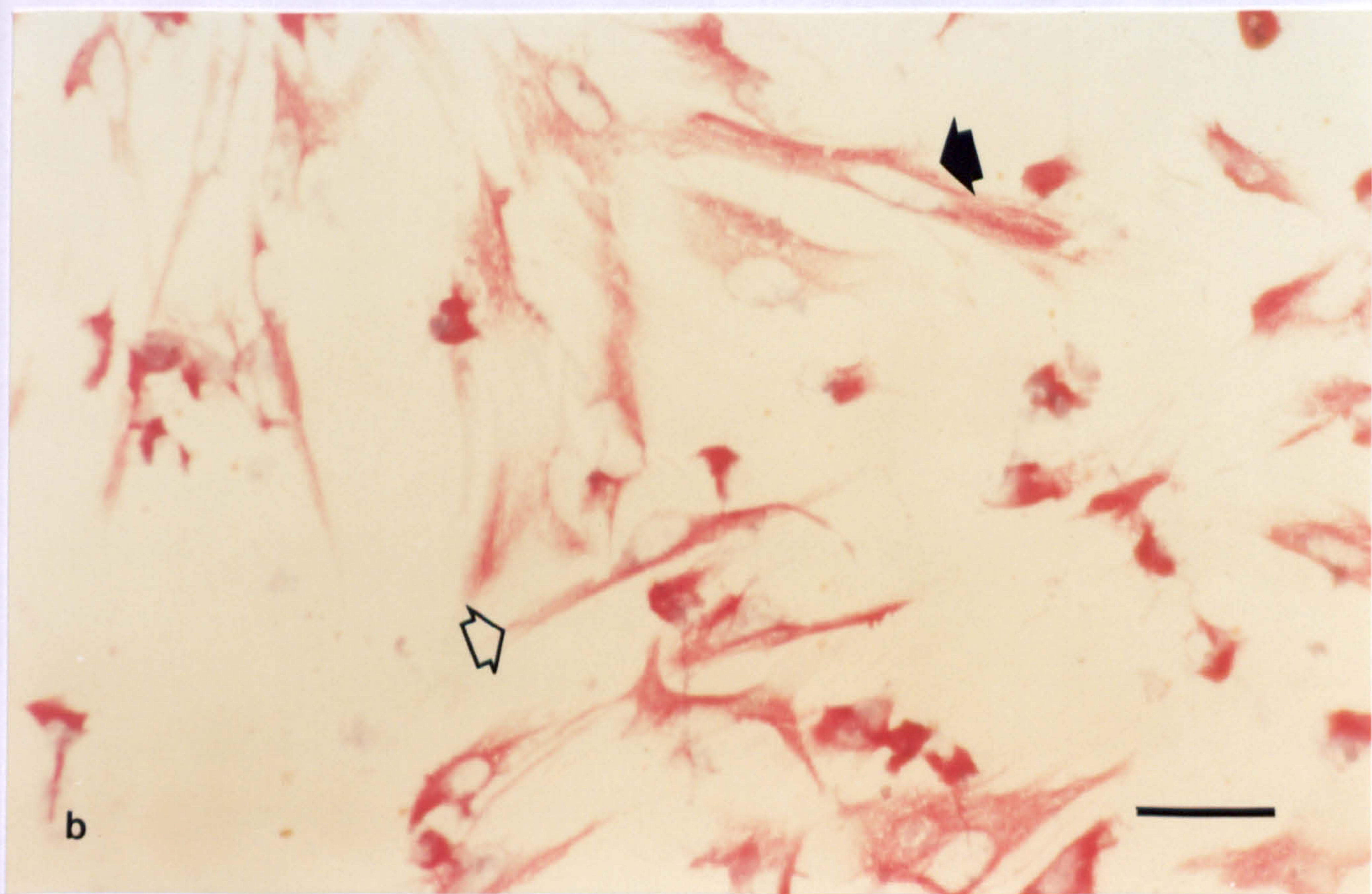
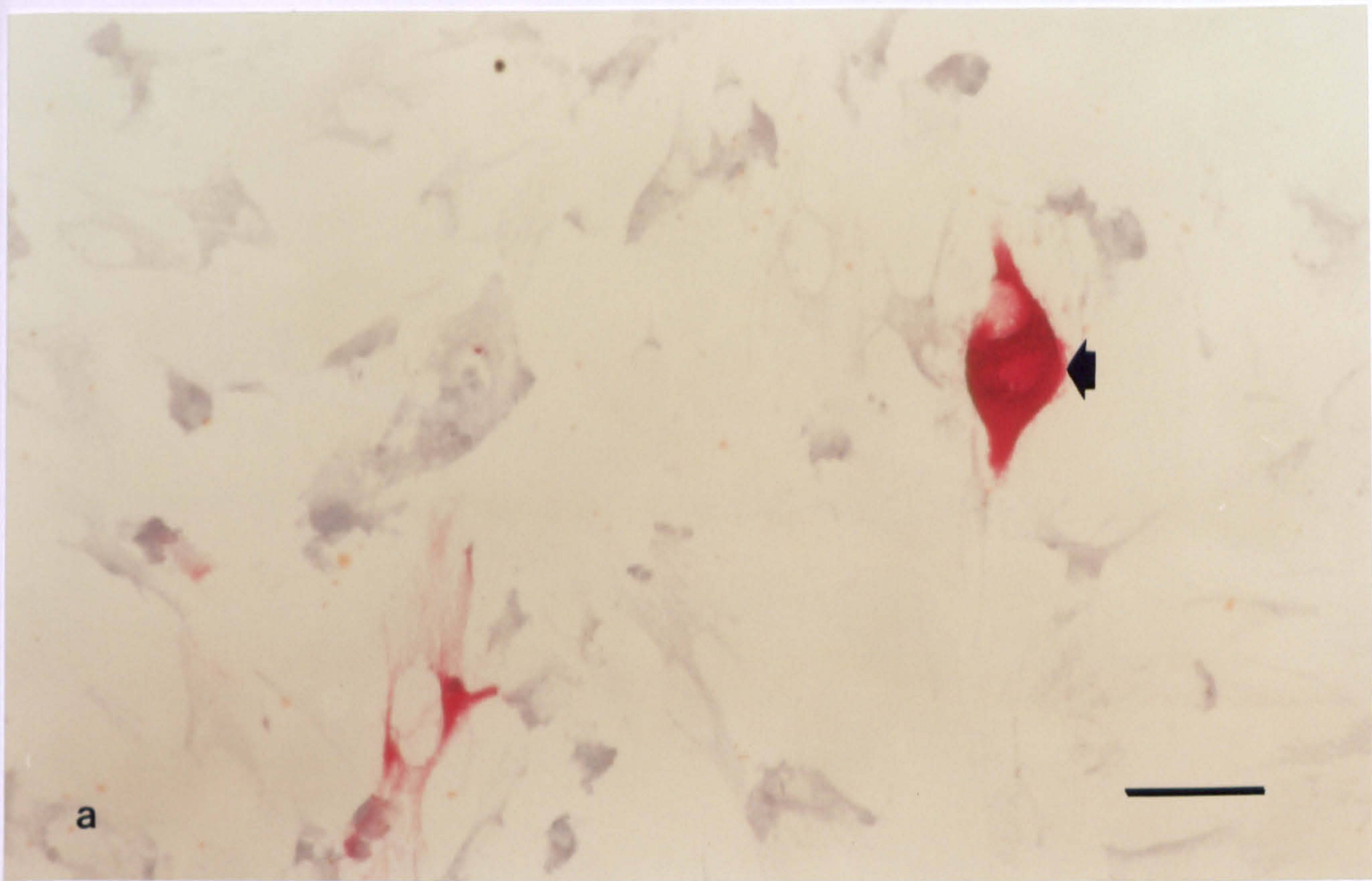
Figures 6.5a,b: Immunocytochemical demonstration of “spun” group cells after 6 days in monolayer culture. (a) demonstrates small number of anti-cytokeratin stained large cells (small arrow) obtained by the “spun” procedure whereas (b) shows abundant anti-vimentin positive cells (arrow) obtained by the same procedure. Note the cytoplasmic projections (empty arrow) that are characteristic for stromal cells.

Staining: (a) Monoclonal anti-cytokeratin - APAAP

(b) Monoclonal anti-vimentin - APAAP

(a) Bar represents 29 μm

(b) Bar represents 30 μm



6.3. RESULTS

In the present study a three-dimensional endometrial stromal cell culture model was used, in order to determine the *in vitro* morphology of endometrial stromal cells obtained from the proliferative and secretory phases of the menstrual cycle. In addition, stromal cell cultures were treated with oestrogen and progesterone to evaluate the effects of steroid hormones on stromal cell morphology at both parts of the cycle *in vitro*. After six days in culture, cells were processed and examined by electron microscopy.

6.3.1. TECHNICAL RESULTS

For confirmation that the stromal and epithelial cell separation methods were successful, cell suspensions (after collagenase digestion) were divided into two tubes and processed as described in section 6.2.2. Immunocytochemical staining of cells that directly cytopun onto glass slides from "settled" cell suspension showed a number of cytokeratin positive large epithelial cells and cell clumps, whereas the "spun" group cell suspension contained fewer cytokeratin positive cells and abundant vimentin positive, smaller stromal cells. Cell morphology after a 6 day culture period was examined using direct phase-contrast microscopical observation and using light microscopy before and after culture on immunostained cells.

Phase-contrast microscopical examination of "settled" cells in culture showed large epithelial cells as well as smaller stromal cells (Figure 6.4a), whereas the "spun" cell culture dish contained fewer epithelial cells and more fibroblast-like smaller cells (Figure 6.4b). These direct phase-contrast microscopical observations also showed that the cell separation procedure did not damage the cell structure. Cells in monolayer culture dishes had the usual cellular appearances, containing cytoplasmic projections and normal nuclear morphology. Following phase-contrast microscopical observations, both "spun" and "settled" cell cultures were stained with anti-cytokeratin for epithelial cells and with anti-vimentin for stromal cells. "Settled" cells showed a considerable number of large anti-cytokeratin positive cells and cell clumps which were defined as epithelial cells. On the other hand, "spun" cells contained fewer anti-cytokeratin positive cells, and abundant anti-vimentin positive cells were observed in this group (Figures 6.5a,b). While anti-vimentin positive stromal cells contained characteristic cytoplasmic projections along

with “spindle” cellular shape, anti-cytokeratin positive epithelial cells demonstrated large cytoplasmic volume, and they also formed cell clumps typical of epithelial cells. These observations indicated that cells after the separation procedure preserved their morphology, and no apparent adverse effect of the process on cell structure was evident.

Following immunocytochemical observations, it was decided to use the second (“spun”) cell separation technique, since this method clearly provided a stromal enriched population of cells at the start of the cultures which was maintained after 6 days *in vitro*. Following 6 days of culture, cells were processed for electron microscopy and examined by using a Phillips 301 electron microscope. A detailed descriptive morphology of the human endometrial stromal cells were determined *in vitro*.

6.3.2. BIOLOGICAL RESULTS

Cells grown for 6 days in a three-dimensional culture model had an appearance of endometrial stromal cells seen *in vivo* at the electron microscope level. They had oval shaped nuclei containing abundant euchromatin material and cytoplasmic projections were also regular features of these cells (Figure 6.2c). Cells were rich in cytoplasmic organelles: rough endoplasmic reticulum (RER) was in its regular arrangement as observed *in vivo*; mitochondria were small in size and had a normal cristae structure; lipid droplets, Golgi apparatus, cytoplasmic vacuoles and vesicles were other cytoplasmic components seen at electron microscopical level *in vitro* and *in vivo*. In summary, endometrial cells grown in this culture system had a similar appearance to those stromal cells observed in human endometrium *in vivo* (Figures 6.2b,c).

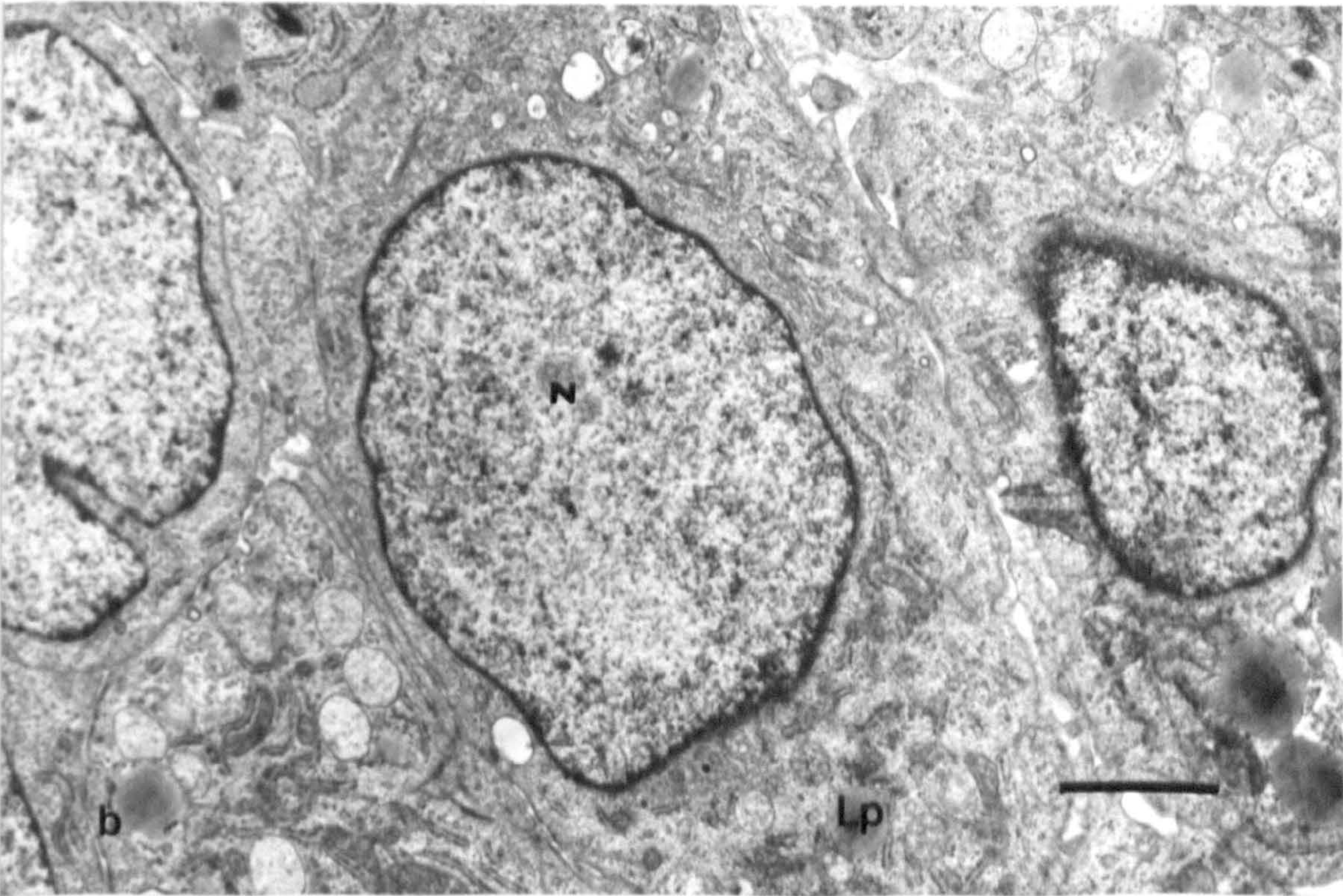
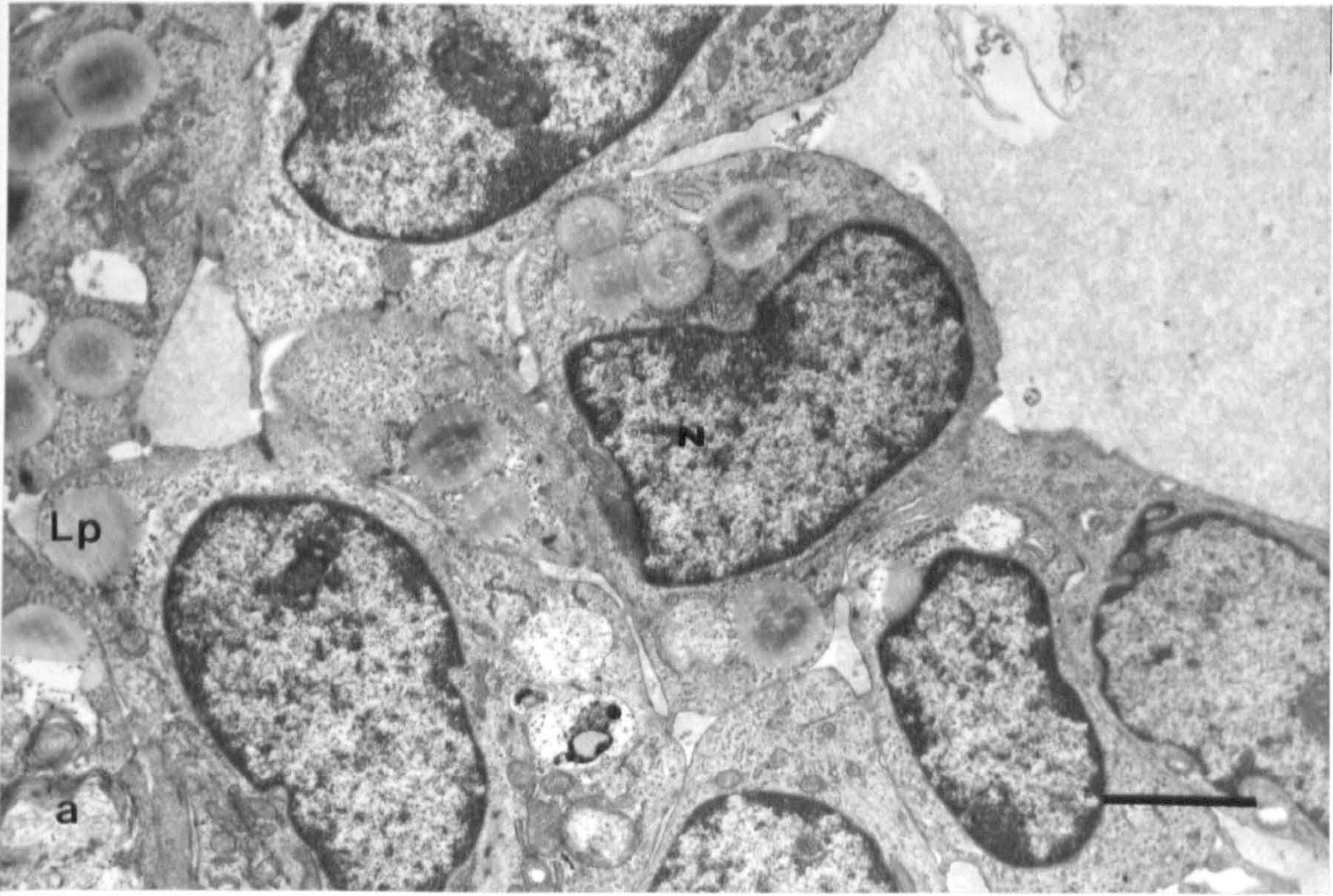
In the present study, stromal cells from two groups of endometrial biopsies (proliferative and secretory phase) were cultured in a three-dimensional system and examined morphologically with and without sex steroid hormone supplementation *in vitro*. Unsupplemented cultured stromal cells obtained from the proliferative phase contained oval shaped nuclei with abundant euchromatic material. Their cytoplasm had numerous projections and organelles with the appearance of normal structure (Figure 6.2c and Figure 6.6a). The cytoplasm also had lipid droplets that are typical for cultured endometrial cells. Intracytoplasmic vesicles and vacuoles were also observed in the unsupplemented proliferative phase group. There were no dramatic structural

Figures 6.6a,b: Unsupplemented group endometrial stromal cells cultured in the 3-D culture system obtained from proliferative phase (a) and secretory phase (b) endometria. Both groups contained oval shaped euchromatic nuclei (N) and regular cytoplasmic organelles and inclusions. (Lp) lipid.

Staining: Uranyl acetate - Lead citrate

(a) Bar represents 1.95 μm

(b) Bar represents 1.75 μm

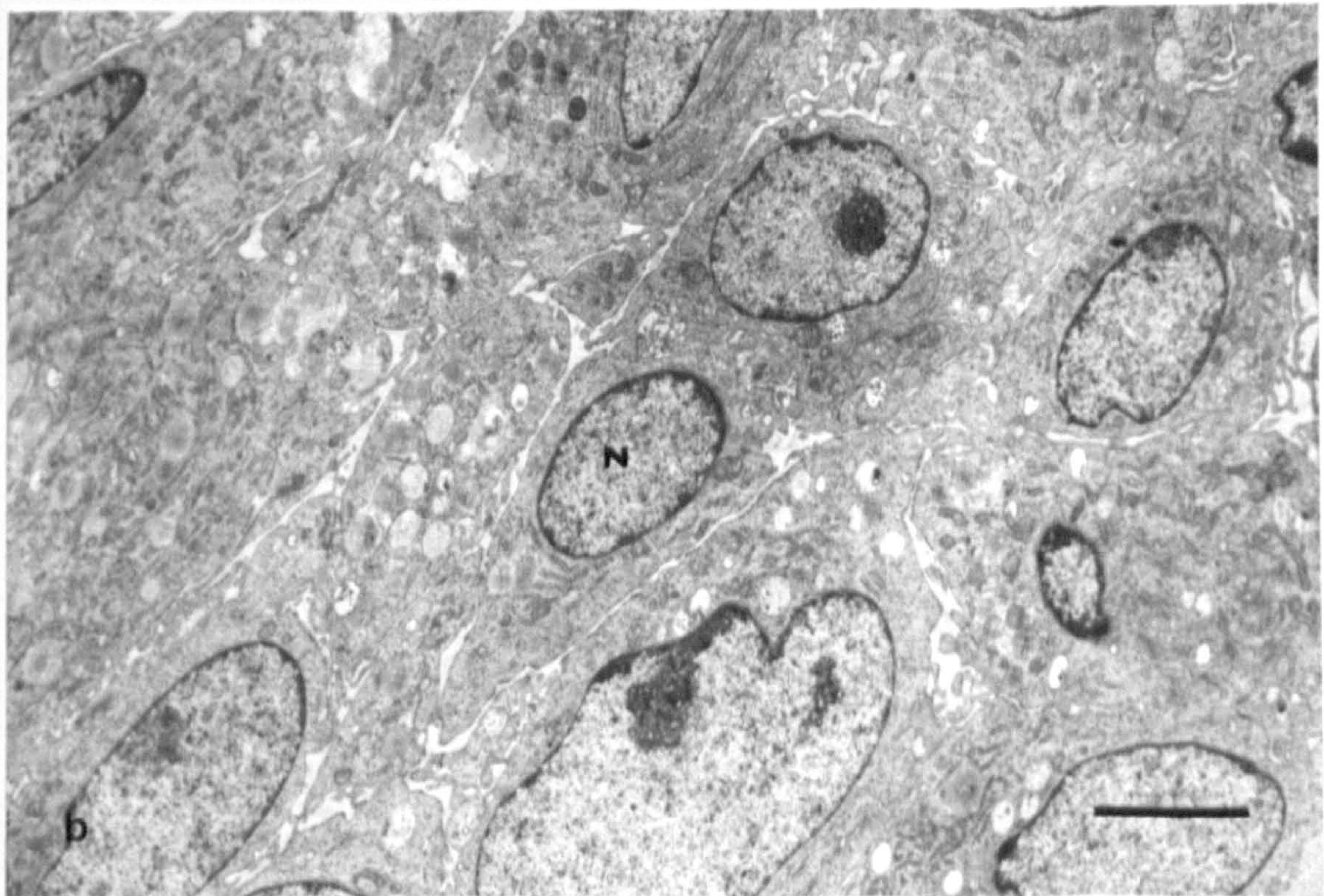
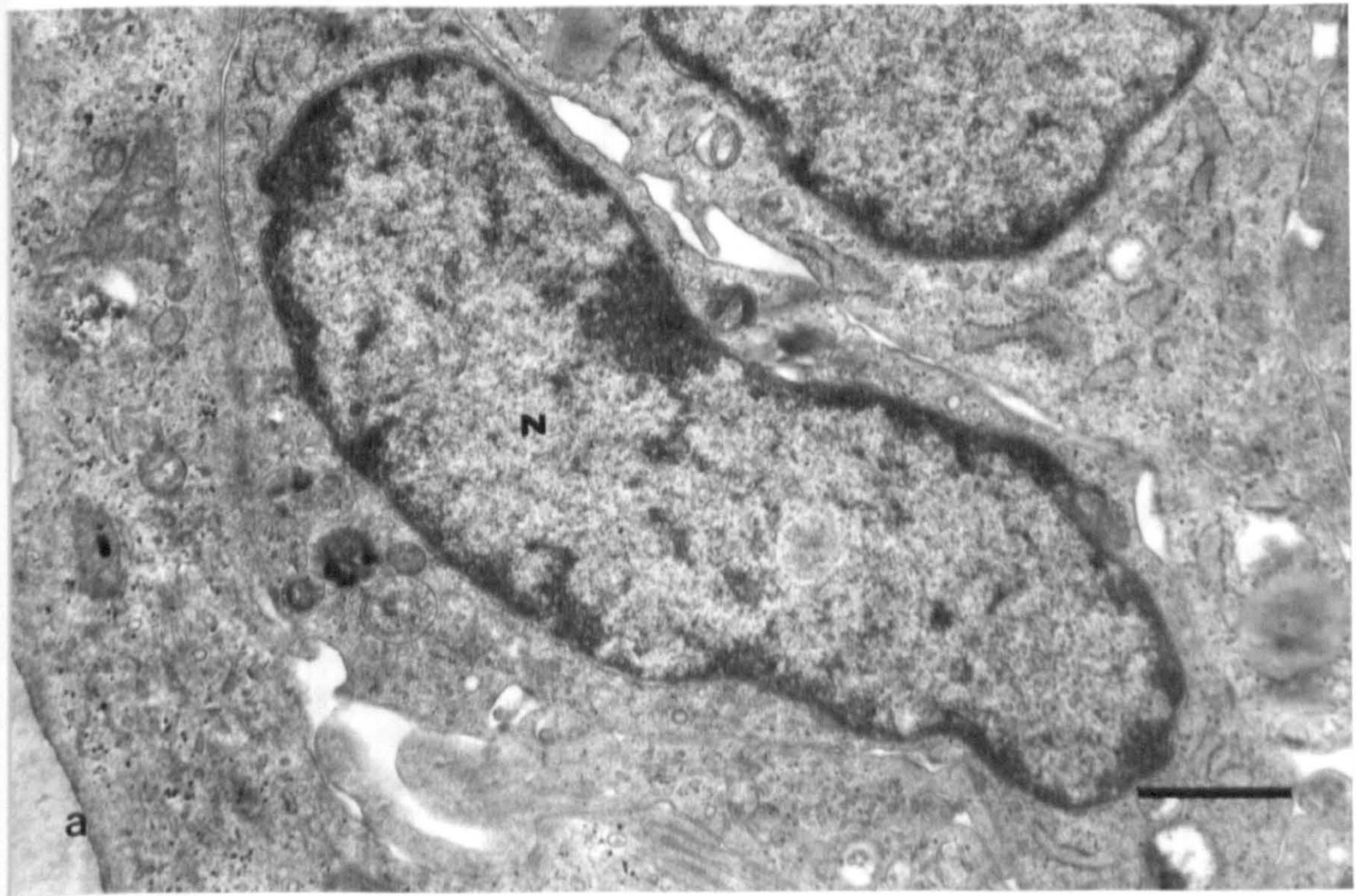


Figures 6.7a,b: Progesterone supplemented stromal cells cultured in the 3-D model system obtained from proliferative phase (a) and secretory phase (b) endometria. There are no substantial structural differences between cultured stromal cells at either stage of the cycle. Oval shaped euchromatic nuclei (N) and normal amount of cytoplasmic organelles are seen in both group cells.

Staining: Uranyl Acetate - Lead Citrate

(a) Bar represents 1.00 μm

(b) Bar represents 3.80 μm

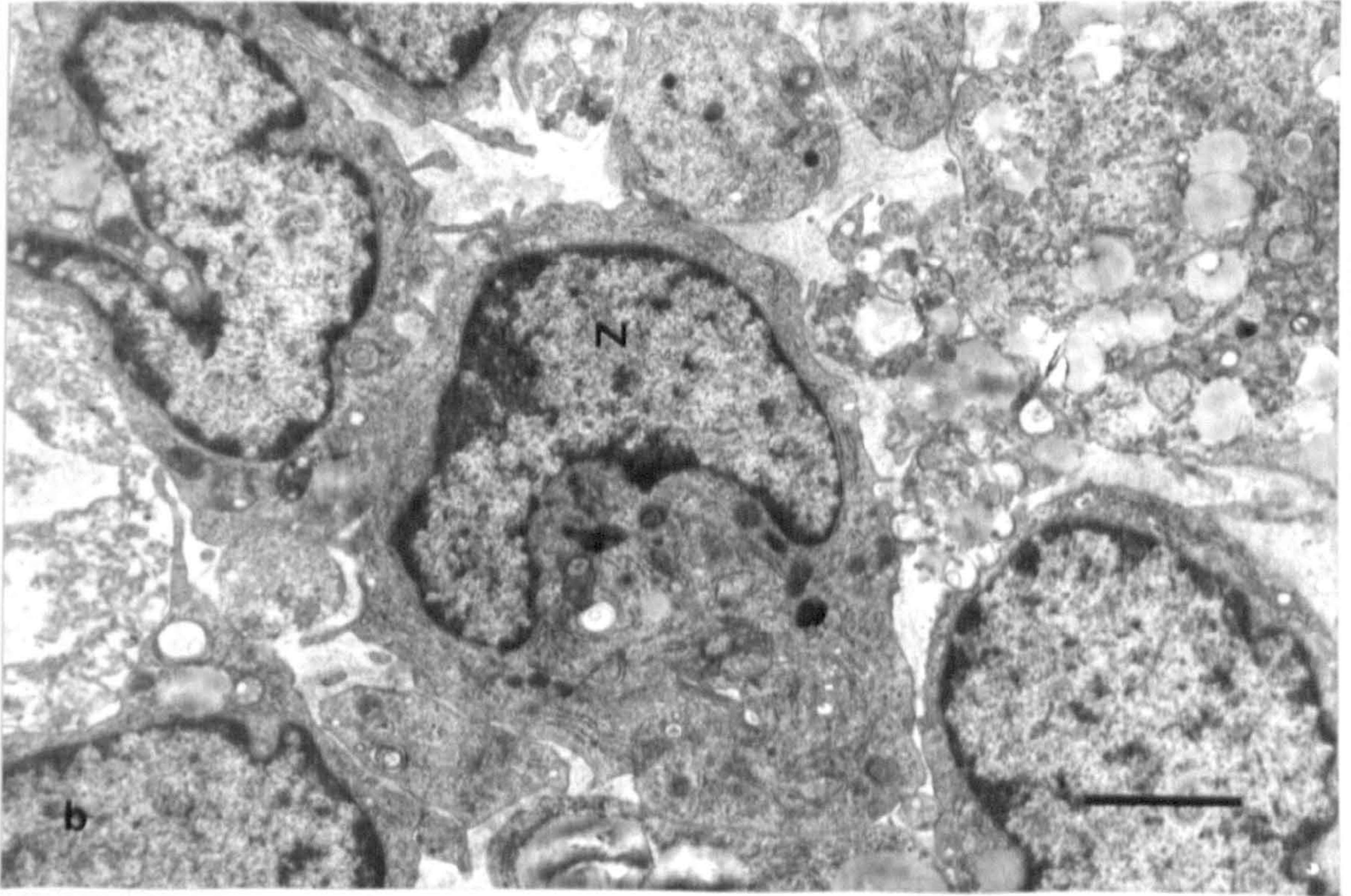
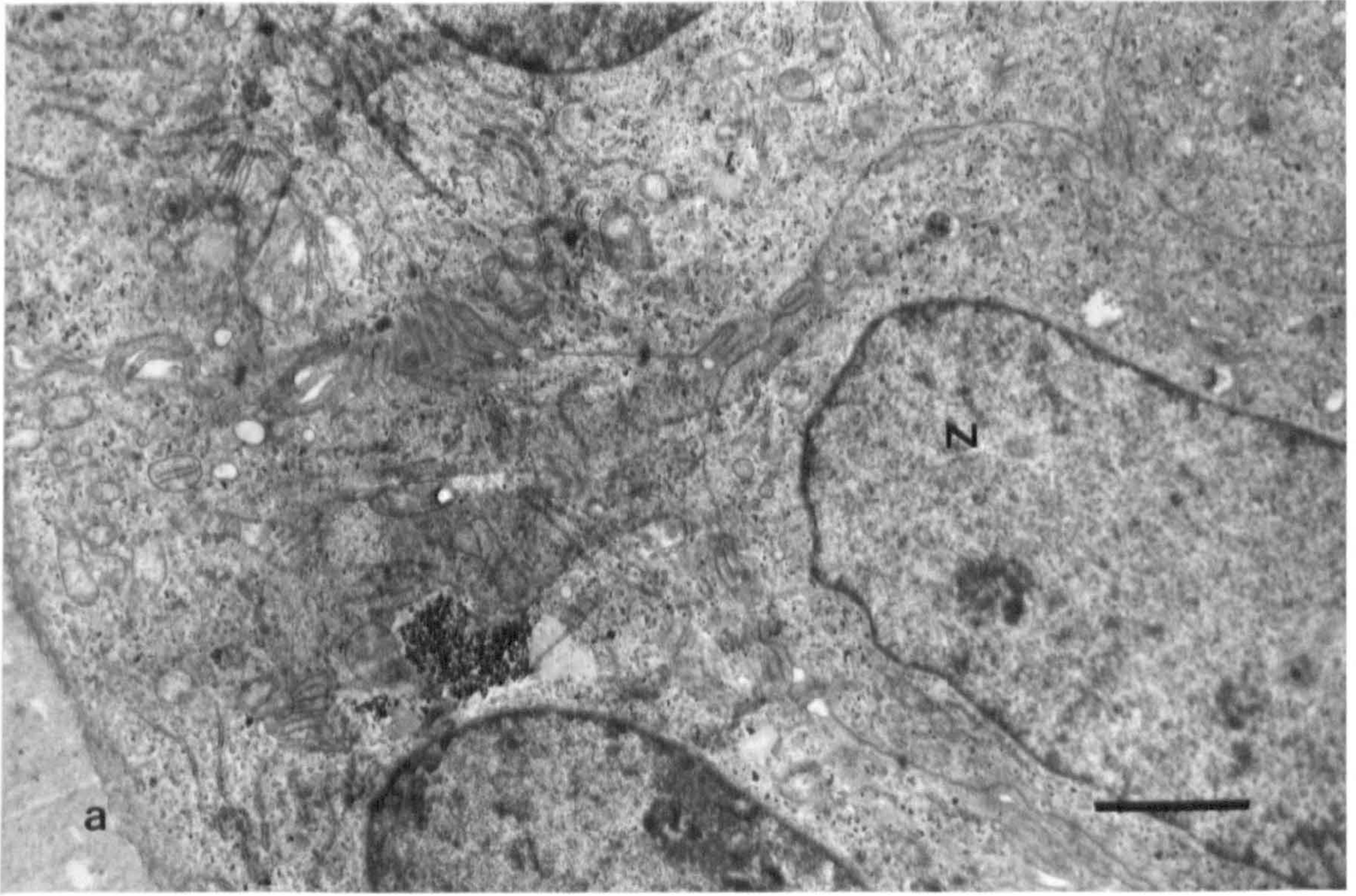


Figures 6.8a,b: Oestrogen supplemented cultured stromal cells obtained from proliferative phase (a) and secretory phase (b) human endometria. While oestrogen supplementation caused no effect on cells obtained at the proliferative phase (which have round euchromatic nuclei (N) and normal cytoplasmic structures), oestrogen administration caused substantial changes in stromal cells obtained at the secretory phase of the menstrual cycle. Irregular shaped heterochromatic nuclei (N) and atrophic organelles were frequently observed in these cells.

Staining: Uranyl Acetate - Lead Citrate

(a) Bar represents 1.75 μm

(b) Bar represents 1.80 μm

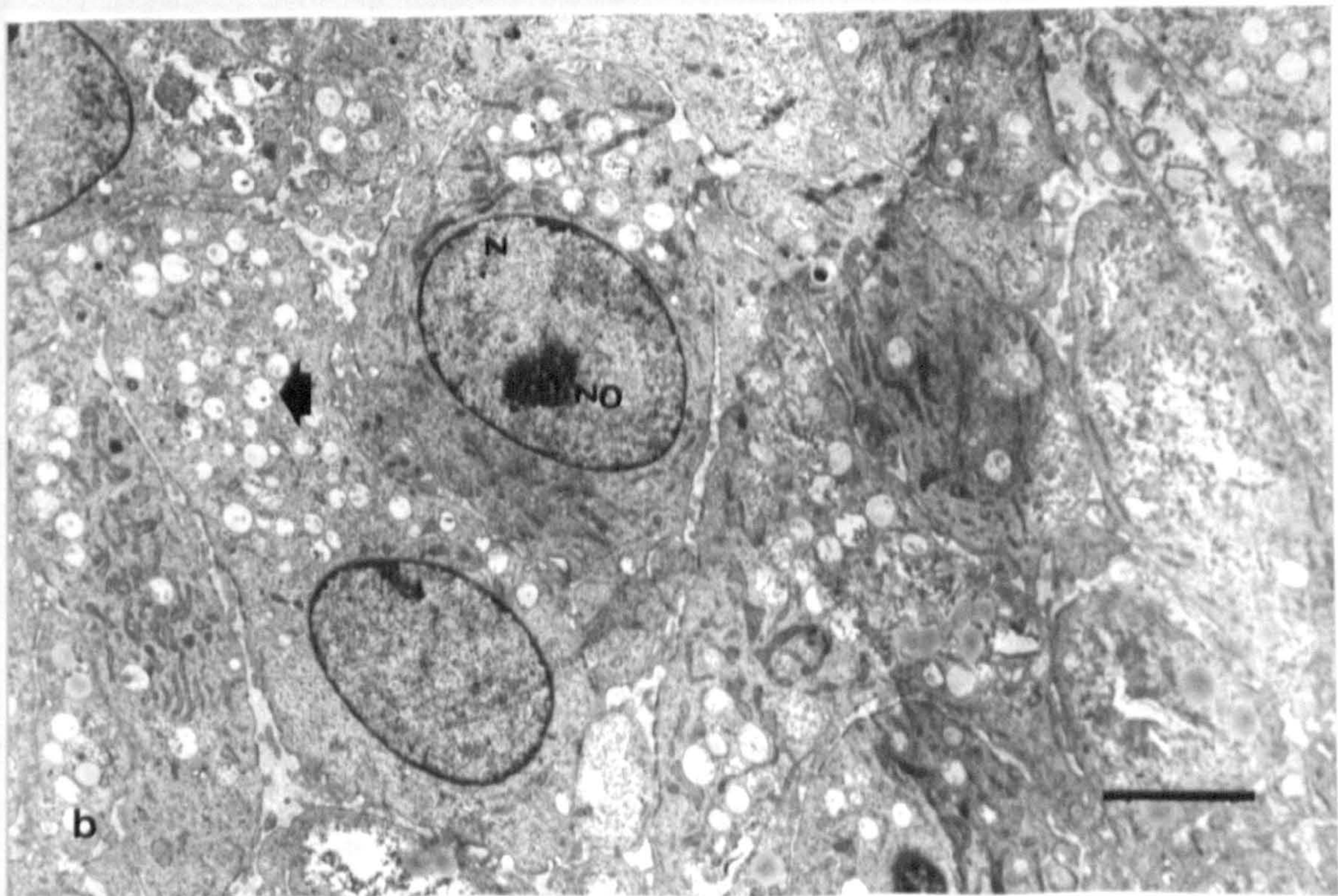
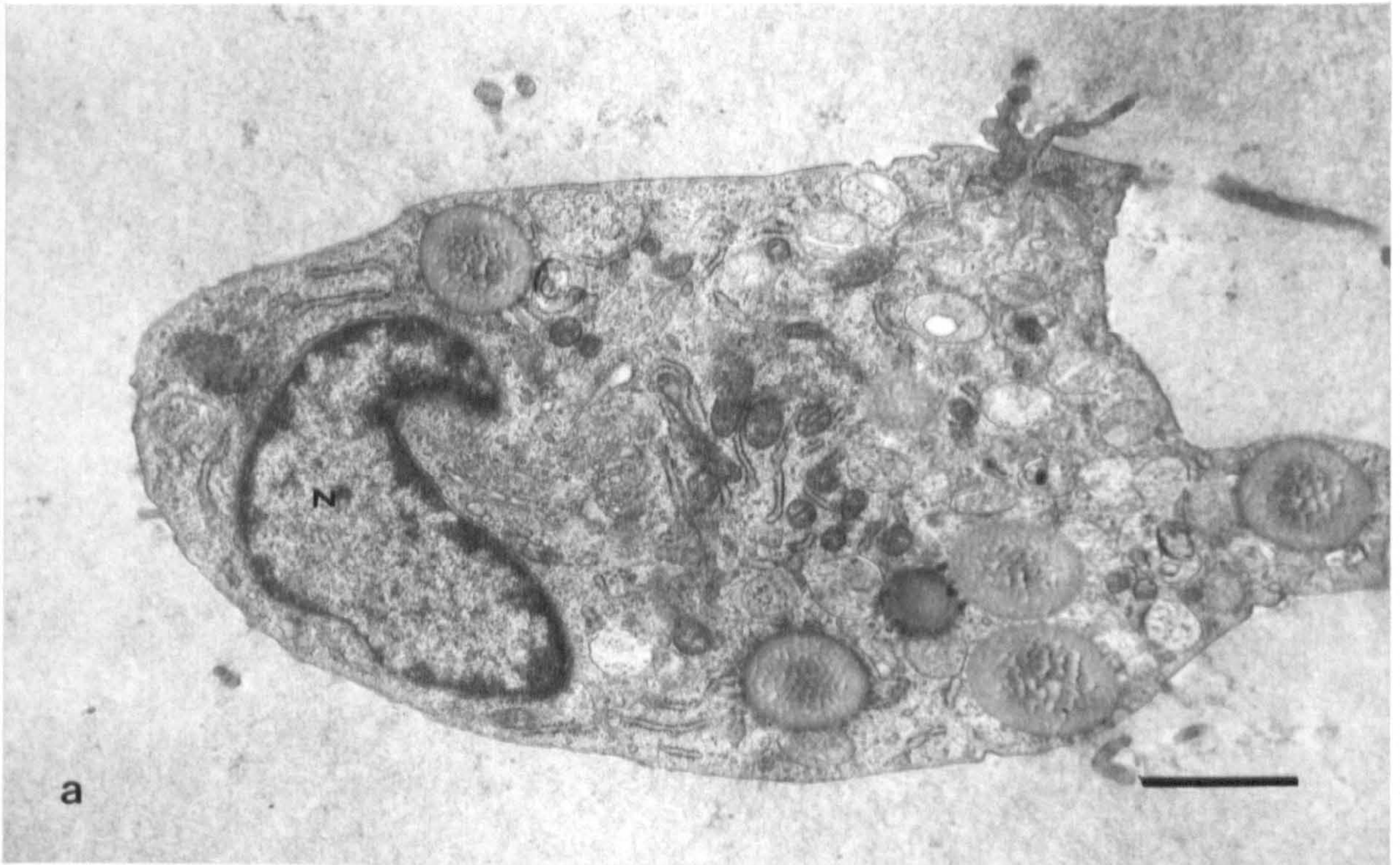


Figures 6.9a,b: Combined oestrogen + progesterone supplemented cultured stromal cells obtained from the proliferative phase (a) and the secretory phase (b) human endometria. While proliferative phase cultured cells contained irregular shaped heterochromatic nuclei and normal amount of organelles, secretory phase cultured stromal cells had rounded nuclei (N) with prominent nucleolus (No) along with apparently increased secretory activity: vesicles (arrow) may be seen in the cytoplasm.

Staining: Uranyl Acetate - Lead citrate

(a) Bar represents 1.05 μm

(b) Bar represents 3.65 μm



differences between proliferative phase and secretory phase unsupplemented group human endometrial stromal cells in culture (Figures 6.6a,b).

The morphology of cultured endometrial stromal cells to which progesterone supplements had been added showed no apparent differences to the unsupplemented cells that were cultured from either proliferative or secretory phase samples. There were also no differences between progesterone supplemented endometrial stromal cells obtained from either proliferative or secretory phases of the menstrual cycle (Figures 6.7a,b).

While oestrogen supplemented cells showed no significant morphological differences from unsupplemented cells in the proliferative phase (Figure 6.8a), stromal cells with the same treatment which were obtained during the secretory phase contained hypertrophic cytoplasmic organelles (Figure 6.8b). Oestrogen supplemented stromal cells that were taken from proliferative phase endometrium had rounded euchromatic nuclei, cytoplasm which contained mitochondria with normal structure and a few intracytoplasmic vesicles were also present (Figure 6.8a). On the other hand, cultured oestrogen supplemented cells obtained from secretory phase endometrium had irregular shaped nuclei with frequent heterochromatic material, their cytoplasm included many vacuoles and lipid droplets, and large number of pinocytotic vesicles along with lysosomal structures were also evident (Figure 6.8b).

While the combination of oestrogen and progesterone supplementation caused no change in cultured stromal cells obtained from the proliferative phase endometrium (Figure 6.9a), cells obtained during the secretory phase of the cycle contained increased numbers of intracytoplasmic vacuoles and vesicles, suggesting an increased secretory activity or internal transport in these cells caused by the combination of sex hormones (Figure 6.9b). Oestrogen + progesterone supplemented proliferative phase stromal cell had irregular shaped small nuclei that contained heterochromatic material, and their cytoplasm had number of small mitochondria along with RER and Golgi apparatus in their normal appearance (Figure 6.9a). However, after combined steroid treatment of cultured stromal cells obtained from secretory phase endometrium, these cells showed rounded euchromatic nucleus with a nucleolus, and their cytoplasm contained extensive "secretory apparatus" and RER (Figure 6.9b), which were characteristic appearance of active secreting cells seen in *in vivo* human endometrium. Furthermore, these cells

treated with the combination of both hormones in the secretory phase had an appearance of stromal cells *in vivo* during late secretory phase of the menstrual cycle.

In summary, immunocytochemistry has confirmed that the endometrial stromal cell separation method was a successful one. Electron microscopical observation of the cultured stromal cells established that the present three-dimensional culture system produced cells with appearance *in vitro* close to those seen *in vivo*. Hormonal supplementation had variable effects depending on the stage of the cycle when the tissue was taken.

6.4. DISCUSSION

Since it is difficult to perform *in vivo* experiments in humans during implantation and gestation of a conception cycle, an *in vitro* model is required. It is crucial to grow cells *in vitro* which are as similar in structure as possible to those *in vivo*. In the present study, a successful endometrial stromal cell isolation method was established, and human endometrial stromal cells were grown in a novel three-dimensional culture model system in order to establish cells *in vitro* with an appearance of normal stromal cells *in vivo*. In addition, the effect of cell culture on stromal cell structure was determined at two different stages of the menstrual cycle (proliferative and secretory phase), and the effects of oestrogen and progesterone hormone supplementation on the stromal cell morphology at these stages were also examined. Since this is a novel method for human endometrial stromal cell culture, technical and biological results of the study will be discussed in separate sections.

6.4.1. TECHNICAL ASPECTS OF THE STROMAL CELL CULTURE MODEL

Cytokeratin and vimentin are intermediate filaments that have been identified on the basis of their protein composition and cellular distribution (Ross *et al.* 1989). These chemically distinct types of intermediate filaments are composed of subunits at different molecular weight. Cytokeratin is of 40 000-65 000 molecular weight and characteristic for epithelial cells, whereas vimentin has a molecular weight of 55 000 and is found in cells of mesenchymal origin (Ross *et al.* 1989). The use of antibodies, that are specific to the filament types, is an accurate and reliable method for the identification of the cell types in tissues and can also be used in the diagnosis of tumour origins. In the present study, vimentin and cytokeratin antibodies were used for the identification of stromal and epithelial cells in human endometrial cultures.

Immunocytochemical observations of human endometrial cells before and after the culturing process revealed that the cell isolation method used in the present study provided a stromal enriched cell suspension. The cell suspension obtained after 5 minutes centrifugation at 500 rpm contained mostly anti-vimentin positive small cells which are defined as the cells with stromal origin. On the other hand, the cells that were obtained by settling the cell suspension for 10 minutes, contained relatively more anti-

cytokeratin positive cells, which were defined as epithelial cells, than the “spun” method. Furthermore, immunocytochemistry results of both cell suspensions after 6 days in monolayer culture showed that cell population contained mostly anti-vimentin positive cells in the cultures obtained using the centrifugation technique, whereas the cells obtained by the cell settling method included relatively larger numbers of anti-cytokeratin positive cells after 6 days culture. In addition to the qualitative examination of the immunostained culture cells, an approximate estimation of stromal and epithelial cell proportions in culture was also determined using an image analysing microcomputer (Quantimet 970, Cambridge Instruments Ltd., UK). Stromal and epithelial cell proportion *in vivo* was estimated (which is 1:5 in favour of epithelial cells; this is the relative volumes of the cells, not their numbers. Since epithelial cells are larger than stromal cells the number distribution would be less than 1 in 5) from volume fraction data obtained previously, and by measuring total area of anti-vimentin positive (stromal) and anti-cytokeratin positive (epithelial) cell areas using the above instrument. A small, systematic random sample of stained cells was used from 2 slides of each preparation.

The final outcome of the endometrial cells after 6 days in culture was a proportion of 1:8 in favour of the stromal cells. Although this estimate of stromal and epithelial cell proportion in culture was very preliminary, cell proportion in culture was determined to help the interpretation of cell separation method. This result suggested that the endometrial cells in culture were mainly stromal cells, and confirmed the cell isolation technique.

These results indicate that the stromal cell isolation method used in the present study was both successful and reliable. The reliability of the cell separation method is especially important for the final outcome of the experiment. The separation of the stromal cells was not intended to be a total separation, but a stromal cell enrichment for use in the 3-dimensional culture experiments. No polarised glandular structures were observed during the electron microscopical examination of the cultured tissue, further confirming the success of cell separation by the method used in the present study.

Several cell separation techniques have been reported in *in vitro* studies dealing with human endometrial cells in culture (Bongso *et al.* 1988; Schatz *et al.* 1990; Negami and Tominaga 1991; Hatayama *et al.* 1994). Bongso *et al.* (1988) centrifuged the whole

endometrial cell suspension at 100 g for 5 minutes, after leaving the tissue 2 hours in 0.25% collagenase in HBSS at 37 °C. They repeated this step once more, and then centrifuged the remaining supernatant (containing stromal cells) at 400 g for 5 minutes, resuspended the cell pellet with medium and cultured the cells in culture flasks. Bongso *et al.* (1988) recognized epithelial and stromal cells by identifying their morphologies using Nomarski's inverted optics, but no immunocytochemical staining for cell type identification was reported. In the present study, the technique used by Bongso *et al.* (1988) was modified by the centrifugation of the whole cell suspension after 2 hours in 0.25% collagenase at 500 rpm for 5 minutes, and further centrifugation of supernatant (containing stromal cells) at 1000 rpm for 5 minutes in order to improve the yield of stromal cells in the final suspension. Also an immunocytochemical method was used in the present study to distinguish the stroma and epithelial cells in order to accurately determine the relative proportion of these cell types after the separation procedure. Results of this method confirmed that at least 85-90% of the cell population were stromal cells.

The filtration of the cell suspension through a stainless steel sieve is another method used for cell isolation process in *in vitro* studies (Holinka and Gurpide 1987; Schatz *et al.* 1990; Negami and Tominaga 1991). Schatz *et al.* (1990) filtered an endometrial cell suspension through a stainless steel sieve, then they backwashed the epithelial cells from the sieve. Further isolation of stromal cells in the filtrate was made by taking advantage of the more rapid adhesion of the stromal cells than the contaminant cells (epithelial and blood cells) to tissue culture plastic. On the other hand, there were 2 reasons in the present study not to use the cell filtration technique: 1) the present study deals with the morphology of the endometrial cells, and filtration process through a metal filter may have an adverse effect on the cell morphology, and 2) the present study did not aim to completely separate epithelial and stromal cells, but to produce a stroma-enriched suspension since pilot studies suggest these give better growth than pure cultures.

Hatayama *et al.* (1994) used a different cell isolation method for the examination of macrophage colony-stimulating factor (M-CSF) production by human endometrial stromal cells. They left the centrifuge tube, containing the dissociated cell suspension, upright for 10 minutes and collected the supernatant excluding the lowermost 2 ml of the

suspension (consisting of epithelial cells). They repeated this procedure several times leaving each cell suspension for 10 minutes. The final cell suspension contained 90% viable stromal cells. Their efforts at stromal cell isolation was a time consuming procedure since they had to spend at least an hour to settle down the epithelial cells and this time could have affected the cells' later viability in culture. In the present study, the settling time for epithelial cells was accelerated by centrifuging the cell suspension at low speed. In the present study, the percentage of stromal cells at the end of the isolation process was at least 85-90%, as determined by using an image analysing microcomputer on antibody stained cultured endometrial cells. It can be seen that the final proportion of stromal cells after cell separation in the present study and that of Hatayama *et al.* (1994) were very similar. However, the method used in the present study is much quicker.

There were some technical difficulties in the present study; for example, the stromal-enriched cell 3-D cultures contained very few cells, since they were grown on small inserts, in very thin layers of Matrigel. This caused great difficulty in the sectioning of Epon blocks since there were so few cells present. Similarly, the electron microscopical evaluation of these sections which contained small number of stromal cells was also very difficult and time consuming since cells were widely spread throughout the blocks, resulting many sections to be cut to observe 1 or 2 cells per section.

In the present study, stromal cells grown in a three-dimensional culture model showed similar appearances to those *in vivo*, at the electron microscope level (Figure 6.2b and c). Oval shaped nuclei with abundant euchromatin and cytoplasmic projections were regular features of these cells. Cells were rich in cytoplasmic organelles; RER was in its regular arrangement, mitochondria were small in size and with a normal structure (all suggesting active, healthy, secreting cells). It is suggested that the three-dimensional culture system used in the present study produced stromal cells with a morphology very similar to that seen *in vivo* and it is therefore a suitable model for *in vivo* conditions in Man.

6.4.2. BIOLOGICAL ASPECTS OF HUMAN ENDOMETRIAL STROMAL CELLS IN CULTURE

Human endometrial cell cultures are used in a wide range of research areas including physiologic (Irwin *et al.* 1989; Schatz *et al.* 1990; Negami and Tominaga 1991), morphologic (Luginbuhl 1968; Liszczak *et al.* 1977; Hill *et al.* 1994) and metabolic (Jacobs and Carson 1991; Watson *et al.* 1994) experiments.

One purpose of using endometrial tissue cultures is to detect the effects of steroid hormones on the production of different substances by human endometrial cells. Irwin *et al.* (1989) examined the effects of oestradiol and progesterone on the production of prolactin (PRL) by human endometrial stromal cells in monolayer cultures. They suggested that the treatment of stromal cells with sex steroids increased the PRL secretion by cultured stromal cells and, since PRL is a marker of decidualization in humans, they further suggested that the physiological doses of sex steroids they used induce the decidualization process of endometrial stromal cells *in vitro*. Negami and Tominaga (1991) examined the effects of PRL on cultured human endometrial cells, and they suggested that PRL acts as a stimulator at low doses and acts as an inhibitor at high doses of the attachment and growth of cultured endometrial epithelial and stromal cells.

Human endometrial cells secrete cytokines in response to ovarian steroid hormones. Hatayama *et al.* (1994) showed an enhanced production of macrophage colony-stimulating factor (M-CSF) by human endometrial stromal cells when they were treated with progesterone in culture. These authors concluded that human endometrial stromal cells secrete M-CSF in a progesterone dependent manner during the process of decidualization.

Receptor binding studies of several factors in endometrial cells is another field that can be performed *in vitro*. Watson *et al.* (1994) determined the binding characteristics of epidermal growth factor (EGF; plays an important role in the regulation of uterine cell growth and differentiation) receptor in isolated human endometrial glands and stromal cells in culture. Study on the effects of cytokines on cultured endometrial cells is another use of cell culture techniques *in vitro*. The effect of interleukin-1 β (IL-1 β ; is synthesized and secreted by human decidua, stimulates IL-6 production and regulates PGE₂ release from endometrial glands - Tabibzadeh 1990) on the production of prostaglandins by

cultured human decidual cells was determined (Kennard *et al.* 1995), and it induced prostaglandin E₂ (PGE₂) and PGF_{2α} production and increased cyclooxygenase-2 (COX-2) levels in a time-dependent manner in cultured human decidual cells.

In addition to these physiological and metabolic studies, human endometrial cell cultures have also been used for morphological purposes. Luginbuhl (1968) determined the effects of tissue culture on human endometrium by using explants in culture dishes. In this early study, tissue explants of human endometrium had a poor survival rate in culture due to technical difficulties. In particular the stromal compartment of endometrium was affected by the relatively crude explant culture procedure. Liszczak *et al.* (1977) performed a morphological study on the effects of ovarian steroids using human endometrial monolayer cultures, and they suggested that the use of monolayer cell culture methods provides a useful model system for the morphological investigation of human endometrium *in vitro*. However, later reports on this issue have revealed that the monolayer cell culture models are not appropriate in terms of morphology and cells obtained with this technique do not resemble to those seen *in vivo* (Hill *et al.* 1993 and 1994; Bentin-Ley *et al.* 1994). Hill *et al.* (1994) used a three dimensional cell culture model using human endometrial epithelial cells. They compared the morphological differences between endometrial epithelial cells cultured in monolayer and the cells cultured in the three dimensional model. The cells in monolayer culture dishes were flat in shape and had poor cellular membrane specializations such as microvilli and cilia, whereas the cells cultured in three dimensional system were polarized [they had prominent basement membrane, apical surface structures (microvilli and cilia) and lateral membrane specializations such as desmosomes and tight junctions], they were columnar in shape, and had organelle rich cytoplasm (Hill *et al.* 1994).

In the present study, the effects of oestrogen and progesterone hormone supplementation on the structure of stromal cells were also determined in a three-dimensional culture model on tissue taken at 2 different stages of the menstrual cycle. Stromal cell morphology in culture seemed to be effected by oestrogen treatment at the secretory phase of the menstrual cycle, whereas no effect of oestrogen was seen at the proliferative phase. This appears contradictory to the situation *in vivo* where oestrogen causes the changes typical of the proliferative stage of the menstrual cycle while not

being present to a great extent in the secretory phase. Stromal cells taken from the secretory phase endometrium had hypertrophic cellular structures such as enlarged vacuoles and vesicles following oestrogen treatment. This result may indicate a hyper-oestrogenic effect, since the endometrial cells had already been primed by oestrogen before the biopsies were taken and would not normally be exposed to such high levels of oestrogen at this stage. However, further studies using several different concentrations of hormone treatment on stromal cells and during several different stages of the cycle are required to confirm this suggestion. While progesterone supplementation alone caused no changes in stromal cell morphology *in vitro* neither at the proliferative nor at the secretory stages, the combination of oestrogen and progesterone supplementation (at the same concentrations) caused a slight apparent increase in the number and the size of the cytoplasmic vacuoles and vesicles (so-called "secretory apparatus") of stromal cells cultured from the secretory phase endometria, suggesting an increased secretory activity in these cells. It may be suggested that the cells treated with combined steroid hormones (oestrogen + progesterone) had the appearance of stromal cells from later stages of the menstrual cycle *in vivo* than these treated with oestrogen alone. Yet again this is a novel culture method examining the effects of sex steroids on stromal cell morphology in a three-dimensional system, and more work is required for a complete understanding of this phenomenon.

Hill *et al.* (1994) reported that endometrial epithelial cells in a three-dimensional culture system tended to have more rounded and euchromatic nuclei when treated with oestrogen than when unsupplemented. However, cells to which progesterone supplements had been added showed no dramatic differences compared to the unsupplemented cells, and the response of cells supplemented with both oestrogen and progesterone together did not show significant differences from those given progesterone alone (Hill *et al.* 1994). However, their study was on human endometrial epithelial cells and the present study was on stromal cells. Therefore it is difficult to make direct comparisons between 2 cell types treated with steroids since they are of different origin, and also their response to steroid hormones are likely to be different.

Irwin *et al.* (1989) grew human endometrial stromal cells in monolayer culture dishes and reported that progesterone supplementation increased proliferation rates of stromal

cells and oestrogen and progesterone combined, further stimulated growth but oestrogen alone had no effect. In addition to the growth rate, they also examined the ultrastructure of cultured stromal cells after treatment with steroid hormones, and found extensively developed Golgi complexes, RER and well-developed prominent gap junctions in the well-developed cytoplasm. They also suggested that oestrogen + progesterone combined, caused stromal cells to resemble those described during decidualization *in vivo*. However, cells that Irwin *et al.* (1989) used for *in vitro* experiments were passaged and subcultured cells and steroid supplementation was made after 1 to 2 weeks in culture. Furthermore, monolayer cell culture is not the best method for ultrastructural observations since the cells have a flat appearance with poorly developed cytoplasmic organelles (Hill *et al.* 1994). In brief, they do not resemble to those seen *in vivo*.

On the other hand, in the present study, primary stromal-enriched cells were cultured 6 days in a three-dimensional culture chamber, and the effects of oestrogen and progesterone on the ultrastructure of stromal cells were examined using these primary cell cultures. In the present study, any change seen in cellular ultrastructure is likely to be due to the effect of the hormonal treatment rather than the differentiation of the primary cells as an effect of long culturing period or due to the distortion of normal cell structure due to monolayer culture (as in the Irwin study).

Holinka and Gurpide (1987) examined the proliferative potential of the human endometrial stroma in monolayer culture. They also compared the effects of different culture media and different hormone treatment regimes on the morphology of the stromal cells at the light microscope level. They found a great proliferative potential of stromal cells in serial subcultures, and further suggested distinct changes in morphology when the cells were exposed to progesterone. They also suggested that hormonal effects on morphology required a relatively long period of exposure (2 weeks) and that these morphologic changes were obtained in primary as well as secondary cultures. As discussed in the Irwin study, long culture period could affect the characteristics of the cell and so make it different from those seen in short term primary cultures. In addition, their study examined the stromal cell morphology only at the light microscope level using a phase-contrast microscope, and no ultrastructural observations were made.

In conclusion, the present study has established a novel three-dimensional culture system for human endometrial stromal cells *in vitro* which can be used as a model for *in vivo* experiments. Stromal cells in this culture model appeared very similar to those observed *in vivo*. In addition, an attempt was made to examine the effects of sex steroid hormone administration on the ultrastructure of the stromal cells in this culture model. While stromal cells from secretory endometria that were treated with oestrogen seemed to have a hypertrophic structure, progesterone alone had no effect on the ultrastructure of the stromal cells at either stages, whereas oestrogen + progesterone combination treatment on tissue taken from the secretory phase produced cells with a close resemblance to those observed in the human endometrial stroma *in vivo*. More studies are needed on this phenomenon for a complete understanding of the behaviour and structure of human endometrial stromal cells *in vitro*.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1. GENERAL DISCUSSION

The mechanisms of implantation and embedding of the embryo into the endometrium under the influence of the ovarian steroid hormones is a highly complex phenomenon which involves hormonal interactions between several organs (the so-called "hypothalamo - pituitary - ovarian - endometrial axis"). The endometrium is probably the most important part of the reproductive tract since it is the site for successful implantation and development of the embryo. While luminal and glandular epithelia play important roles in the apposition, adhesion and initial invasion of the blastocyst, it is the endometrial stroma which takes the main role in development of the decidual tissue and the blood vessels of the maternal placental tissue which are likely to be crucial in maintaining a successful pregnancy.

A total of five experiments have been reported in the present thesis, ranging from studies on the fertile and infertile human endometrial stroma *in vivo* to *in vitro* models of endometrial tissue. Chapter 2 dealt with the morphological changes that occur in endometrial stroma (including blood vessels) of normally ovulating fertile women during the mid to late luteal phase of the menstrual cycle (which is the time that stromal cells differentiate and form decidual tissue). In later chapters, the effects of ovulation induction and ovulation enhancement treatments (clomiphene citrate and growth hormone) on the endometrial stromal and vascular structures of normal fertile women and of women with several fertility problems (Turner's syndrome and idiopathic POF) have been reported. In addition, quantitative stromal and vascular structural differences were evaluated between women with persistent unexplained recurrent miscarriage and women with previous history of unexplained recurrent miscarriages but a subsequent successful pregnancy in order to provide descriptive data on any possible "endometrial factor" in this field of infertility, which is still unclear.

Objective stereological methods were used on a wide range of features in order to determine the changes in endometrial stromal and vascular morphologies between fertile and infertile women, and also to determine the effects of several infertility treatments on these compartments. In the last chapter of the thesis, a novel three-dimensional *in vitro* culture model was established which can be used as a model for *in vivo* conditions and

the effects of steroid hormones on the morphology of the stromal cells grown in this culture system were investigated qualitatively at light and electron microscope levels.

The 2nd chapter of the present thesis reported quantitatively and qualitatively the morphological changes that occur in stromal and vascular compartments of the normal fertile human endometrium during mid to late stages of the luteal phase. This part of the study was especially important since there is a lack of published information on the descriptive morphology of human endometrial stroma and blood vessels, and crucial events such as the differentiation of stromal cells (as occurs in decidualization) and changes in blood vessels that occur during this stage of a conception cycle.

In humans, decidualization takes place during the post-implantation period of a conception cycle under the influence of progesterone. The stromal cells display a similar type of modification during a non-conceptional menstrual cycle at around LH+7 which is called pseudo-decidualization, and these cells form the maternal decidual part of the developing placenta in case of a conception. Decidual cells are believed to play crucial roles in the later stages of implantation and in the maintenance of pregnancy through the control of trophoblast invasion (Pijnenborg *et al.* 1980), nourishment of the blastocyst (Keams and Lala 1983), endocrine (PRL) secretion (Maslar *et al.* 1980), and protection of the embryo from maternal immune rejection (Golander *et al.* 1981). Despite these crucial functions, little is known about the morphological changes that take place in the differentiation process of stromal cells into decidual cells (see earlier) and this remains an important area for further study. In the present study, stromal cells were evaluated regardless of whether they were pseudo-decidual cells or undifferentiated stromal cells. However, other cell types such as white blood cells and smooth muscle cells were excluded when the morphometrical measurements were performed.

Qualitative light and electron microscopical examination of the endometrial glandular epithelium throughout the luteal phase demonstrated large amounts of glycogen-like material in the subnuclear region of epithelial cells at day LH+4 of the cycle in normal fertile endometrium. At this time stromal cells also contained glycogen-like material. These observations could indicate that both compartments of the human endometrium are well prepared for the support and nourishment of a possible - "to be implanted"-embryo before the implantation process. These findings were also consistent with

previous studies on this subject which have shown that maximum subnuclear glycogen accumulation in glandular epithelial cells occurs around day LH+4 of the luteal phase (Dockery et al. 1988a). However, there appears to be no published reports on the presence of glycogen-like material in human endometrial stromal cells throughout the luteal phase, and the present study appears to be the first one demonstrating this material in human endometrial stromal cells at the ultrastructural level.

The present study showed that stromal cells underwent dramatic changes throughout the luteal phase. Stromal cell nuclear profile minor axis and profile axial ratio along with the mean profile diameter steadily increased towards the end of the cycle, indicating a wider and therefore rounder stromal cell nuclear shape during this time of the cycle. This, combined with the observation of an unchanged nucleus to stromal cell volume fraction suggested an increased cytoplasmic volume of the stromal cells in the late stages of the luteal phase. In addition, volume fractions of "secretory apparatus" to cell and RER to cell also increased towards the end of menstrual cycle. These quantitative observations were also consistent with the qualitative examination of the stromal cells during this time; stromal cell cytoplasm demonstrated increased amounts of organelles along with the presence of abundant collagen fibres in the extracellular matrix, indicating an increased activity of stromal cells around the time that decidualization process would take place.

Endometrial vascular morphology differed during the times examined; while the volume fraction of nucleus to endothelial cell decreased, the volume fractions of all cytoplasmic organelles (mitochondria, "secretory apparatus", RER) to endothelial cell significantly increased towards the end of the menstrual cycle. In addition, the vascular basement membrane became dramatically thicker in the later luteal phase than in early stages. The thickness of the blood vessel basement membrane is one feature which can affect diffusion between blood and surrounding tissue. Thus increased vascular basement membrane thickness towards the end of the cycle may indicate a decreased level of diffusion or transport through the endothelial tissue and the basement membrane at this time. Basement membranes are continuously eroded and renewed in a cyclical fashion (Wracko 1982), and it is either an increased rate of basement membrane renewal or a decreased rate of basement membrane erosion that caused the increase in blood vessel

basement membrane thickness towards the end of the menstrual cycle. The interaction between the production of ovarian steroids, the dynamics of the blood vessels of the functional layer, the permeability of the capillary endothelial cells and the development of oedema in the endometrial stroma are highly complex phenomena which are still incompletely understood. Therefore in the present thesis, the exact reason for these vascular changes that occurred towards the end of the luteal phase remains speculative.

In conclusion, the present study established a full description of stromal and vascular morphology throughout the luteal phase of normal fertile women, and thus provides baseline data for future work on human endometrial morphology.

The present thesis has reported the effects of an anti-oestrogenic drug (clomiphene citrate) on the endometrial stromal and vascular morphologies in normal fertile women. CC acts as an oestrogen antagonist in the hypothalamic site where it appears to block oestrogen receptors (Huppert 1979). It is used for induction of ovulation in anovulatory women via blockage of oestrogen receptors so causing a continuous secretion of gonadotrophins which eventually induce ovulation, and care must be taken to avoid hyperstimulation when using CC treatment. However, its' effects on the different compartments of the reproductive tract are unclear. Its' anti-oestrogenic action may cause adverse effects directly on the endometrium thereby reducing its' beneficial effects on ovulation. Most previous reports on the effects of CC on endometrial morphology have been performed in women being treated for infertility, and it is not possible to exclude any underlying pathology from their results. However, the present study examined the effects of CC on the endometrial stromal and blood vessel structures using the same group of women, which were of proven fertility so any effects seen in these endometrial compartments should be the result of CC treatment and not any underlying pathology.

Results have shown that treatment with CC did not cause major changes in glandular and stromal features whereas blood vessels seemed to be slightly affected by the treatment. Although the exact reason for structural changes in blood vessels following CC treatment is unclear, some speculative suggestions could be made. Eicosanoids (including prostaglandins, leukotrienes and related substances) are potent biological mediators which are produced by variety of tissues including those in the endometrium

and decidua (Shaw *et al.* 1994). These substances act as local hormones with a major influence on vasomotor tone, as mediators of vasoconstriction, vasodilatation and vessel permeability (Shaw *et al.* 1994). Prostaglandin (PG) dehydrogenase inactivates PGs by oxidising the 15-hydroxyl group, and thus plays a key role in controlling their effective local concentrations (Kelly *et al.* 1994). This enzyme is thought to be under the control of steroid hormones and is found in human endometrium at high levels in the secretory phase (Downie *et al.* 1974). It has been shown that the amount of PG dehydrogenase is increased during the secretory phase of the menstrual cycle following treatment with CC (Kelly *et al.* 1994). It could be speculated that slightly changed endometrial vascular morphology after CC treatment may be related to prostaglandin inactivation by CC in endometrium, which plays an important role in the permeability of the vessels, and has both vasoconstrictor and vasodilator functions.

It may be concluded that CC has no adverse effect on normal human endometrium in terms of morphology. In addition, the present study also provided a full profile of normal fertile human endometrial structure under the influence of an anti-oestrogenic drug, and thus filled a gap in this field of fertility treatment.

The fourth chapter of the present thesis has dealt with the effects of growth hormone treatment, with and without hormone replacement therapy, on endometrial stromal and vascular compartments in 2 groups of patients; those with Turner's syndrome and those with idiopathic premature ovarian failure (POF). These two groups were chosen since both had no ovarian function but the cause of this failure was very different in the 2 groups. GH has been used for the treatment of children with hypopituitarism and short stature, and also has been used to treat patients with Turner's syndrome to improve final height achieved (Wilton 1987). More recently, GH has been used to augment the response of the human ovary to stimulation by menopausal gonadotrophins (Homburg *et al.* 1990). It is likely that GH will be used more often in the future for the treatment of infertility. A review of the literature on GH action in the reproductive tract indicates that GH, along with gonadotrophins, the IGF system (with its ligands, receptors and binding proteins) and ovarian steroids have very complicated interactions and thus effects on the reproductive tract through the pituitary - ovarian - endometrial axis. However, despite its ovulation enhancing effect, the exact mechanism of GH action in fertility treatment

remains unknown. Chapter 4 of the present thesis aimed to investigate the effects of GH supplementation on human endometrial stromal and vascular morphologies in infertile women undergoing HRT and thereby throw some light on the mechanisms of GH action.

While there were no major changes in overall endometrial morphology between any of the untreated and GH treated infertile groups, features differed significantly between the fertile day LH+6 group and infertile groups with or without GH treatment, suggesting an impairment in the development of endometrium especially in Turner's syndrome patients. However, idiopathic POF individuals seemed to have more normal endometrial morphology and a better response to GH and HRT treatments than the Turner's syndrome group. GH administration has been reported to increase the cytosolic and nuclear oestrogen receptor concentrations in guinea-pig uterus (Bezecny *et al.* 1992). The present data strongly suggest that GH treatment may have substantial effects on the uterus, especially on the endometrium, as well as having influences on the ovulation enhancement in ovary.

Miscarriage is defined as "the expulsion or extraction of an embryo or fetus, weighing 500g or less, from its mother" (WHO 1977). In recurrent miscarriages, women manage to achieve pregnancy, but on 3 or more consecutive occasions lose the embryo before it is viable. Several reasons (including anatomical, genetic and immunological disorders) have been implicated for repeated pregnancy losses, however a large percentage of recurrent miscarriage cases still remain unexplained. The studies reported in the 5th chapter of the present thesis have been designed to determine if an "endometrial factor" exists in unexplained persistent pregnancy losses. Miscarriage is not a failure of implantation, but it is the rejection of an already implanted embryo. Therefore stromal and vascular compartments, rather than glandular and luminal epithelia, are likely to be major endometrial contributors for the rejection of an embryo. Taking this hypothesis as a starting point, the 5th chapter aimed to examine the endometrial and vascular morphologies in two groups of women; the first group was recruited from women with persistent unexplained pregnancy losses, and the second group consisted of similar women with a history of previous recurrent miscarriages but at least one successful pregnancy.

Results demonstrated no morphological differences between successful and unsuccessful pregnancy groups in stromal and vascular structures, however the coefficients of variation of some features were higher in unsuccessful group whereas in some features they were higher in the successful pregnancy group. These results suggested the possible existence of subgroups within the groups. Both recurrent miscarriage groups differed significantly from the day LH+6 fertile group, indicating an abnormal endometrial development at the stromal and endothelial cell levels in these individuals. It is suggested that examination of these compartments at later stages of the menstrual cycle may be more appropriate since most morphological and physiological changes in the stroma take place when the placenta is developing in a conception cycle and which coincides with the period that rejection of an embryo from the endometrium often occurs.

The precise cytological events that occur during interaction between embryo and endometrium are not entirely known, and direct *in vivo* microscopic studies of the structural changes that occur in human endometrial epithelium and stroma during implantation and gestation are virtually impossible to study in humans for ethical and technical reasons. *In vivo* animal experiments remain limited since the processes of implantation in animals is very different in detail from those in Man. A reliable and accurate *in vitro* model system is clearly required. Therefore the last chapter of present thesis was designed to establish a novel *in vitro* model system of human stromal cells and to use it to examine the effects of hormonal supplementation on the cultured cells and where possible to relate it to *in vivo* conditions reported in previous chapters.

In the 6th chapter, immunocytochemical observations of human endometrial cells before and after the culturing process revealed that the cell isolation method used in this experiment provided a stroma-enriched cell suspension that contained abundant anti-vimentin positive cells. In addition, electron microscopical observations following the culture of this cell suspension in a 3-dimensional culture system on an artificial ECM showed stromal cells with a close resemblance to those seen *in vivo*. Furthermore, oestrogen supplementation of these cultures produced cells with degenerative cellular structures whereas progesterone alone caused no changes, and oestrogen + progesterone combined treatment caused increased secretory changes in cultured stromal cells. In conclusion, a successful stromal cell isolation technique and a 3-D culture technique were produced in this preliminary experiment which has established a starting point for the study of human endometrial stromal cells in *in vitro* conditions which are very close to *in vivo* conditions.

The common theme of the present thesis was to evaluate the ovarian sex steroid hormone action in human endometrial stroma at the morphological level. While oestrogen is responsible for tissue proliferation throughout the proliferative phase, progesterone causes secretory changes in human endometrium during the luteal phase. In normally cycling fertile women, changing levels of oestrogen and progesterone throughout the menstrual cycle are reflected by sequential changes in endometrial structure and function (Johannisson *et al.* 1982, 1987; Dockery and Rogers 1989), leading to the successful implantation of the blastocyst into a receptive endometrium. Therefore in the second chapter of the present thesis, extensive structural changes were made of the changes in stromal cells and blood vessels which occur in the normal human endometrium during the mid to late luteal phase (when progesterone is the dominant hormonal profile). These observations were consistent with, but an extension of, previous reports (More *et al.* 1974a; Dockery *et al.* 1990). A significant correlation has been shown between plasma progesterone levels and the mean area of endometrial capillaries (Peek *et al.* 1992) further supporting the dependence of endometrial tissue on ovarian hormones. This also agreed with the findings of the present study.

Decidualization is a crucial event that occurs in post-implantation human endometrium. During this process stromal cells undergo extensive differentiation which is reflected by expression of new products (Huang *et al.* 1987; Clemmons *et al.* 1990; Tabanelli *et al.* 1992) and extensive morphological changes (Lawn *et al.* 1971; Tekelioglu-Uysal *et al.* 1975; Bijovsky and Abrahamson 1992). This differentiation process of endometrial stromal cells is under the control of progesterone, probably in concert with relaxin (Bryant-Greenwood 1991) and growth factors (Irwin *et al.* 1991). Biochemically, stromal cells at this stage of differentiation produce prolactin and ECM proteins (Maslar *et al.* 1980), and express oestrogen and progesterone receptors (Leawitt and Takeda 1986) which provide the mechanism for these stromal cell functions. A similar differentiation process (pseudo-decidualization) also occurs in non-pregnant human endometrium in the late luteal phase of the cycle (Flamigni *et al.* 1991). The time period examined in the present study coincides with the time that pseudo-decidualization occurs, therefore increased morphological profiles of human endometrial stroma observed in the present study throughout this period may well indicate this differentiation process in non-conception cycle.

The anti-oestrogenic drug (CC) treatment (often used clinically to induce ovulation) may cause structural changes in human endometrium via changes in the oestrogen receptor concentrations and actions in this compartment thereby causing a discrepancy between

ovulation (70%) and conception rates (30%) in women treated with CC (Wu 1984). Infertile women are not the best subjects to evaluate this effect because of their underlying pathologies, which may itself affect the influence of the drug treatment. There are two groups of reports dealing with the effects of CC treatment; the first group used infertile subjects and found adverse effects of CC treatment on oestrogen and progesterone receptor (ER and PR) concentrations (Koike *et al.* 1994) and endometrial structure (Benda 1992; Bonhoff *et al.* 1993); the second group used fertile subjects and found no effect of CC on ER and PR concentrations (Hecht *et al.* 1989) and endometrial morphology (Li *et al.* 1992). The present thesis also used normal fertile women to evaluate the effects of CC on human endometrial stromal and vascular morphologies and found no apparent effect, being consistent with the second group of reports.

A possible synergistic action of GH and steroid hormones for the enhancement of ovulation induction has been suggested (Homburg *et al.* 1988, 1990). However, this synergistic mechanism of GH and oestrogen may also have direct effects on endometrial structure and function. To examine this possibility, women with intact ovaries are not the best subjects to use since GH may exhibit its effects via hypothalamo-pituitary-ovarian axis in women with functional ovaries. Therefore in the present study, two groups of women (Turner's syndrome and idiopathic POF) with dysfunctional ovaries were used to eliminate the possibility of the indirect effect of GH and steroids via ovaries. Results of the present study showed no structural changes in these women either before or after GH treatment along with HRT. However, the Turner's syndrome group seemed to be poor responders to treatment, which also agreed with previous reports (Li *et al.* 1991b; Rogers *et al.* 1992).

Unexplained recurrent miscarriage is that where all factors which can currently be diagnosed (such as chromosomal anomalies and immunological factors) have been excluded, leaving the endometrium as the likely 'problem'. This may well be related to endocrine influences and it has been closely related to luteal phase defect by several authors (Tulppala *et al.* 1991; Serle *et al.* 1994; Dalton *et al.* 1995). Serle *et al.* 1994 found retarded endometrial development in women with recurrent miscarriage when compared to the control group. On the other hand, in the present study unchanged human endometrial morphology between successful and unsuccessful pregnancy groups were found. However, high coefficients of variance within groups indicated the existence of subgroups. Therefore more caution must be used for the definition of inclusion criteria into groups in the case of recurrent miscarriage.

7.2. CONCLUSIONS

The first aim of the present study was to establish baseline data on the morphology of fertile human endometrial stromal and vascular compartments during the mid to late luteal phase of the cycle. This was successfully done using objective morphometry and appropriate staining methods.

It has been reported that ovulation induction by CC has a success rate of 70%, whereas the pregnancy rate remains at about 30% (Wu 1984). This could be the result of an adverse effect of CC administration on endometrium, however the present study demonstrated that the CC treatment had no substantial effects on stromal morphology, and thus this is unlikely to be the cause of the discrepancy between ovulation and fertilization rates.

The effects of GH treatment with or without HRT on human endometrial stroma and blood vessels were evaluated in two groups of sub-fertile women with non-functioning ovaries. The use of GH to enhance ovulation in infertile women is relatively new and its structural effects on the endometrium are relatively unknown. Results showed that GH treatment caused no substantial effects on endometrial structure in the experimental groups, although both of these groups were dramatically different from the day LH+6 control endometrium. Of the 2 experimental groups studied, Turner's syndrome subjects showed no change either with or without HRT, women with idiopathic POF did respond to GH treatment in some ways.

The present study was also designed to evaluate the endometrial stromal and vascular structural differences between persistent unexplained recurrent miscarriage subjects and a group with previous similar history of unexplained recurrent miscarriage, but at least one successful pregnancy. Results demonstrated no significant differences between groups at the level of endometrial stromal and blood vessel morphology, however high coefficient of variations indicated the existence of subgroups within groups, indicating that better diagnosis of this condition is required to improve experimental investigation into structural events.

The last chapter of the present study aimed to establish an *in vitro* 3-dimensional culture model for human endometrial stromal cells and to evaluate the effects of sex

steroids on stromal cell in culture, both of these aims were successful and this model can be used for further *in vitro* studies.

FUTURE WORK

Future research on this subject area could be seen along the following lines:

Absolute volumes of human endometrial stroma, blood vessels and stromal and endothelial cell can be determined at different stages of the menstrual cycle using appropriate stereological methods such as volume weighted mean volume. This may be important since they can be used as reference points for calculations of absolute volumes of different cellular components including mitochondria, RER and "secretory apparatus".

In the present study, the morphological evaluation of human endometrial stromal compartment in unexplained recurrent miscarriage patients suggested that the investigation of this compartment during later stages of the menstrual cycle and on well defined recurrent miscarriage subjects (to avoid the effects of different aetiologies within the groups) could be more appropriate.

The effects of growth hormone treatment on human endometrial development must be examined using fertile human endometrial tissue, since the underlying pathologies of infertile subjects could affect the results of the experiment . To do this, the *in vitro* 3-dimensional cell culture system, that was successfully established in the present study, could be used as a model for human endometrium *in vivo*.

Also the effects of ovulation induction regimes (such as the clomiphene citrate treatment) could be performed on cultured endometrial cells *in vitro*, as could progesterone receptor blockade using, for example, RU 487.

The effects of steroid hormone supplementation at several different dosages on stromal cell morphology could be examined using the 3-dimensional cell culture method. In addition, the influence of different drug treatments on human endometrial stromal cells can also be investigated *in vitro*.

The implantation procedure in humans is impossible to study *in vivo*, but further development of the 3-D culture system established in the present thesis, can be modified by growing endometrial epithelial cells on top of the stromal cells, and human embryos can be put on cells in culture. However, such studies may have ethical and practical difficulties, such as obtaining the human embryos, and such studies also require precise timing and planning as well as a good experimental design such as amount and

proportion of sex steroids, endometrial biopsy time, proportion of stromal and epithelial cells in culture and so on.

The decidualization process of the human endometrial stromal cells in late luteal phase of the cycle is still an unclear phenomenon, and a new understanding of this subject can be established by using different amounts and proportions of oestrogen and progesterone hormone supplementations on stromal cells in the present culture model.

The angiogenic process during the formation of placental tissues in pregnant human endometrium is another subject that more attention must be paid. To overcome the difficulties for *in vivo* human experiments, *in vitro* culture methods for blood vessel endothelial cells could be established.

APPENDICES

APPENDIX 1

FIXATION

- **PREPARATION OF 3% GLUTARALDEHYDE SOLUTION IN PHOSPHATE BUFFER**

Add 12 ml of 25% glutaraldehyde stock solution to 88 ml of 0.1M sodium phosphate buffer (pH 7.4).

- **PREPARATION OF 0.1M SODIUM PHOSPHATE BUFFER**

1. Dissolve 3.56 grammes of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 100 ml of distilled water (solution A).
2. Dissolve 3.12 grammes of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 100 ml of distilled water (solution B).
3. Mix 36 ml of solution A with 14ml of solution B (0.2M phosphate buffer).

Add 50 ml distilled water to the 0.2M phosphate buffer (0.1M phosphate buffer).

LIGHT MICROSCOPY

- **PREPARATION OF JB4 RESIN**

Catalysed JB4 solution A: Dissolve 0.45 grammes of catalyst in 50 ml of JB4 solution A and stir for 30 minutes at room temperature.

JB4 embedding solution: Mix 25 ml of catalysed JB4 solution A and 1 ml of JB4 solution B and use within 10-15 minutes.

ELECTRON MICROSCOPY

- **PREPARATION OF EPON RESIN**

10.16 gm Agar 100 Resin(Agar, Essex, UK).

7.50 gm MNA (Methyl Nadic Anhydride) (Agar, Essex, UK).

6.25 gm DDSA (Dodecenyl Succinic Anhydride) (Agar, Essex, UK).

0.72 gm BDMA (n-Benzyldimethylamine) (Agar, Essex, UK).

- **PREPARATION OF GELATINE METHENAMINE SILVER (GMS) SOLUTION**

1. 50 ml of 3% aqueous methenamine.

2. Add 5 ml of 5% aqueous silver nitrate, drop by drop with constant mixing.

3. Make up the solution to 95 ml by adding 40 ml distilled water.

4. Add 5-6 ml of 5% sodium tetraborate and mix.

This solution must be made fresh just before use and kept in the dark.

APPENDIX 2

• PREPARATION OF MATRIGEL COATED MILLICELL INSERTS

Matrigel is stored at -20 °C and thawed at 4 °C. Procedure is carried out on a cold block to avoid setting too soon.

- 1. Millicell inserts are removed from the packing and placed into the well.**
- 2. Approximately 50 µl of Matrigel is placed in the insert ensuring that the bottom is totally covered with matrigel.**
- 3. Approximately 0.5 ml of cell suspension is added into the insert and the same amount of DMEM-F12 media is added around the insert.**

• PREPARATION OF DMEM-F12 CULTURING MEDIA

All ingredients are collected together, all the bottle tops are loosen and all the bottle necks are flamed in order to prevent possible contamination.

In a sterile bottle add:

2.5 ml of Foetal Calf Serum (Imperial, UK)

2.5 ml of Nu Serum

1.0 ml of Streptomycin/Penicillin (Life Technologies, Paisley, Scotland)

1.0 ml of L-glutamine (Life Technologies, Paisley, Scotland)

3.0 ml of Sodium Bicarbonate (Life Technologies, Paisley, Scotland)

2.0 ml of HEPES

88 ml of Dulbecco's Hams F12 media (Life Technologies, Paisley, Scotland)

Final solution media is filtered through a Millipore filter into another sterile bottle.

• PREPARATION OF THE SUBSTRATE-FAST RED COMPLEX FOR CYTOKERATIN AND VIMENTIN IMMUNOSTAINING

2 mg Naphtol AS Phosphate

0.2 ml Dimethylformamide

9.8 ml 0.1 M Tris buffer (pH 8.2)

10 µl 1 M Levamisole

10 mg Fast Red TR Salt

Filter the final solution directly onto the slide.

APPENDIX 3

• WORKED EXAMPLE OF VOLUME FRACTION CALCULATIONS

Volume fraction measurements of endometrial glandular, stromal and vascular features were performed by counting the points of the related features on superimposed grid points (Weibel 1979). Following determination of total points that have fallen on the feature (e.g. stroma), this number is divided by total number of points that have fallen on the whole feature (e.g. endometrium). Mean value of each equation gives the volume fraction value of that feature (e.g. volume fraction of endometrium occupied by stroma). Example given below was taken from block number J792 (recurrent miscarriage individual) of the volume fraction measurements of glandular and stromal features at the light microscope level.

Total number of points on Glands: 234

Total number of points on Stroma: 513

Total number of point on Endometrium: 747

- The volume fraction of endometrium occupied by glands: $234/747 = 0.313$**
- The volume fraction of endometrium occupied by stroma: $513/747 = 0.687$**

APPENDIX 4


• WORKED EXAMPLE OF HARMONIC MEAN BASEMENT MEMBRANE THICKNESS MEASUREMENT (Hirose *et al.* 1982)

Table demonstrates the calculation of harmonic mean apparent basement membrane width (l_h) (data were taken from GH Group - J782).

Class number	A		B		C		D x C	
	Lower limit (mm)	1/Lower limit (mm ⁻¹)	Class midpoint (mm ⁻¹)	Number of observations	D	D	D	D
0	1.50	0.667	0.574	19	10.906			
1	2.09	0.480	0.413	42	17.346			
2	2.90	0.345	0.297	31	9.207			
3	4.02	0.249	0.214	30	6.420			
4	5.60	0.179	0.154	11	1.694			
5	7.77	0.129	0.111	3	0.333			
6	10.8	0.0927	0.0797	0	0			
7	15.0	0.0667	0.0574	0	0			
8	20.9	0.0480	0.0413	0	0			
9	29.0	0.0345	0.0297	0	0			
				Σ	136	45.906		

$$l_h = \Sigma D / \Sigma(C \times D) = 136 / 45.906 = 2.963 \text{ mm}$$

$$T_h = 8/3\pi \times l_h (2.963) \times 10^6 / \text{magnification} (25 \ 164) = 99.96 \text{ nm}$$

- T_h - harmonic mean thickness 8/3 π - Correction factor for harmonic thickness estimation
- 10^6 - is used to convert the final value from millimetres to nanometers
- ♦ The logarithmic ruler used for harmonic mean basement membrane thickness measurements (in actual size): 

APPENDIX 5

• WORKED EXAMPLE OF ARITHMETIC MEAN BASEMENT MEMBRANE THICKNESS MEASUREMENT (Hirose *et al.* 1982)

An ordinary ruler is used to measure the orthogonal intercepts of the grid fallen on blood vessel basement membrane. Data were taken from Growth Hormone Group (J782).

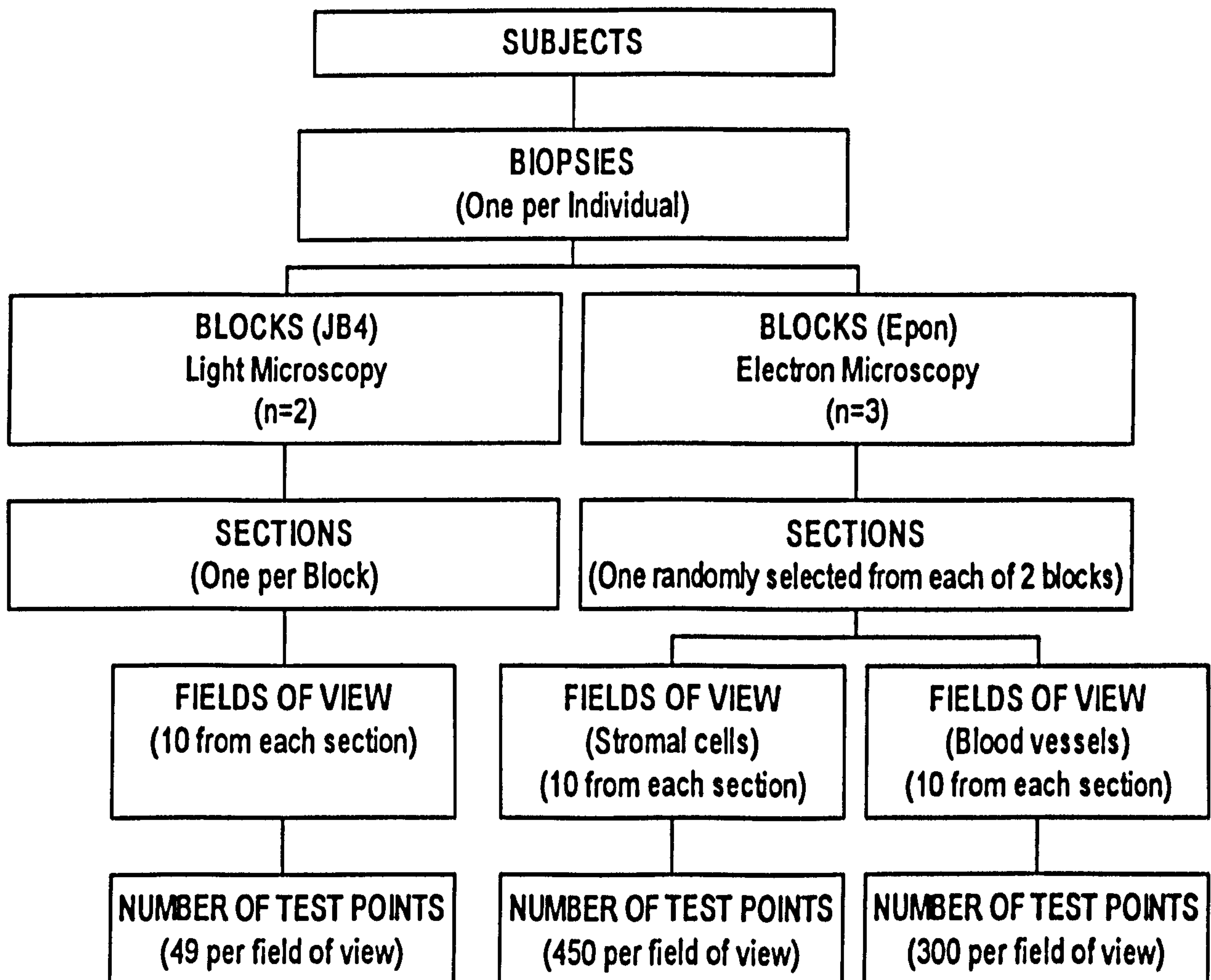
$$\ell_a = \Sigma \text{ length (mm)} / \Sigma \text{ number of observations}$$

$$\ell_a = 492 / 137 = 3.591 \text{ mm}$$

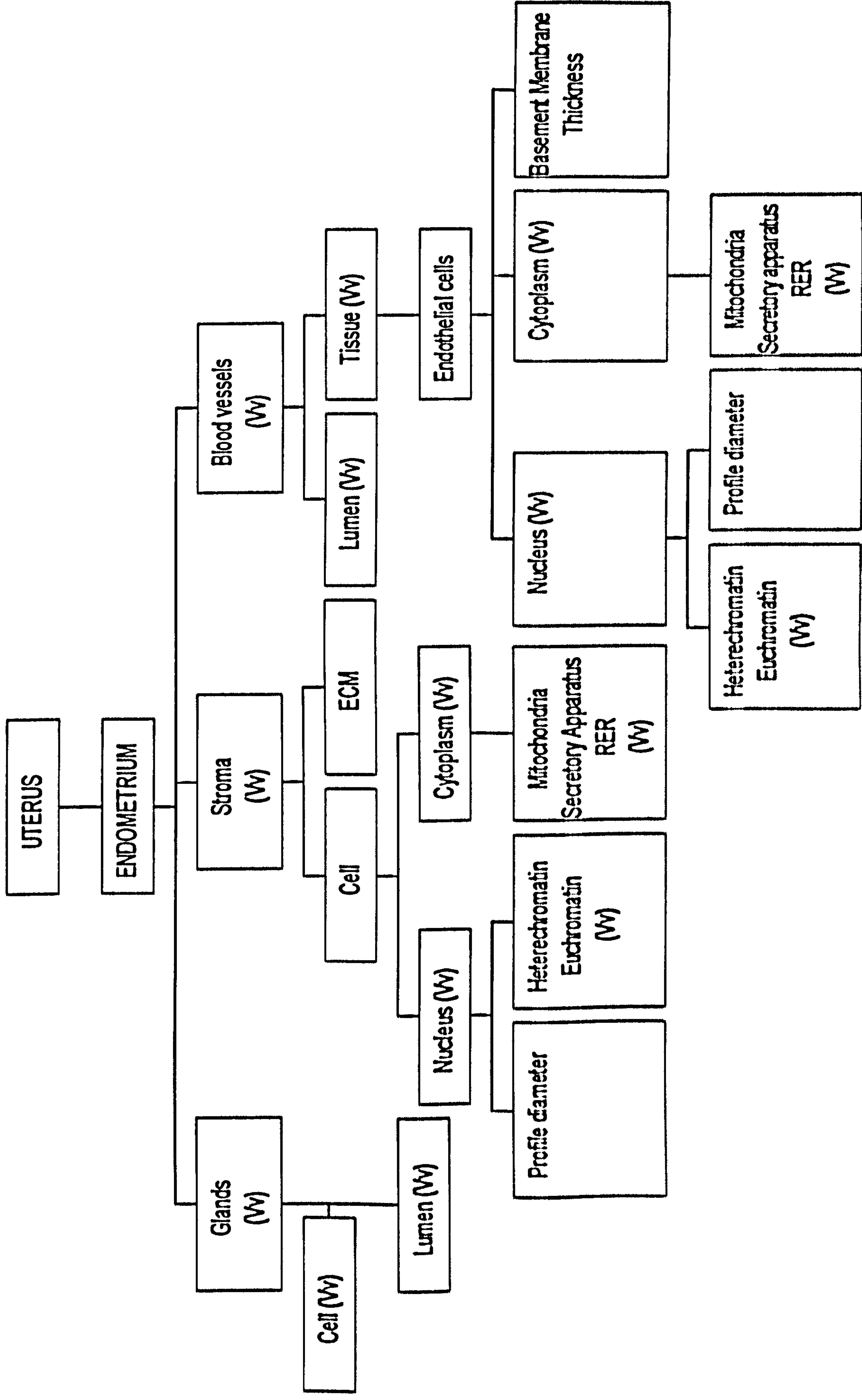
$$T_a = \ell_a (3.591) \times \pi/4 (0.79) \times 10^6 / \text{magnification (25 164)} = 112.08 \text{ nm}$$

- * T_a - Arithmetic mean thickness
- * ℓ_a - Mean arithmetic width of the blood vessel basement membrane
- * $\pi/4$ - Correction factor for arithmetic mean thickness estimation
- * 10^6 - is used to convert the final value from millimetres to nanometers

APPENDIX 6: Sampling tree showing the sampling steps used in for Vv estimates in the present experiment. The number of individuals per group differed for each set of experiment, and this information, along with the numbers of block, micrograph and fields used is given in the appropriate Materials and Methods section of each chapter.



APPENDIX 7: Morphometric model of human endometrium.



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