The effect of platelet releasate on oocytes has a detrimental impact on early mammalian embryos.

Francesca Martin

MSc by Thesis – Medical Sciences

The University of Hull and The University of York

Hull York Medical School

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Abstract

Background: Blood-derived products are a growing area of therapeutic treatments for a diverse range of conditions, including hair loss, scarring, muscle injury, wounds, arthritis, and symptoms related to the menopause. Platelet-rich plasma (PRP) is one blood product being trialled on the menopausal ovary. Emerging data from ongoing trials suggest that PRP injection might promote resumption in ovarian function, and an increase in both spontaneous and assisted conception. However, knowledge of the direct cellular effects of PRP on oocytes and early embryos is limited.

Hypothesis & Aim: We hypothesised that platelet releasate would influence embryo development. This study aimed to determine the extent to which the embryo is affected by releasate. We further hypothesised that oocytes (mature and dead) and cumulus cells would activate platelets. This study aimed to determine the extent to which oocytes and cumulus cells were able to induce platelet activation.

Method: Oocytes were collected from abattoir-derived bovine ovarian tissue and incubated for 22 hours in in the presence of either 10% platelet releasate (derived from whole human blood) or 10% foetal calf serum (FCS) (control). A proportion of oocytes were selected for nuclear staining to establish maturation status. The majority of the oocytes were fertilised with bull sperm and allowed to develop into embryos. The number of embryos that reached the 2-cell stage by day 2 (cleavage rate) and blastocyst stage by day 9 (blastocyst rate) were recorded, as were a variety of blastocyst endpoints: blastocyst metabolism, mitochondrial function, and expression of the epigenetic marker, H3K27me3. Separately, the effect of oocytes on platelets was investigated. Mature oocytes, dead (unexpanded) oocytes, culture media, and cumulus cells were added to PRP to determine their capability to activate platelets via a series of aggregations.

Results: Platelet releasate had no discernable effect on metaphase II rate in oocytes matured *in vitro*. There was no significant difference between the cleavage rate of platelet releasate and FCS derived oocytes, however the blastocyst rate of platelet releasate derived embryos was significantly lower than that of the FCS group. No significant difference was found in lactate release and pyruvate depletion of platelet

releasate and FCS derived embryos. However, glucose depletion was significantly reduced in platelet releasate derived embryos in comparison to the FCS control. Oxygen consumption rate, used as a measure of mitochondrial function, did not differ significantly between the groups, and neither did H3K27me3 expression – both platelet releasate derived embryos and FCS derived embryos expressed the modification. No significant difference in platelet activation of PRP was observed between mature oocytes, dead oocytes, culture media, and cumulus cells exposure, although repeats were inconsistent.

Conclusion: Oocyte exposure to platelet releasate did not lead to differences in nuclear maturation, the oocytes' capability to be fertilised was similar between the releasate group and FCS control. The number of oocytes that were able to cleave after fertilisation was unaffected by prior supplementation with platelet releasate. However, platelet releasate derived embryos were significantly less likely to develop into blastocysts. In comparison with the FCS control, platelet releasate derived embryos that did develop into blastocysts, had a reduced consumption of glucose but a normal pyruvate consumption and lactate production, they also had a normal oxygen consumption rate and normal H3K27me3 expression. Neither mature oocytes, nor dead oocytes, nor cumulus cells, showed ability to activate platelets to a further extent than culture media.

While platelet-derived factors injected into the ovary might stimulate resumption of ovarian activity, the oocytes exposed to such factors may have diminished developmental competence once they form embryos.

Publications and Conferences

In conjunction with writing the Introduction section of this thesis, a literature review was co-authored; Atkinson L, Martin F, Sturmey RG. Intraovarian injection of platelet-rich plasma in assisted reproduction: too much too soon? Hum Reprod. 2021 Jun 18;36(7):1737-1750.

Sections of this thesis research have been presented in poster format, titled “Platelet releasate significantly reduces the development of early mammalian embryos”, at the Fertility 2022 conference.

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Author's Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources, these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources.

Abbreviations

Acid Citrate Dextrose (ACD)

Adenosine Diphosphate (ADP)

Adenosine Triphosphate (ATP)

Anti-Mullerian Hormone (AMH)

Antimycin A and Rotenone (A/R)

Antral Follicle Count (AFC)

Assisted Reproductive Technology (ART)

Bone Morphogenic Proteins (BMPs)

Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)

Endothelial Growth Factor (EGF)

European Society of Human Reproduction and Embryology (ESHRE)

Foetal Bovine Serum (FBS)/Foetal Calf Serum (FCS)

Follicle Stimulating Hormone (FSH)

Gonadotropin-Releasing Hormone (GnRH)

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

Hormone Replacement Therapy (HRT)

In Vitro Fertilization (IVF)

In Vitro Maturation (IVM)

Interferon alpha (IFN-α)

Interleukin (IL)

Luteinising Hormone (LH)

Nicotinamide Adenine Dinucleotide (Hydrogen) (NAD(H))

Oocyte Maturation Inhibitor (OMI)

Oocyte-Cumulus Complex (OCC)

Ovarian Stem Cell (OSC) Ovarian Surface Epithelium (OSE)

Oxygen Consumption Rate (OCR)

Paraformaldehyde (PFA)

Phosphate Buffered Saline (PBS)

Platelet-Poor Plasma (PPP)

Platelet-Rich Plasma (PRP)

Platelet-Derived Growth Factor (PDGF)

Polyvinylpyrrolidone (PVP)

Poor Ovarian Responders (POR)

Premature Ovarian Failure (POF)

Premature Ovarian Insufficiency (POI)

Primordial Germ Cells (PGCs)

Protease-Activated Receptor 1 (PAR1)

Stem Cell Factor (SCF)

Stromal Cell-Derived Factor-1 (SDF-1)

Thrombin Receptor Activated Peptide-6 (TRAP-6)

Trimethylation of Histone 3 Lysine 27 (H3K27me3)

Tumour Necrosis Factor alpha (TNF-α)

Vascular Endothelial Growth Factor (VEGF)

Chapter 1 Introduction

1.1 Female Fertility

1.1.1 Gametogenesis

During the second week of embryo development, primordial germ cells (PGCs) are formed, and in week 4 they migrate to the developing gonads. PGCs undergo mitotic divisions during this time, increasing their numbers. At the female gonad (the ovary), PGCs differentiate into oogonia and again divide via mitosis to form “nests” (Larose *et al.*, 2019), some develop into primary oocytes (Sánchez and Smitz, 2012). At month 5 of development, a female foetus will have the most germ cells of her lifetime – approximately 7 million. It is at this point that atresia begins, where many primary oocytes and the majority of oogonia degenerate. By month 7, the surviving primary oocytes have all entered prophase of meiosis I and most have formed primordial follicles (primary oocyte surrounded by layer of pre-granulosa follicular cells). Many oocytes which have not formed primordial follicles will undergo apoptosis (Sánchez and Smitz, 2012).

The primary oocytes enter the diplotene stage of prophase I, and then arrest near the time of birth (dictyate phase). A peptide factor called Oocyte Maturation Inhibitor (OMI), produced by the pre-granulosa cells, arrests their meiotic development at prophase I until puberty is reached and a pre-ovulatory hormone surge occurs and inhibits OMI activity (Channing *et al.*, 1980; Tsafriri and Pomerantz, 1986). Under 1 million primary oocytes remain at the point of birth, with many becoming atretic during childhood, leaving approximately 300,000 at the commencement of puberty (Woodruff, 2008) – although fewer than 400 oocytes will actually be ovulated (Park *et al.*, 2022).

1.1.2 Menarche

The term menarche describes the onset of the menstrual cycle, marked by the first menstrual bleed – a crucial moment within female puberty. For ovulation and menarche to commence, the hypothalamic-pituitary-ovarian axis must mature. This maturation includes: an increase in the release of Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH) from the pituitary gland; the ovaries recognising and responding to these gonadotropins by producing oestrogen and progesterone; and

positive feedback regulation of the hypothalamus and pituitary gland by oestrogens (Bablis, Pollard and Monti, 2006).

Menarche is thought to be closely related to the attainment of a critical level of body fat (Frisch, 1987), and could explain why there has been historical variance in the age of menarche (Baker, 1985). Prior to puberty, metabolic changes occur, including rising levels of serum Leptin to meet a threshold which triggers reproductive ability (Matkovic *et al.*, 1997). Leptin inhibits neuropeptide Y, which controls gonadotropin-releasing hormone (GnRH) neuronal activity in the hypothalamus. As Leptin levels increase, neuropeptide Y is inhibited, allowing GnRH levels to increase. Leptin also affects GnRH levels through kisspeptin and pro-opiomelanocortin pathways (Hausman, Barb and Lents, 2012).

The initiation of puberty is related to the pulsatile release of GnRH, which acts on the anterior pituitary to increases the pulse amplitude of FSH and LH, the first endocrinological indicator of puberty (Spaziani *et al.*, 2021) (as depicted in Figure 1.1).

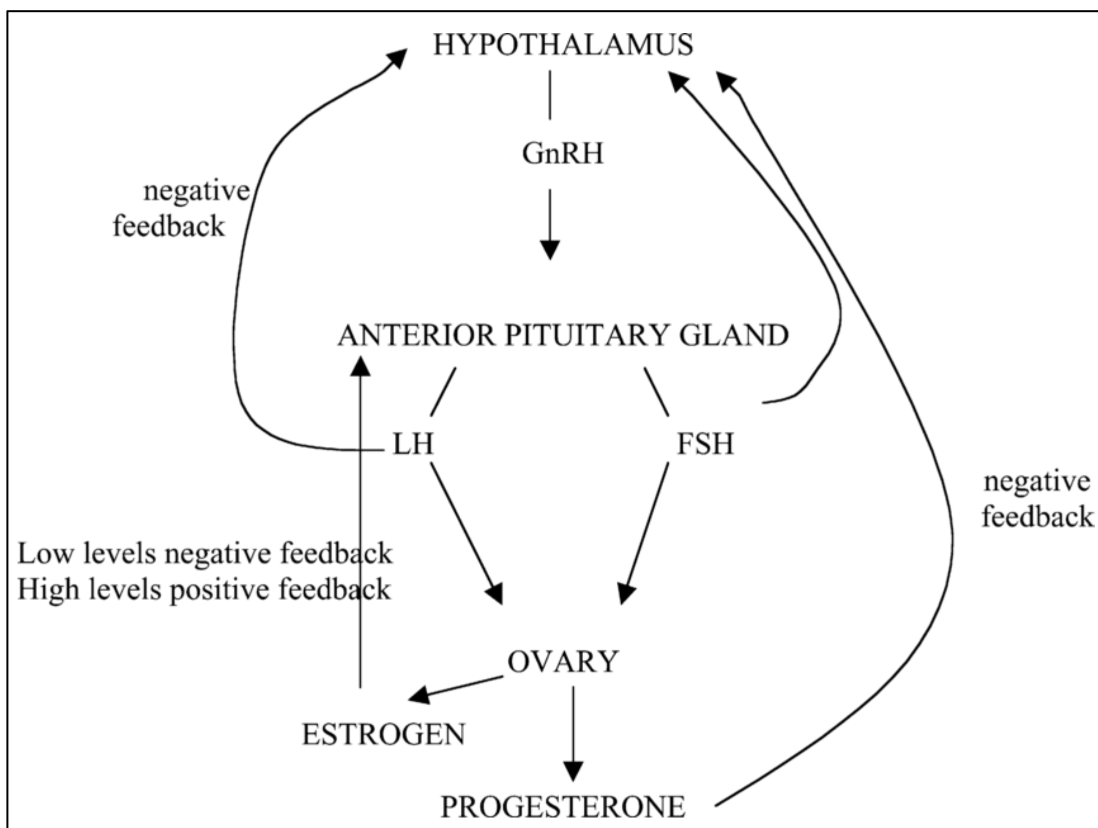

Figure 1.1. Overview of the hormonal feedback system that controls the menstrual cycle (adapted from Bablis, Pollard and Monti, 2006).

In response to regular production of LH and FSH, the ovary begins to consistently synthesise oestrogens which support the development of the ovarian follicle and maturation of the oocytes within. Ovarian oestrogens also regulate the production of gonadotropins from the pituitary gland, and the integrated feedback mechanism is described as the hypothalamic-pituitary-ovarian axis, as shown in Figure 1.1. Once this axis is established, ovarian oestrogens produced by the granulosa cells within the follicle, will drive the development of a dominant ovarian follicle, leading to the ovulation of a mature oocyte, and subsequent menses (Holesh, Bass and Lord, 2022).

1.1.3 The Ovarian Cycle

The ovarian cycle is the foundation of female fertility. It consists of three broad phases: the *follicular* phase, *ovulation*, and *luteal* phase.

The pool of growing follicles established at puberty is maintained by the supply of primordial follicles, of which 15-20 each month are recruited to begin maturation. This process of folliculogenesis can take 6 months to a year, and typically only 1 follicle is selected to be the dominant follicle which escapes atresia and is ovulated, so a continuous monthly supply of primordial follicles is critical (Sánchez and Smitz, 2012). This process of recruitment gives rise to extensive oocyte loss, as most follicles are not selected to be dominant follicles and therefore undergo atresia (as shown in Figure 1.2). It is thought that each ovary can lose up to 1000 oocytes per ovulatory cycle, consisting of oocytes within primordial stage to early-antral stage follicles – causing a monthly depletion of oocyte reserve which accelerates with age as oocyte quality declines, ultimately ending in the menopause when no oocytes remain (Holesh, Bass and Lord, 2022).

During the follicular phase the ovarian follicle matures and grows from a primary follicle to a pre-ovulatory follicle. The granulosa cells proliferate, forming multiple layers of granulosa. The theca layer forms, and both the theca and granulosa proliferate further (see Figure 1.2).

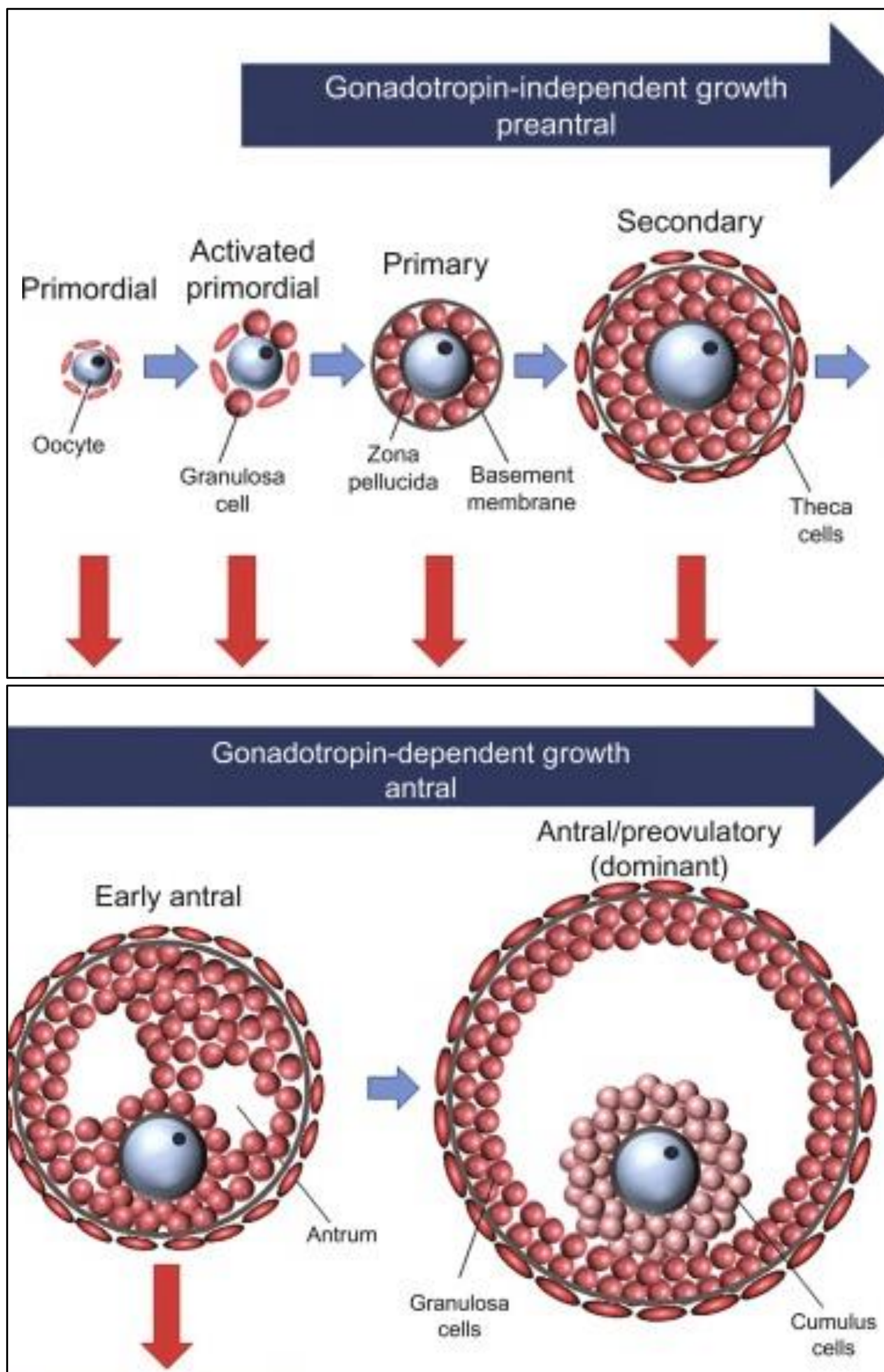

Figure 1.2. Illustration of follicular development from primordial to preovulatory stage (Findlay *et al.*, 2019). Red arrows indicate possibility of follicular atresia.

FSH from the pituitary gland promotes the formation of follicular fluid – establishing the antrum, and under FSH the granulosa cells differentiate into two populations – cumulus granulosa cells which surround the oocyte, and mural granulosa cells which line the follicle wall (as shown in Figure 1.2). Under FSH, undifferentiated granulosa cells differentiate into mural granulosa cells and express LH receptors (Turathum, Gao and Chian, 2021).

Whilst the follicle is developing, the oocyte within is also maturing. A “pre-ovulatory surge” of FSH and LH levels support the oocyte’s development from primary to secondary stage and propels the follicle into the pre-ovulatory stage, completing the follicular development process. LH acts on the follicle’s LH receptors, causing mural granulosa cells to dissociate by reducing gap junctions between cells, whilst FSH causes the oocyte-cumulus complex (OCC) to expand (Son *et al.*, 2011; Turathum, Gao and Chian, 2021). The LH surge induces germinal vesicle membrane breakdown (within which contains the oocyte’s 46 chromosomes) an the nucleus resumes meiosis I and arrests at metaphase II, now a mature oocyte with 23 chromosomes – capable of being fertilised (Turathum, Gao and Chian, 2021).

Within a few hours of the LH surge the cumulus mass expands as it produces hyaluronic acid, the follicle swells, and its wall becomes thin (Turathum, Gao and Chian, 2021). The follicular phase ends at ovulation, when the follicle ruptures due to plasmin and collagenase enzyme action (Thibault and Levasseur, 1988), and releases the mature OCC into the peritoneal cavity, where it is collected by the fimbriae (Li and Winuthayanon, 2017). The beating of the ciliated tubal wall is caused by smooth muscle contraction, regulated by prostaglandins and their receptors, of which expression is influenced by oestradiol (an oestrogen) and potentially progesterone (Huang *et al.*, 2015). This contraction moves the OCC, which is viable to be fertilised by spermatozoa for up to 24 hours, along the oviduct to the ampulla, and begins its descent towards the uterus (Huang *et al.*, 2015; Mahé *et al.*, 2021).

In response to the LH surge, luteinisation of follicular cells in the dominant follicle occurs, and the process enters the luteal phase. Luteinisation comprises the theca interna cells developing into small luteal cells, and the granulosa cells into large luteal cells which switch from expressing FSH-receptors to LH-receptors. These cells form the corpus luteum, a hormone-secreting mass of luteal cells, which produce progesterone in response to LH (Holesh, Bass and Lord, 2022).

If implantation occurs, the corpus luteum receives a signal – which may be derived from the conceptus, between day 5 and day 9, preventing corpus luteum regression and thus sustaining progesterone and oestradiol secretion during the first phases of pregnancy (Holesh, Bass and Lord, 2022). Without the signal to the corpus luteum, it will remain in

the ovary for 2-14 days and then degenerate by luteolysis into the corpus albicans – scar tissue. This cessation of progesterone production leads to the commencement of the follicular phase again as FSH production is no longer inhibited, and so the level increases (Lesoon and Mahesh, 1992).

1.1.4 The Uterine Cycle

Concomitant with the ovarian cycle, the uterus also undergoes cyclical changes. The uterine cycle can be split into three broad phases: *menses*, the *proliferative* phase and the *secretory* phase.

Menses and the proliferative phase both occur during the ovaries' follicular phase, with menses marking the first day (Figure 1.3). Menses is the degradation of the uterine lining, whereby the top two thirds of the endometrium are shed in the absence of progesterone (Jabbour *et al.*, 2006), marked by blood and tissue loss for typically 5-7 days.

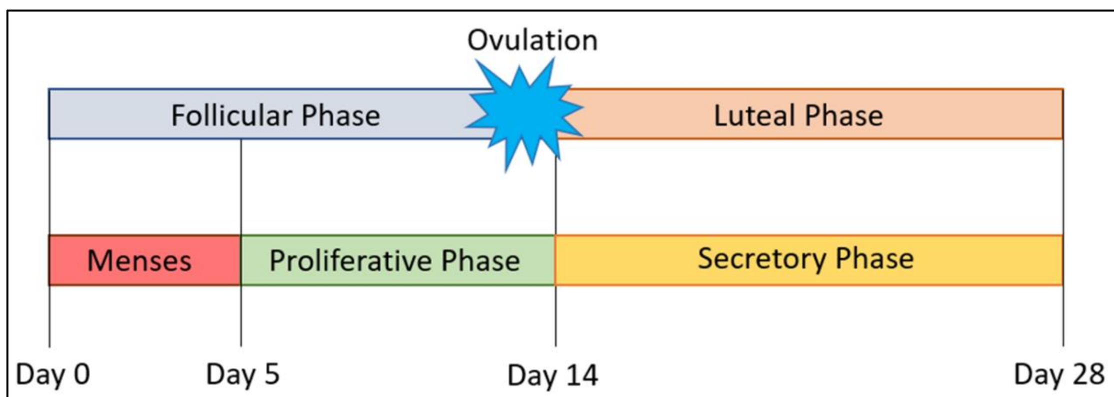

Figure 1.3. Representation of the ovarian and uterine cycle from day 0 to day 28, with ovulation marking the end of the follicular phase on ~day 14.

Upon cessation, the lining begins to repair and develop (proliferative phase), increasing in blood supply and thickness as a result of a rising oestradiol level (Jabbour *et al.*, 2006). This oestrogen production from the ovary is a result of the anterior pituitary's increased FSH production during the ovarian follicular phase (Figure 1.1). Tissue regeneration from the underlying basal layer is oestrogen-dependent and involves interaction between the immune system and endocrine system. An influx of leukocytes covers the exposed surface, spiral form arterioles develop as the epithelial, endothelial, and stromal cells regenerate (Jabbour *et al.*, 2006). The blood supply provided to the endometrium by the microvasculature also creates an environment that can support

the placenta if implantation and pregnancy occur during the secretory phase (Strassmann, 1996).

After ovulation occurs the secretory phase begins, driven by the rising levels of progesterone secreted by the luteal cells of the corpus luteum (during the ovarian luteal phase). The progesterone production causes a pause in tissue proliferation of the uterine lining by converting oestradiol into oestrone (a less biologically active type of oestrogen) and by supposedly reducing oestrogen receptor expression (Critchley *et al.*, 2020). The endothelium differentiates and maintains its thickness for a further week in preparation for the implantation of an embryo. When there is no implantation event, the lining begins to thin in sync with the degradation of the corpus luteum and the associated decline in progesterone level (Critchley *et al.*, 2020). Once complete, the lining again begins to degrade and shed out of the uterus – signifying the re-entry into the menses phase.

1.1.5 Ovarian Exhaustion

Ovarian exhaustion refers to when the ovary diminishes its reserve of follicles and ceases to ovulate oocytes. Typically, this begins with a transition from normal ovulation to oligo-ovulation (perimenopause) and then anovulation (Kailas, Sifakis and Koumantakis, 2005), resulting in the menopause – which signals the end of a woman's reproductive lifetime at approximately 50 years of age. When ovulation and menstruation cease in women under the age of 40, it is regarded as premature menopause or premature ovarian insufficiency/failure (POI/POF) (Nelson, 2008). The main causes of premature menopause are pharmacological (for example, chemotherapy treatment), surgery, but mostly idiopathic (Rossetti *et al.*, 2017). Because menopause refers to the exhaustion of the ovarian reserve of follicles, it is irreversible and leads to infertility. This is especially devastating when menopause is premature and before a woman has completed her family. Whilst the symptoms of menopause, such as temperature irregularity, mood swings, and brain fog can be treated by using medications such as Hormone Replacement Therapy (HRT) (Nelson, 2008), the loss of fertility cannot (Hegazy, 2020).

The climacteric (perimenopause) describes the phase of life immediately before menopause and is characterised by a reduced, but not exhausted ovarian reserve

(Taechakraichana *et al.*, 2002). Perimenopause is a transitional period typically characterised by irregular menses, due to a loss of ovarian cyclicity, which can last for up to 5 years before ovarian reserve is exhausted and menopause is reached (Nelson, 2008). There is hope for restoring fertility in women experiencing perimenopause, as a reduced supply of functional ovarian follicles is still present in the ovary, so ovulation and therefore conception remains theoretically possible – although the monthly probability of conception in women as young as their mid-forties, can decline by 50% (Kailas, Sifakis and Koumantakis, 2005).

As more women delay childbearing, they consequently move closer to perimenopause at the time of trying to conceive, this has led to the rise in couples seeking fertility treatment and the increase in the ages of women seeking such assistance. Between 2006 and 2016, the proportion of women over 40 undergoing autologous IVF (using their own eggs) increased from 20% to 25% in Australia (Vollenhoven and Hunt, 2018). The success of fertility treatments in perimenopausal women is largely dependent on the ovarian reserve of the individual; if functional follicles are not present to generate oocytes, then donor eggs may be recommended to the patient.

1.2 Infertility

Infertility is described as the inability to conceive. Infertility can be primary – where a patient has never conceived a child and is having difficulty conceiving currently, or secondary – where a patient has conceived in the past but is currently struggling to conceive (NHS, 2020). Approximately 84% of couples across all ages and fertility statuses will conceive within a year of trying, increasing to 92% within 2 years and 93% within 3 years according to NICE, 2018.

The causes of infertility can be broadly categorised into three: female factor, where the underlying cause is attributed to the female partner; male factor, where the underlying failure to conceive is related to the male partner; and idiopathic, where there is no clearly diagnosed pathology, and both partners appear fertile on the basis of standard fertility testing (see Figure 1.4). However, these broad classifications are overly simplistic, since approximately 40% of couples present with a combination of male and female factor (NICE, 2017).

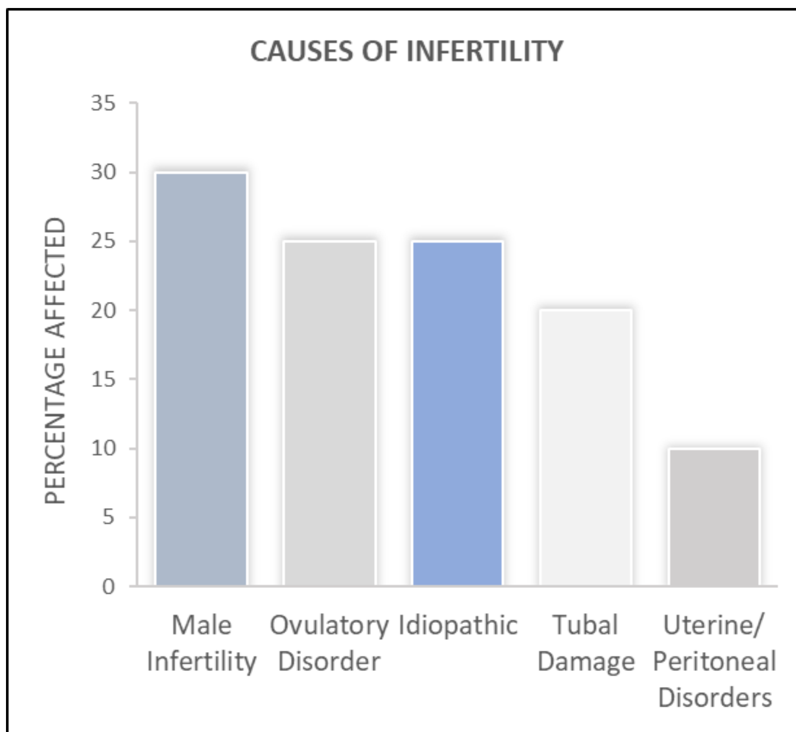

Figure 1.4 The percentage of couples experiencing different causes of infertility, adapted from NICE (2018). 30% of couples experience male infertility, although some estimates place this to be nearer 50% (Brugh and Lipshultz, 2004), 25% have ovulatory disorders, 20% have tubal damage, 10% have uterine or peritoneal disorders, and 25% have no identifiable cause of their infertility.

Although there is roughly an even split in causes of primary infertility across the sexes, significant efforts are made to classify causes ascribed to the woman, probably because of the expanded role of the female in maintaining pregnancy. Importantly, every woman will at some stage in their lifetime experience infertility since the number of gametes within the ovary is finite. When the gamete pool has been depleted, ovarian exhaustion occurs, which manifests as “the menopause”.

1.2.1 Female Factor Infertility

Female factor infertility is subclassified due to the clinical investigations that are carried out when a woman reports subfertility. Accurate diagnoses can explain why ovulation, fertilisation, or implantation have so far been unsuccessful.

Disorders of the major internal reproductive organs can lead to reduced fertility. For example, scarring or fibroids may be present within the fallopian tubes or within the uterine cavity, surgical procedures can be performed to remove fibroid tissue (Zepiridis, Grimbizis and Tarlatzis, 2016). In the case of a fallopian tube with an occlusion, blockages can be removed in procedures such as salpingostomy or laparoscopic

adhesiolysis (Khalaf, 2003; Ng and Cheong, 2019), thus permitting the passage of sperm into the fallopian tube to fertilise the ovulated oocyte.

In addition, uterine fibroids may be removed by laparoscopic myomectomy, leading to a more suitable environment for an embryo to implant (Grube *et al.*, 2019). In some cases, more profound surgery may be required. Salpingectomy describes the complete removal of one or both fallopian tubes, and can be necessary in certain cases of ectopic pregnancy where salpingostomy (the surgical opening of the fallopian tube to remove the conceptus without removing the whole tube) is not possible (Farquhar, 2005). In such cases, the chance of natural fertilisation can be reduced (de Bennetot *et al.*, 2012) or eliminated completely if bilateral salpingectomy was performed. However, assuming no other infertility diagnoses, *in vitro fertilisation* (IVF) can be a possible solution to the infertility (Bredkjaer *et al.*, 1999).

Similarly, endometriosis causes endometrial tissue to grow on and around the ovaries and fallopian tubes, leading to endocrine disturbance, thereby disrupting ovulation (Macer and Taylor, 2012; Vercellini *et al.*, 2014). This again may require surgical intervention to remove endometrial tissue from the ovaries and repair tubal damage, however evidence of long-term success is limited, as is hormonal treatment of endometriosis (NHS, 2019). In women with less severe endometriosis and no other diagnoses, IVF can show similar success rates as in women with other causes of infertility (Senapati *et al.*, 2016; González-Comadran *et al.*, 2017).

Ovulatory disorders arising from diminished levels of gonadotropins being secreted can result in infertility. Without adequate levels of FSH and LH, stimulation and control of the cyclic changes of the ovary can be dysregulated. For women who are not ovulating regularly, or at all, medication can be administered to induce ovulation; a commonly administered drug is clomiphene citrate (Clomid) (Perales-Puchalt and Legro, 2013). Clomiphene citrate works as an antioestrogen, blocking oestrogen receptors on the anterior pituitary gland and preventing oestrogen, produced by the ovary, from binding. This inhibition causes a negative feedback response by the pituitary, resulting in increased FSH and LH production, which stimulates follicular development and ovulation (Brown and Farquhar, 2016). In some cases, ovulation-induction can be

enough assistance required to lead to successful pregnancy (Sovino, Sir-Petermann and Devoto, 2002), but others may need IVF.

As previously discussed, female infertility can also result from ovarian insufficiency or failure (due to normal perimenopause and menopause, premature menopause, or as a result of medical treatments like chemotherapy) which cannot currently be treated to restore fertility. Once the supply of ovarian follicles has been exhausted, IVF using donor eggs would be recommended. However, there is hope of treating sub-fertile women experiencing perimenopause who may still have a reserve of follicles, albeit limited, in the ovaries.

1.3 Ovarian Rejuvenation

1.3.1 Overview

New theories and therapies are emerging in attempt to rejuvenate the ovary to ovulate remaining oocytes, to give patients an alternative to donor eggs. In cases of ovarian exhaustion, ‘ovarian rejuvenation’ refers to the induction of follicular growth to stimulate ovulation. There are currently two theories as to how this can be achieved.

One hypothesis proposed the presence of a latent stem cell population discovered in mature ovaries, (Tilly and Telfer, 2009), which experimentally have the capacity to differentiate into functional follicles and generate oocytes, however the clinical application of this remains unknown. Virant-Klun *et al.* (2008) analysed the ovarian surface epithelium (OSE) and isolated putative ovarian stem cells in female patients (20 postmenopausal and 5 with premature ovarian failure) with no naturally present follicles or oocytes. The cells identified through OSE-scraping expressed a range of early embryonic developmental markers, and proliferated *in vitro*, developing into oocyte-like cells (expressing transcription markers associated with early oocytes). This finding suggests that it could be possible to rejuvenate the ovary into developing new follicles from this oocyte source in the ovary, instead of relying on donor eggs or the very few remaining “established” follicles a patient may have.

The second theory is that existing ovarian follicles can be supported to grow by increasing the vascularisation of the ovary. The ovary and therefore the follicles receive greater blood supply and hence more growth factors and cytokines. This provides an

ideal environment within the ovary for follicular development, which ideally should allow perimenopausal women to produce dominant follicles and ovulate suitable remaining oocytes.

1.3.2 Platelet Rich Plasma Therapy

There is mounting interest in performing ovarian rejuvenation *in situ*, one way being the injection of platelet rich plasma (PRP) into the ovary. A handful of case reports have described the use of PRP injections into the ovary in humans (Sfakianoudis *et al.*, 2018; Farimani *et al.*, 2019; Pantos *et al.*, 2019; Sills and Wood, 2019; Hsu *et al.*, 2020), and there is now growing interest in the use of this approach to stimulate ovarian function.

1.4 Platelet Rich Plasma

1.4.1 Background and Usage

Platelet rich plasma (PRP) is a product of blood after removal of red blood cells and platelet poor plasma (PPP), plasma fluid with low platelet content. PRP contains a high concentration of platelets and an array of growth factors, cytokines, and small molecules. Platelets (seen in Figure 1.5) are a core contributor of haemostasis and are known to synthesise proteins in response to activation, typically injury. They also facilitate inflammation, angiogenesis and tissue repair through the release of growth factors and cytokines from intracellular granules (Sánchez-González, Méndez-Bolaina and Trejo-Bahena, 2012).

At the site of vascular injury, platelets adhere to collagen, which, along with thrombin, activate the platelets and stimulate the recruitment of more platelets. Platelets become activated when an agonist, like thrombin, binds to its corresponding receptor on the platelet surface, such as Protease-Activated Receptor 1 (PAR1) (Lisman, Weeterings and de Groot, 2005). This activation induces granule movement towards the platelet membrane, where the granule membrane and platelet membrane fuse – allowing the platelet contents to be released (Blair and Flaumenhaft, 2009). In the laboratory, alternatives can also be used to stimulate platelets, such as Thrombin Receptor Activating Peptide-6 (TRAP-6), which is a commonly used PAR1 agonist (Dziedzic *et al.*, 2020).

Platelet activation leads to morphological changes to the cytoskeleton, from their usual disc shape when resting, to the development of pseudopodia (see Figure 1.5 images), allowing them to adhere to each other via fibrinogen strands and form a mesh-like structure over a site of vascular injury.

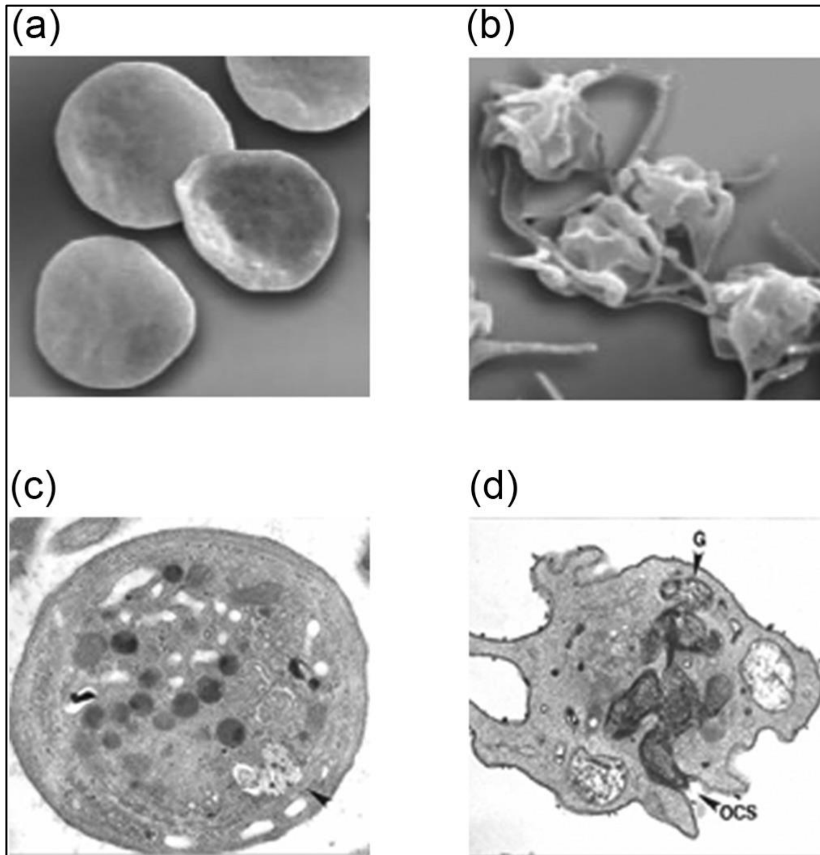

Figure 1.5. Scanning electron micrograph images, (a) and (b), and transmission electron micrograph images, (c) and (d), of resting and activated platelets respectively (Kannan, Ahmad and Saxena, 2019).

The clinical utility of PRP has been investigated in a range of specialities for regenerative purposes, such as to support muscle and ligament repair (Hurley *et al.*, 2019) and aid wound healing (Suthar *et al.*, 2017). Osteoarthritis in the knee was found to be potentially effectively treated using injections of autologous PRP (derived from the patient's own blood) (Han *et al.*, 2019), and osteoarthritic pain was said to be improved after PRP treatment of hands and feet (Evans *et al.*, 2020). Further studies have investigated PRP's efficacy in treating conditions within dermatology (Zhang *et al.*, 2018), such as scarring, hair, and vitiligo. PRP has been found to induce differentiation in a range of cell types, supposedly due to the activity of cytokines and growth factors secreted by the platelets upon activation and platelet degranulation (Etulain, 2018).

Platelet activation (before injection or within the injection site) facilitates platelet α granules to fuse to the platelet outer membrane and release their protein contents – such as cytokines and both anti and pro inflammatory factors (Golebiewska and Poole, 2015). Meanwhile, dense granules provide small molecules like ADP, ATP and calcium ions, which aid in platelet recruitment (Rendu and Brohard-Bohn, 2001; Chen, Yuan and Li, 2018). Lysosomes provide hydrolases which can degrade the circulating platelet aggregate (Rendu and Brohard-Bohn, 2001) and assist with remodelling the thrombus via proteolytic activity (Meng *et al.*, 2015). In human atherosclerotic lesions subjected to activation by thrombin, platelets were found to release over 300 proteins, some being monocyte chemoattractants and smooth muscle cell growth factors, potential contributors to the pathology by promoting the development of vessel adhesions (Coppinger *et al.*, 2004), demonstrating the necessity for platelet activity to be tightly controlled. It is notable that, as per the literature, different variations of proteins are released by platelets depending on the stimulant (type of agonist) used for activation (Zhang *et al.*, 2019).

PRP aids in healing/repair as the factors secreted from platelet granules have pro-inflammatory (stimulating the immune system), proangiogenic (stimulating the recruitment of blood vessels) and proliferative (stimulating cell replication) properties, which contribute to the recruitment of further cytokines, growth factors and immune cells (Sánchez-González, Méndez-Bolaina and Trejo-Bahena, 2012). There is overlap between factors secreted by platelets, and factors secreted by follicular cells which have shown to have a beneficial effect on folliculogenesis and oogenesis.

A type of factor which could affect female fertility is Bone Morphogenic Proteins (BMPs). BMP6 naturally circulates in the plasma and is produced by bone morphogenic mesenchymal stem cells (Vukicevic and Grgurevic, 2009). It has also been found to have a protective effect on cumulus cells – preventing staurosporine-induced apoptosis (Hussein *et al.*, 2005). BMP2 is another factor found in circulating blood plasma (Zhang *et al.*, 2015), but it has also been discovered that cumulus cells with a higher expression of BMP2 were associated with good quality oocytes and subsequent embryos, compared with cumulus cells expressing a lower level of BMP2 (Demiray *et al.*, 2017).

Interleukin 8 (IL-8) is produced by platelets and a variety of tissues, and work to control inflammation by regulating leukocyte migration and targeting immune cells such as neutrophils (Shahzad *et al.*, 2010; Chen *et al.*, 2020). It is also present in follicular fluid, with its concentration being highest in larger follicles, although its concentration in large follicles of young women was higher than that of equal sized follicles of older women (Malizia *et al.*, 2010). In human preimplantation embryos, a higher rate of IL-8 in embryo culture media was associated with higher implantation rates, higher pregnancy rates, and higher live birth rates (Huang *et al.*, 2017).

Endothelial growth factor (EGF) is a prominent protein secreted from platelets upon stimulation (Chen *et al.*, 2018), but it has also been found to be required for luteinising hormone-mediated cumulus expansion (Tekepetey, Daniel and Yuzpe, 1995), a crucial component of the ovulation process. In rats, EGF caused oocytes enclosed within both large and small antral follicles to mature, the researchers suggested that EGF may be able to prevent follicular inhibitors being transferred to the oocyte (Dekel and Sherizly, 1985).

Vascular Endothelial Growth Factor (VEGF) is an angiogenic factor transported and released by platelets (Verheul *et al.*, 1997). Stromal Cell-Derived Factor-1 (SDF-1) is also expressed by platelets, and is involved in promoting cell differentiation and tissue regeneration (Stellos *et al.*, 2008). Both VEGF and SDF-1 are also secreted by granulosa cells, and are linked with ovarian angiogenic development and follicular growth, with low levels of these factors in follicular fluid being associated with a low rate of oocyte recovery (Nishigaki *et al.*, 2019). This study also noted a positive association between concentration of SDF-1 and day 5 blastocyst rate.

The studies mentioned provide some foundational knowledge which could explain why the factors present in PRP, or secreted by the platelets upon activation, have had the beneficial effects noted in human and animal studies of intraovarian PRP injection.

1.5 Current State of Knowledge

Thus far, there have been a number of publications claiming to explore the effect of PRP on ovarian function. While delivering important new insight, many of these studies have been limited and thus consensus of whether PRP is effective at treating ovarian exhaustion is still lacking. Sfakianoudis *et al.*, 2020 described how when PRP is injected into the ovary directly, the resumption of the menstrual cycle is stimulated in climacteric women, they reported data from 4 pilot studies (menopause, perimenopause, POI, poor ovarian responders (POR)), noting improvements in markers of ovarian function in women referred to as POR. Autologous PRP injection into the ovary of women with low ovarian reserve was found to improve several reproductive markers – follicle stimulating hormone (FSH), anti-Mullerian hormone (AMH), and antral follicle count (AFC) (Melo *et al.*, 2020). Aside from the purported benefits this therapy has for patients, it is also worth noting the clinical benefits – autologous PRP is readily available from the patient’s own blood, the preparation of PRP is quick and inexpensive, and most clinics will already have the centrifuges required for preparation.

A small number of studies have been conducted using animal models, including rat. One study by Bakacak *et al.* (2016) found that PRP, when administered to the peritoneal cavity, had a protective effect on the ovary and prevented ischemia and reperfusion damage in rats inflicted with ovarian torsion. The study also noted VEGF concentration in the peritoneal cavity was significantly higher in PRP-treated rats than those without PRP treatment, suggesting PRP administration is sufficient in raising the local concentration of VEGF. Although, one limitation of the study was the absence of a sham treatment group as a control, to compare between PRP administration and saline administration.

Another rodent study, by Ozcan *et al.* (2020), found that PRP had a protective effect on ovaries exposed to cyclophosphamide (an immunosuppressive chemotherapy (Ogino and Tadi, 2022)). After cyclophosphamide exposure, ovaries treated with PRP had a significantly higher serum AMH level than those treated with sodium chloride. The number of primordial, primary, and antral follicles were significantly higher in the PRP treated group compared to the sodium chloride group. Meanwhile, the number of atretic follicles was significantly lower in the PRP group compared to sodium chloride,

suggesting that without PRP treatment the follicles could have been destined for atresia as a result of the cyclophosphamide exposure. A similar number of follicles entering atresia was noted in the cyclophosphamide + PRP group as well as 2 groups which were not exposed to cyclophosphamide – instead just injected with sodium chloride or PRP every 7 days. This indicates that PRP treatment after cyclophosphamide exposure can reduce the extent of follicular atresia down to a level expected of non-cyclophosphamide exposed ovaries.

A unique advantage of this study compared to others, is its use of a sham treatment group. Intraperitoneal injection with sodium chloride was used as a control against PRP injection, indicating that the difference in results between the groups was due to PRP treatment, not an unintentional consequence of the needlestick injury. It is also relevant to mention that the PRP used was prepared using blood from male rats (of the same breed), so the possibility that female derived PRP may invoke different results cannot be excluded.

In a bovine study, Cremonesi *et al.* (2020) found that intraovarian PRP injection resulted in a statistically significant increase in follicle number, and number of high grade blastocysts, compared with cows not injected. The right ovaries of the cows were treated as the control – they were not injected, whereas the left ovaries were injected with PRP. This is advantageous as it eliminates the possibility of inter-animal differences. Additionally, the PRP used was autologous – derived from the individual cow for which it will be administered back into.

Another bovine study by Ramos-Deus *et al.* (2020) also reported beneficial effects of PRP on *in vitro* embryo production. The group found that PRP supplemented maturation media did not increase cumulus expansion, with both the 5% and 10% PRP groups having lower cumulus expansion (the 10% PRP group having the lowest rate of expansion) than the Foetal Bovine Serum (FBS) control group. However, although the group supplemented with 5% PRP did not have a significantly higher cleavage rate than the FBS control, it was significantly higher than the 10% PRP group. Also, the blastocyst rate of the 5% PRP group was significantly higher than that of the 10% PRP group and the FBS control. These results suggest that there may be an optimum level of

supplementation, and that a higher percentage of PRP supplementation may be less effective than a milder supplementation.

A study where PRP was supplemented into media containing primordial follicles, from the healthy ovaries of 3 deceased human donors, showed an increase in follicle size and 10th day viability compared to using FBS, FBS + PRP, or human serum albumin as controls (Hosseini *et al.*, 2017). This shows that PRP can successfully support *in vitro* development of human ovarian follicles. However, it does not address the effect PRP has, if any, on the oocytes or how PRP supplementation may impact subsequent embryos.

Whilst the data gained so far is promising regarding ovarian rejuvenation, the safety of PRP injections into the ovary and the knowledge on mechanism remains limited, and to date, the question of whether PRP supplementation alters oocyte and subsequent embryo physiology remains unknown.

A common shortfall in preclinical studies has been inappropriate controls. For example, Melo *et al.* (2020) did not include a sham injection for the control group. The absence of this means that it is impossible to exclude the conclusion that local injury in response to the needlestick may have contributed to the effects described in the PRP-treated group of women (e.g. higher antral follicle counts). It is conceivable that a time-limited function of the ovary could be redeemed by the ovarian inflammatory response induced by the mild injury caused by the injection alone – potentially due to the recruitment of platelets and their cytokine activity to the region. This may increase the vascularisation of the ovary, albeit temporarily, which could contribute to the facilitation of spontaneous ovulation and increased oocyte yield observed in some studies (Sfakianoudis *et al.*, 2018; Cakiroglu *et al.*, 2020; Hsu *et al.*, 2020; Melo *et al.*, 2020). This should be investigated to determine if this is the case using a sham saline injection, and then compare it to results obtained from PRP-treated women.

It is also of note that the Melo *et al.* (2020) study was non-randomised, introducing a potential socioeconomic selection bias whereby only patients who could afford the PRP treatment received it. This factor suggests that these patients were also in a financial situation that afforded them access to other health benefits, such as time away from work, private and accessible healthcare, gym/exercise sessions, and higher quality food

– which could have positive effects on fertility (Sharma *et al.*, 2013; Mumford *et al.*, 2020).

Injection with platelet poor plasma (PPP) could also be used as a control in clinical trials and basic research, to compare against PRP to identify if the effects seen in studies are due to the platelet concentration of the injection material, or if there are potentially contents of the plasma that could be causing the clinical results observed.

There is poor standardisation across PRP studies regarding the preparation of PRP, notably the type of anticoagulant used, and the concentration of the anticoagulant. It is poorly understood what effect citrate-based anticoagulants have on the ovary, and whether different concentrations determine if an effect is detected. Wellen *et al.* (2009) reported that alterations in gene expression due to an increase in histone acetylation could be observed when high concentrations of citrate are used, as it has been thought to increase the synthesis of acetyl-CoA via ATP citrate lyase. A change in gene expression of oocytes could lead to detrimental implications for the subsequent embryos.

Additionally, the activation status of the platelets in PRP, and the method by which the platelets are activated (calcium, thrombin, PAR1 agonist etc) is poorly standardised across studies, whether this can influence the results is unclear. As previously mentioned, the agonist used to activate the platelets can result in different concentrations of cytokines and growth factors being secreted from the platelets.

Despite these limitations, the PRP-treated patients who opted for IVF had higher quality-scoring embryos than patients pursuing IVF without being treated with PRP, which suggests that something beneficial could be happening at the cellular level within the oocyte or follicle as a result of PRP ovarian injection – which should be explored.

1.6 This Study

1.6.1 Hypothesis

There will be a measurable effect on bovine oocyte and embryo development under human platelet releasate exposure, during *in vitro* maturation, compared with the foetal calf serum (FCS) control.

1.6.2 Aims

To address this hypothesis, the study has 3 specific aims:

1. To test whether incubation with PRP alters the maturation of mammalian oocytes
2. To test whether mammalian oocytes interact with platelets that might lead to their aberrant activation
3. To discover whether embryos generated from oocytes incubated with PRP have any measurable phenotypic alterations

1.6.3 Study Impact

This study will address the current unknown physiological impact growth factors and cytokines, released by platelets in PRP, have on oocytes and embryos, potentially giving a more well-informed view for clinicians in an ART setting.

Chapter 2 Methodology

2.1 Supplier List

2.1.1 Acros Organics

D-ABA Acros Organics 142140050

Isoleucine Acros Organics 166170250

Leucine Acros Organics 125121000

Phenylalanine Acros Organics 130310250

2.1.2 Agar Scientific

Paraformaldehyde 16% solution Agar Scientific AGR1026

2.1.3 Analox

Glucose standard 5mM/L Analox GMRD-010

Lactate standard 5mM/L Analox GMRD-079

Pyruvate standard 0.45mM/L Analox GMRD-140-E

2.1.4 Cell Signaling

Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb Cell Signaling #9733

2.1.5 Ferring

Menopur (75IU FSH, 75IU LH) Ferring G03GA02

2.1.6 Fisher

Antibiotic antimycotic (Ab-Am) Fisher 11580486

Magnesium sulphate Fisher M/1050

Methanol Fisher M/4056/17

Sodium hydroxide Fisher S4920/60

2.1.7 Fresenius

Embryo-tested water Fresenius Kabi

2.1.8 Gibco

Antibiotic-Antimycotic 100X Gibco 15240096

GlutaMAX II 100X Gibco 35050061

2.1.9 Invitrogen

Alexa Fluor 594 Goat Anti-Rabbit Invitrogen A11012

2.1.10 IVF Bioscience

BO-SemenPrep™

2.1.11 Millipore

B-Mercaptoethanol Millipore 444203

2.1.12 Roche

Hexokinase/G-6-P Roche 127-825

Lactate dehydrogenase (LDH) Roche 107 042

NADH Di sodium salt Roche 10 128 023 001

NAD Roche 127 981

NADP Roche 128 040

2.1.13 Sigma-Aldrich

Adenosine triphosphate (ATP) Sigma A6419

Alanine Sigma A-7627

Amino acid (AA) standards Sigma Aas18

Antimycin A Sigma A8674

Apo-Transferrin bovine Sigma T1428

Arginine Sigma A-5131

Asparagine Sigma A-4284

Aspartate Sigma A-4534

Benzyl alcohol Sigma 39971

Benzyl benzoate Sigma 68183

Bovine serum albumin fatty acid free Sigma A3803

$CaCl_2 \cdot 2H_2O$ Sigma C7902

Carbonyl cyanide 4- (trifluoromethoxy)phenylhydrazone (FCCP) Sigma C2920

Coenzyme Q10 (Co-Q10) Sigma 07386

Cycloheximide Sigma C7698

Cysteine Sigma C-1276

D-Glucose Sigma G6152

D-Glucose anhydrous Sigma RDD016

(N,N-)Dimethylformamide (DMF) Sigma 227056

Dithiothreitol (DTT) Sigma D0632

EDTA Sigma ED4SS

Epidermal Growth Factor from murine submaxillary gland Sigma E4127

EPPS Sigma E9502

Essential amino acids Sigma M7145

Fibroblast Growth Factor from bovine pituitary Sigma F3133

Foetal calf serum Sigma F9665

Glacial Acetic Acid Sigma CHE1018

(L-)Glutamate Sigma G-1251

(L-)Glutamine Sigma G8540

Glycine Sigma G6388

Heparin sodium salt Sigma H3393

HEPES sodium salt Sigma H3784

HEPES Sigma H3375

Histidine Sigma H-8125

Hydrazine sulphate Sigma H3376

Kanamycin sulfate Sigma K4000

Lysine Sigma L-5626

Magnesium chloride hexahydrate Sigma 246964

Magnesium sulfate Sigma M7506

M199 Sigma M0650

Methionine Sigma M-9625

Mineral oil Sigma M8410

Phtaldialdehyde Reagant (OPA) Sigma P0532

Penicillin G Sigma P4697

Penicillin/Streptamycin Sigma P0781

Polyvinyl alcohol Sigma 341584

Potassium chloride (KCl) Sigma P5405

Potassium dihydrate phosphate (KH2PO4) Sigma P5655

Proline Sigma P-0380

Serine Sigma S-4500

Sodium bicarbonate ($NaHCO_3$) Sigma S6014

Sodium chloride (NaCl) Sigma S5886

Sodium DL-lactate solution Sigma L1375

Sodium pyruvate Sigma P2256

Streptomycin Sigma S1277

Tetrahydrofuran (THF) Sigma 34865

Threonine Sigma T-8625

Tryptophan Sigma T-0254

Tyrosine Sigma T-1020

Valine Sigma V-0500

2.2 Platelet Releasate Preparation

All blood samples were collected and processed in accordance with NHS REC Ref 21/SC/0215. All bovine embryos were created and used in accordance with Hull York Medical School Ethical Review Board (18 42).

Whole human blood from healthy volunteers was taken into a 10ml syringe containing 2ml ACD (acid citrate dextrose) (see Table 2.1) to prevent coagulation of the blood. The blood was centrifuged at 90 x G for 10 minutes, to separate the blood components (as illustrated in Figure 2.1).

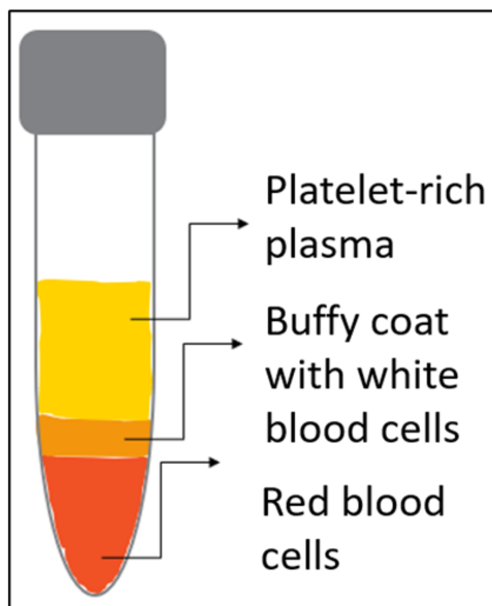

Figure 2.1. Illustration of the separation of blood components after centrifugation.

The platelet rich plasma was aspirated into a clean tube. The remaining blood was centrifuged again at 120 x G for 10 minutes, and plasma removed and added to the previous plasma. Citric acid (0.3M; 20µl per 1ml plasma) was added to the plasma and

the tube inverted, to reduce the plasma pH and maintain the platelets in a quiescent state.

The PRP was then centrifuged at 800 x G for 12 minutes, to sediment the platelets. The supernatant, which was classified as platelet-poor plasma, was aspirated and discarded. The platelet pellet was resuspended in 2ml platelet wash buffer and centrifuged at 800 x G for 10 minutes (see Table 2.2). The platelets were then resuspended in Tyrode's buffer (Table 2.3), counted with a Coulter counter, and adjusted to a final count of $1x10^9$ platelets/ml. They were then allowed to rest for 40 minutes before further use.

For collecting releasate in from activated platelets, 5µl of the platelet agonist TRAP-6 (3mM) was added to 500µl of PRP, and the platelets allowed to aggregate to 70%. Aggregation was measured using a Chronolog Model 490 4+4 aggregometer, and the aggregation software AGGRO/LINK®8. Non-aggregated liquid was aspirated, placed into Eppendorfs and put on ice before being centrifuged at 9500 x G for 10 minutes. The supernatant was removed (platelet releasate) and stored at -80˚C in 45-50µl aliquots until use.

Table 2.1. Preparation of ACD. pH 6.4 (solution filter-sterilised through 0.22µm membrane and stored at 4°C).

Chemical	***Molarity***	***Concentration/L***
D-Glucose (anhydrous)	113.8 mM	20.5 g/L
Tri-Na acetate	29.9 mM	8.79 g/L
NaCl	72.6 mM	4.24 g/L
Citric acid	2.9 mM	0.59 g/L

Table 2.2. Preparation of wash buffer. pH 6.5 (solution filter-sterilised through 0.22µm membrane and stored at 4°C).

Chemical	***Molarity***	***Concentration/ 100 mL***
Citric acid	0.036 mM	0.757 g/100ml
EDTA	0.01 mM	0.380 g/100ml
D-Glucose	0.005 mM	0.090 g/100ml
KCl	0.005 mM	0.037 g/100ml
NaCl	0.09 mM	0.526 g/100ml

Table 2.3. Preparation of Tyrode's buffer. pH 7.4 (solution filter-sterilised through 0.22µm membrane and stored at 4°C).

Chemical	***Molarity***	***Concentration/L***
NaCl	150 mM	8.77 g/L
HEPES (Na^+ Salt)	5 mM	1.30 g/L
NaH_2PO_4 (anh)	0.55 mM	0.086 g/L

$NaHCO_3$ (anh)	7 mM	0.59 g/L
KCl	2.7 mM	0.20 g/L
$MgCl_2$ (Hexa (6) hydrated)	0.5 mM	0.10 g/L
D-Glucose (anhydrous)	5.6 mM	1.01 g/L

2.3 *In vitro* Embryo Production

Isolated bovine reproductive tracts were collected from a local abattoir and delivered to the laboratory for processing within 90 minutes of slaughter. Tissue came from healthy animals of unknown provenance, slaughtered for purposes unrelated to the study. The ovaries were excised and washed twice in 0.2% Antibiotic-Antimycotic solution (800µl in 400ml PBS) warmed to 39°C. Ovarian follicles were aspirated into warmed 39°C holding media (see Table 2.4) supplemented with 0.2ml heparin per 50ml media.

2.3.1 *In vitro* Maturation

Under a stereomicroscope, oocytes with several layers of complete, compact cumulus were selected and washed through warmed 39°C holding media without heparin, and then through wells containing bovine maturation media (see Table 2.5), which had been previously equilibrated for at least 2 hours at 39°C in 5% CO_2 in air. Oocytes were allocated randomly into two groups, each containing 25-50, and put into an Eppendorf tube containing 360µl 39°C bovine maturation media, again equilibrated for at least 2 hours at 39°C in 5% CO_2 in air.

Group 1 oocytes were cultured in medium containing 10% FCS (40µl in 360µl medium); this group served as the control. Group 2 oocytes were cultured in medium containing 10% platelet releasate (40µl in 360µl medium). Oocytes were incubated for 22 hours at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity.

Table 2.4. Preparation of holding media.

Sterile water	80ml	81%
199 medium 10x	10ml	10%
Stock B	2ml	2.0%
Stock H	6ml	6.1%
Stock K	0.1ml	0.01%
BSA Fr V	0.2ml	0.02%

Table 2.5. Preparation of bovine maturation media.

Sterile water	8ml	70%
199 medium 10x	1ml	8.7%
Stock B	1ml	8.7%
EGF:FGF	0.1ml	0.87%
FSH:LH	0.05ml	0.44%
Glutamax	0.1ml	0.87%
Maturation additives	0.222ml	1.9%
FCS (optional)	1ml	8.7%

2.3.2 *In vitro* Fertilisation

After maturation, oocytes were washed through FERT TALP medium (see Table 2.6) and put into wells with 350µl FERT TALP (25-50 per group).

One straw of bull semen, from a bull of proven fertility, was thawed and put into a tube with 2ml of IVF BioScience BO-SemenPrep preparation media. The semen was centrifuged for 5 minutes at 328 x G, the supernatant was aspirated and discarded. The sperm pellet was resuspended in 2ml more IVF BioScience BO-SemenPrep media, and centrifuged again for 5 minutes at 328 x G. The supernatant was aspirated and discarded, and the pellet resuspended in 200µl IVF BioScience BO-SemenPrep media. The final suspension was warmed to 39˚C ahead of insemination.

In an Eppendorf, 10µl of sperm was diluted with 190µl of sterile water. Sperm was counted using a haemocytometer, and concentration calculated. Oocytes were co-incubated with the appropriate volume of sperm to give 2 million sperm/ml, the well volume was made up to 500µl with FERT TALP.

2.3.3 Embryo Culture

Gamete co-incubation occurred for 22 hours, after which, presumptive zygotes were transferred to a 10ml falcon tube containing 2ml HEPES SOF (see Table 2.7) and vortexed vigorously to denude them of cumulus cells. Denuded presumptive zygotes were washed in SOFaaBSA (see

Table 2.8), before being cultured in 20µl SOFaaBSA droplets in groups of ≤ 20 (droplet dish equilibrated for at least 2 hours in hypoxic gassed incubator, 5% CO_2, 5% O_2, bal N_2). Cleavage rate, defined as the number of embryos to have completed as least one cell division, was recorded on day 2. Blastocyst rate, defined as number of embryos to have initiated cavitation, was recorded on day 7-9. Data were expressed as percentages.

Where appropriate, day 7 and day 8 blastocysts were washed through modified HEPES-SOF wash drops, then cultured individually in 5µl modified HEPES-SOF media (see Table 2.9) for ~24 hours (droplet dish equilibrated for at least 2 hours in hypoxic gassed incubator, 5% CO_2, 5% O_2, bal N_2). 3 droplets of this media were kept blank (no blastocysts cultured within them), to be used for later analysis. Blastocysts were removed after 24 hours and humanely discarded, the droplet dish was sealed with parafilm and stored at -80˚C until metabolic analysis.

Table 2.6. Preparation of FERT TALP medium.

Sterile water	14.4ml	71%
10xTL	2.0ml	10%
Stock B	2.0ml	10%
Stock C	0.16ml	0.8%
Stock D	0.24ml	1.2%
Stock L	0.6ml	3%
Stock M	0.2ml	1%
Stock BSA FAF	0.4ml	2%
Penicillamine/Hypotaurine	0.2ml	1%
Heparin	20µl	0.1%

Table 2.7. Preparation of HEPES SOF medium.

Sterile water	7.1ml	71%
Stock S2	1.0ml	10%
Stock B	0.2ml	2%
Stock H	0.8ml	8%
Stock C	0.1ml	1%
Stock D	0.1ml	1%
Stock L	0.1ml	1%
Stock M	0.1ml	1%
Stock G	0.25ml	2.5%
Pen/Strep	60µl	0.6%
Stock BSA Fr V	0.2ml	2%

Table 2.8. Preparation of SOFaaBSA medium.

Sterile water	5.59ml	55.9%
Stock S2	1.0ml	10%
Stock B	1.0ml	10%
Stock C	0.1ml	1%
Stock D	0.1ml	1%
Stock L	0.1ml	1%
Stock M	0.1ml	1%
Stock G	0.25ml	2.5%
Stock GLN	1.0ml	10%
Non-Essential AA	0.1ml	1%
Essential AA	0.2ml	2%
Pen/Strep	60µl	0.6%
Stock BSA FAF	0.4ml	4%

Table 2.9. Preparation of modified HEPES SOF medium for analysis.

ET Water	To volume (100ml)
NaCl	0.629g/100ml
KCl	0.053g/100ml
KH_2PO_4	0.016g/100ml
$NaHCO_3$	0.21g/100ml
Glucose	0.009g/100ml
Glutamine	0.0029/100ml
Pyruvate	0.0035g/100ml
$CaCl_2.2H_2O$	0.025g/100ml
$MgCl_{12}.6H_2O$	0.010g/100ml
Pen/Strep	0.5995ml (0.6%)
Amino acids 50x	2ml (2%)
BSA	0.799g/100ml

2.4 Metabolic Analysis

The individual droplets of blastocyst spent media were used to measure the concentration of glucose, lactate and pyruvate, using enzyme coupled fluorescent assays.

In the "glucose assay", glucose within the spent medium is converted to glucose 6-phosphate and further metabolised to 6-phosphogluconate, within this process NAD undergoes reduction to form NADH. NADH is a fluorophore which emits a fluorescent signal that is detected by a plate reader. The more glucose present in a sample, the more NADH produced and therefore the stronger the signal.

To determine lactate concentrations, the lactate is metabolised to pyruvate, again with NADH being a by-product of the conversion. The higher the level of lactate in the sample, the stronger the fluorescent signal from NADH. The measurement of pyruvate exploits the same reaction but in reverse, pyruvate is converted back to lactate in an oxidation reaction that sees NADH converted to NAD. The higher the level of pyruvate in the sample, the lower the level of NADH and therefore the weaker the fluorescence.

A 96-well plate was used for each metabolic assay. 9µl of glucose, lactate or pyruvate cocktail was added to each well of the plate, and placed into a BMG FluroStar plate reader to measure the fluorescence before the standard concentrations were added. Then standard glucose, lactate and pyruvate samples of known concentrations were prepared and put into a 96-well plate using 3 wells per concentration (1µl). The plate was covered in parafilm to prevent evaporation and incubated (10 minutes for glucose, 30 minutes for lactate, and 3 minutes for pyruvate). The plate was put in the plate reader and the fluorescence measured. By subtracting the "pre" measurement from the "post" measurement, a standard curve representing the fluorescence at various known concentrations was generated. Only where a correlation co-efficient of >0.99 was obtained were standard curves used for quantification.

Using a new 96-well plate, a similar process was followed, but using individual blastocyst media instead of standard samples. Blank droplets (droplets of media that have never contained blastocysts) were also used to later account for the concentration of metabolites already present in the media. Again, the "pre" measurement was

subtracted from the “post” measurement, and each sample’s fluorescence intensity was converted to concentration (mM) using the standard curve’s equation of the line.

2.5 Oxygen Consumption Rate

Oxygen consumption rate (OCR) offers an insight into how mitochondria (the main supplier of an embryo's ATP) are functioning within the developing early embryo. To measure the different components of oxygen consumption, several mitochondrial inhibitors were used: oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and antimycin A and rotenone (A/R).

Oligomycin inhibits ATP-synthase by blocking its proton channel, which is needed for ADP conversion to ATP via oxidative phosphorylation. The OCR during this period of ATP-synthase inhibition, demonstrates the extent to which oxygen consumption is attributed to ATP production (ATP coupled respiration). FCCP enables the free flux of protons across the inner membrane of the mitochondria, thus altering the proton gradient and disrupting ATP synthesis which relies on the proton gradient. The OCR during exposure to this inhibitor is regarded as the maximal respiration. Antimycin A inhibits complex III, and rotenone inhibits complex I within the mitochondria, disrupting mitochondrial respiration. The OCR during A/R exposure represents the proportion of non-mitochondrial respiration. The difference between non-mitochondrial respiration and ATP coupled respiration is termed proton leak, describing the extent of oxygen consumption attributed to the proton gradient (Muller *et al.*, 2019).

The day before the assay was performed, a XFp Sensor cartridge was hydrated with calibrant, 200µl per well and 400µl per moat, and placed in a non-CO_2 incubator overnight at 37˚C.

On the day of analysis the wells were filled with 180µl HEPES SOF and incubated at 37˚C in air. Inhibitors were prepared: oligomycin (10µM), FCCP (37.5µM), and A/R (25µM), each made up of 3µl stock in 297µl HEPES SOF, and stored on ice until use. The cartridge was set-up in the arrangement shown in Figure 2.2, with 20µl HEPES SOF, 22µl oligomycin, 24.5µl FCCP, and 27µl A/R added to the injection wells at 10% of the final well volume. The sensor cartridge was loaded onto the Seahorse XFp for calibration to take place.

An equal number of blastocysts from the FCS and releasate groups were then placed into their corresponding wells (B and C respectively, as in Figure 2.2). The plate was then loaded into the Seahorse XFp for assay.

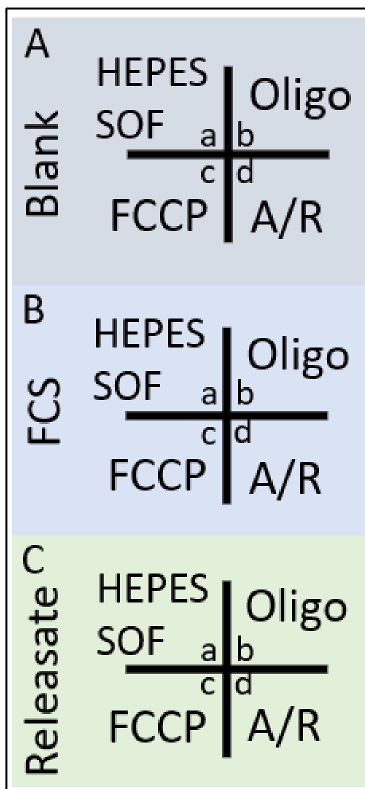

Figure 2.2. Cartridge arrangement, showing experiment groups and inhibitor wells.

2.6 Immunofluorescent Imaging of Blastocysts

To determine if there was a difference in epigenetic modifications exhibited by the blastocysts in each group, immunofluorescence was performed to assess the level of trimethylation on lysine 27 of histone 3 (H3K27me3). The experiment had 4 groups: FCS, FCS control (no primary antibody exposure), releasate, releasate control (no primary antibody exposure), to demonstrate that without primary antibody binding, fluorescence will not be observed.

Day 7-9 blastocysts were fixed in 4% PFA for 10 minutes at room temperature, after which, they were washed in PBS-Triton (0.15%) at room temperature over the course of 30 minutes, to dissolve the membrane – making it permeable to the antibody. The blastocysts were blocked in goat serum (3%) in PBS-Triton (0.15%), for 30 minutes at room temperature, to prevent non-specific binding. The blastocysts were added to primary antibody (C36B11 Rabbit mAb #9733) in blocking solution in a 1:1000 dilution (as per the product datasheet) and left overnight, rocking at 4˚C. Separate groups of blastocysts were incubated in the absence of primary antibody (negative controls).

The blastocysts were then washed in PBS-Triton for 4 hours at room temperature with fresh PBS-Triton every hour. Following this, blastocysts were put into secondary antibody (goat, anti-rabbit, Alexa Fluor 594, A11012) in blocking solution (1:1000 dilution) for 1 hour at room temperature. From this point the blastocysts were protected from light to prevent photobleaching of fluorophores. The samples were again washed in PBS-Triton for 1 hour at room temperature.

The samples were gradually dehydrated in successive concentrations of methanol in PBS-Triton, starting with 25% methanol for 10 minutes, 50% for 10 minutes, 75% for 10 minutes, and then 100% for 1 hour. BABB (Benzyl Alcohol:Benzyl Benzoate from Sigma Aldrich, in a 1:2 dilution) was mixed with methanol (50:50). The samples were incubated in the BABB:MeOH for 10-15 minutes at room temperature, before being moved into 100% BABB for 10-15 minutes. The blastocysts, within their groups, were put into imaging chamber wells with BABB, and imaged immediately using the Zeiss Axio Observer Z1 microscope.

2.7 Determination of Nuclear Maturation Status of Oocytes

The oocytes were supplemented with FCS or releasate (section 2.3.1) during 22-hour *in vitro* maturation, after which time, a representative sample of oocytes from each group were selected and denuded of cumulus cells via vortex alone or with the addition of hyaluronidase (80IU/ml).

The denuded oocytes were washed through 100µl droplets of 39°C PBS-PVP (0.1%), then fixed in 100µl droplets of 4% PFA (100µl 16% PFA in 300µl PBS) for 5-10 minutes at room temperature. They were then washed through 100µl droplets of PBS-PVP followed by PBS (both at room temperature), and stored in PBS at 4˚C.

The oocytes were stained with 50µg/ml Hoechst in PBS, at room temperature for 30 minutes. Following this, they were washed in PBS-Tween (0.1%) and then put in chamber wells containing 30µl PBS. The eggs were imaged using the Zeiss Axio Observer Z1 microscope. The number of eggs in metaphase I and metaphase II were determined based on the nuclear staining and the presence of an extruded polar body (indicating MII).

2.8 Platelet-Oocyte Aggregation

2.8.1 Platelet Preparation and Dose Response

A 10ml sample of whole human blood from a healthy volunteer was obtained on the day of the experiment. Blood was collected into tubes containing 124mM Sodium Citrate (1:10, anticoagulant:blood) using a standardised phlebotomy technique and obtained by a trained phlebotomist. Whole blood was transferred into two 15ml Falcon tubes, 5ml per tube, and centrifuged at 190 x G for 15 minutes at room temperature. The supernatant (PRP) was aspirated into a fresh Falcon tube. The remaining blood was centrifuged again at 190 x G for a further 15 minutes, the supernatant was added to the PRP tube. The clear layer remaining above the blood was pipetted off into Eppendorf tubes and centrifuged at 1700 x G for 5 minutes, the supernatant (platelet-poor plasma) was put in a fresh Eppendorf tube.

A dose response curve was produced by activating 300µl of PRP with 3 different concentrations of TRAP-6 amide (TRAP-6) (0.3, 1 and 3µM), prepared from a 4mM TRAP-6 stock and PBS, to establish which concentration was most appropriate.

2.8.2 Substrate Preparation

After 18 hours of *in vitro* maturation in media supplemented with FCS (control), 5 oocytes with expanded cumulus, and with 5 oocytes with no cumulus expansion (deemed dead oocytes) were selected. Both sets of oocytes were vortexed to remove cumulus cells, the oocytes then put into Eppendorf tubes with 30µl of spent maturation media (creating the Mature Eggs and Dead Eggs groups). 30µl of just spent media was collected (Media), and 30µl of cumulus cells within media was collected (Cumulus). All 4 groups were kept heated to 39°C in the 5% CO_2 incubator, until ready for aggregation (<1 hour).

2.8.3 Aggregation Experiment

Aggregation was determined by light transmission through the samples, demonstrating the opacity of the suspension, which correlates with the extent of platelet aggregation (Koltai *et al.*, 2017).

4 cuvettes were filled with 300µl of PRP. 30µl of each substrate was added to the bottom of the cuvette, to ensure substrate-PRP interaction. The aggregation was

measured for 20 minutes, then 3.3µl of 3µM TRAP-6 was added to each cuvette – acting as a positive control, to confirm that the platelets remained capable of aggregating when activated. The data was exported to Microsoft Excel for processing.

2.9 Statistical Analysis

2.9.1 *In vitro* Embryo Production

All analysis was performed in IBM SPSS. A Mann-Whitney U test was performed in IBM SPSS to determine statistically significant difference between the groups. This nonparametric test was chosen as the data was assumed to not be normally distributed, due to sample size and there being 2 independent groups. Details of n numbers and replicates are presented in figure legends, figures prepared in Microsoft Excel.

2.9.2 Metabolic Analysis

All analysis was performed in IBM SPSS. A Mann-Whitney U test was performed in IBM SPSS to determine statistically significant difference between the groups. This nonparametric test was chosen as the data was assumed to not be normally distributed, due to sample size and there being 2 independent groups. Details of n numbers and replicates are presented in figure legends, figures prepared in Microsoft Excel.

Additionally, glycolytic index was calculated from the glucose and lactate data for each embryo. The glycolytic index for both groups did not indicate normality when tested using Kolmogrov-Smirnov and Shapiro-Wilk. Therefore, a Mann-Whitney U test was performed to determine statistically significant difference between the groups.

2.9.3 Oxygen Consumption Rate

A Mann-Whitney U test was performed in IBM SPSS to determine statistically significant difference between the groups. This nonparametric test was chosen as the data was not assumed to be normally distributed due to the sample size (n=4), and there being 2 independent groups. Details of n numbers and replicates are presented in figure legends, figures prepared in Microsoft Excel.

2.9.4 Immunofluorescent Imaging of Blastocysts

A Mann-Whitney U test was performed in IBM SPSS to determine statistically significant difference between the groups, this nonparametric test was selected as normality testing was performed using Kolmogrov-Smirnov and Shapiro-Wilk, indicating data was

not normally distributed. Details of n numbers and replicates are presented in figure legends, figures prepared in Microsoft Excel.

2.9.5 Determination of Nuclear Maturation Status of Oocytes

A Mann-Whitney U test was performed in IBM SPSS to determine statistically significant difference between the groups. This nonparametric test was chosen as normality was not assumed due to the small sample size (n=3), and there being 2 independent groups. Details of n numbers and replicates are presented in figure legends, figures prepared in Microsoft Excel.

2.9.6 Statistical Analysis

All analysis was performed in IBM SPSS. The data was tested using Kolmogrov-Smirnov and Shapiro-Wilk, both indicating the data was not normally distributed. As the dose response experiment and the aggregation experiment both included more than 2 groups, the Kruskal Wallis One Way ANOVA test was selected to determine statistical significance. Details of n numbers and replicates are presented in figure legends, figures prepared in Microsoft Excel.

Chapter 3 Results

3.1 The Effect of Platelet Releasate on Oocyte Metaphase II Rate

In order to assess whether platelet releasate affected oocyte maturation *in vitro*, a representative sample of oocytes treated with 10% platelet releasate, and representative sample of oocytes treated with 10% FCS were stained to assess nuclear status. Metaphase II is end-phase of oocyte maturation, when the first polar body has been extruded and the chromosomes assemble on the spindle, at which point meiosis arrests. Meiosis only concludes if the oocyte is activated, typically during fertilisation.

The data indicated that rates of attainment of MII was not affected by the presence of platelet releasate (51.2%±23.2 control, compared with 43%±26.2 releasate). The data was obtained from 19 FCS and 29 releasate derived oocytes obtained from 3 oocyte collections. Although the releasate group appeared to have a lower metaphase II rate than the FCS control, this difference had no statistical significance (Figure 3.1). Nuclear maturation status was determined by the presence or absence of two fluorescent signals (one for nucleus and one for polar body), as shown in Figure 3.2 and Figure 3.3.

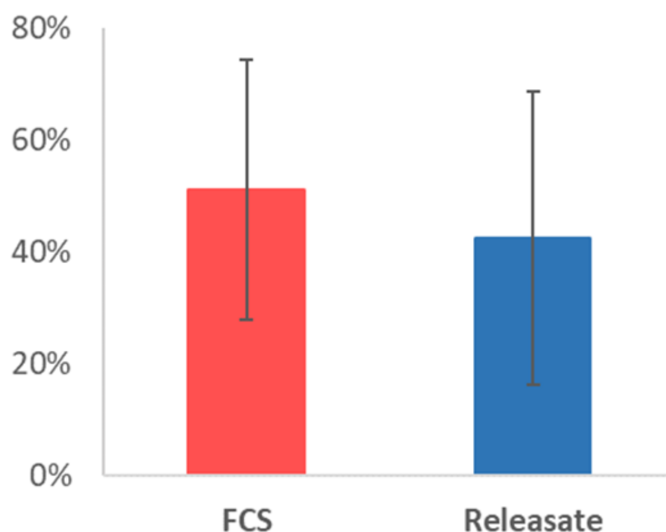

Figure 3.1. The mean metaphase II rate derived from eggs cultured with platelet releasate compared with the FCS control. Mean values were calculated from n=3 data, the error bars represent standard deviation. (**P=0.700**).

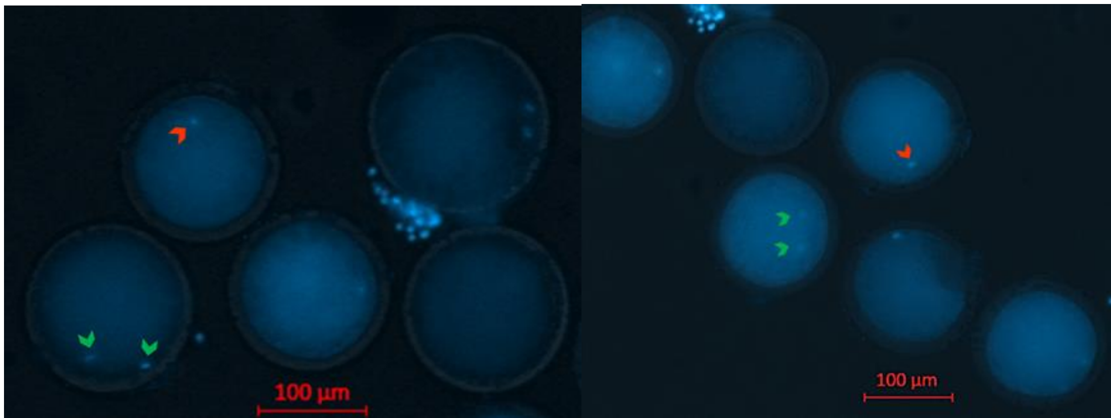

Figure 3.2. Images of FCS derived oocytes stained with Hoechst, showing oocytes in metaphase I and metaphase II taken using Zeiss Axio Observer Z1 microscope. Red arrows represent single nuclear stain (metaphase I), green arrows represent double nuclear stain (metaphase II – nuclei plus a polar body).

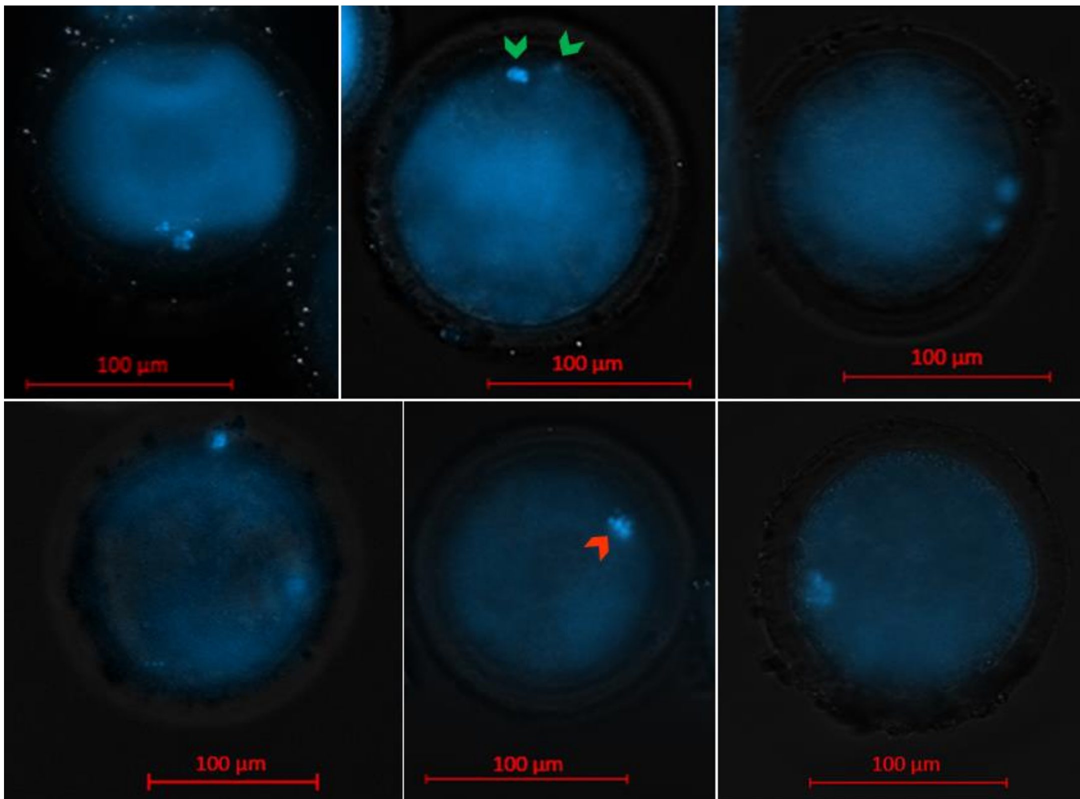

Figure 3.3. Images of releasate derived oocytes stained with Hoechst, showing oocytes in metaphase I and metaphase II taken using Zeiss Axio Observer Z1 microscope. Red arrows represent single nuclear stain (metaphase I), green arrows represent double nuclear stain (metaphase II – nuclei plus a polar body).

3.2 The Effect of Platelet Releasate on Embryo Development

Experiment 2 tested whether treating bovine oocytes with 10% human platelet releasate during *in vitro* maturation caused subsequent differences in the number of cleaved embryos and blastocysts post-fertilisation in comparison to oocytes treated with 10% FCS. To determine this, the number of cleaved embryos at 48 hours post-insemination was recorded for the FCS control and platelet releasate groups. A cleaved embryo was judged to be any embryo which clearly displayed more than 1 cell (typically ranging from 2 to 6 cells on day 2, as shown in

Figure 3.4).

A total of 526 FCS and 546 releasate derived oocytes from 14 oocyte collections were inseminated. Figure 3.5 shows that there is a slight, but non-significant difference in mean cleavage rate (FCS: 68.04%±10.82, releasate: 60.89%±13.75; P=0.164). This data demonstrates that platelet releasate supplementation during *in vitro* maturation does not influence the proportion of oocytes successfully forming embryos.

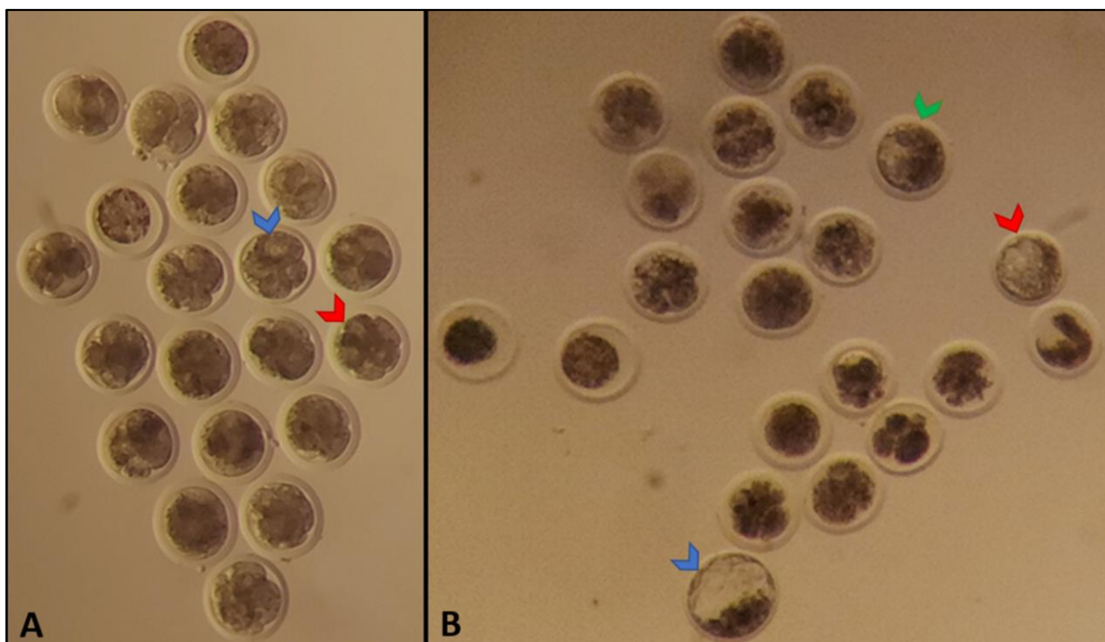

Figure 3.4. Representative image through stereomicroscope of **A.** presumptive zygotes, 48 hours post-fertilisation, showing numerous cleaved embryos. Blue arrow shows a 4-cell embryo, red arrow indicates a 6-cell embryo, and **B.** embryos 7 days post-fertilisation, showing 3 blastocysts. Blue arrow indicates an expanded blastocyst with a defined inner cell mass, red and green arrows show early blastocysts with developing blastocoel cavities.

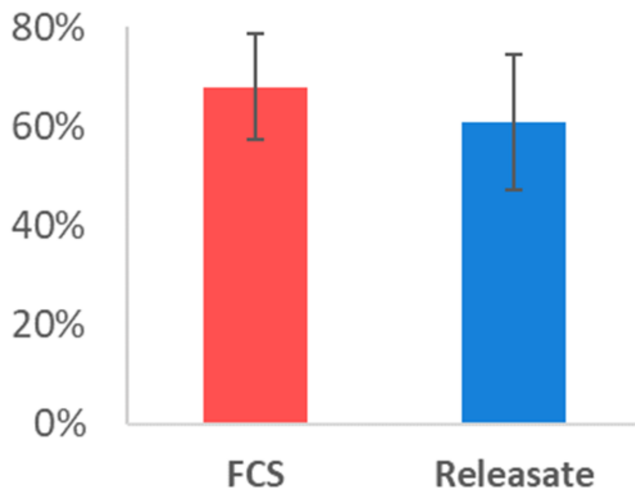

Figure 3.5. The mean cleavage rate derived from eggs cultured with platelet releasate compared with the FCS control. Mean values were calculated from n=14 data, the error bars represent standard deviation. (**P=0.164**).

The embryos were subsequently scored on days 7 and 9 (post-fertilisation) and the blastocyst rate was recorded out of both the total number of oocytes inseminated, and out of the number of cleaved embryos. A blastocyst was judged to be an embryo which had developed a trophectoderm, blastocoel and inner cell mass, although the inner cell mass can be difficult to identify in early blastocysts (as shown in

Figure 3.4 and Figure 3.7).

Both measurements of blastocyst rate showed a statistically significant detrimental effect in the releasate group, as displayed in Figure 3.6. The mean blastocyst rate for the FCS control group was 10.56%±4.47, compared to 5.21%±4.89 for those embryos derived from oocytes treated with platelet releasate (P=0.006). When expressed as blastocyst rate per cleaved embryo, 15.46%±5.86 of cleaved embryos reached blastocyst stage in the FCS control group, whereas in the releasate group, only 8.40%±7.04 pf embryos formed blastocysts (P=0.002). These data indicate that exposing oocytes to platelet releasate during *in vitro* maturation significantly diminishes blastocyst formation.

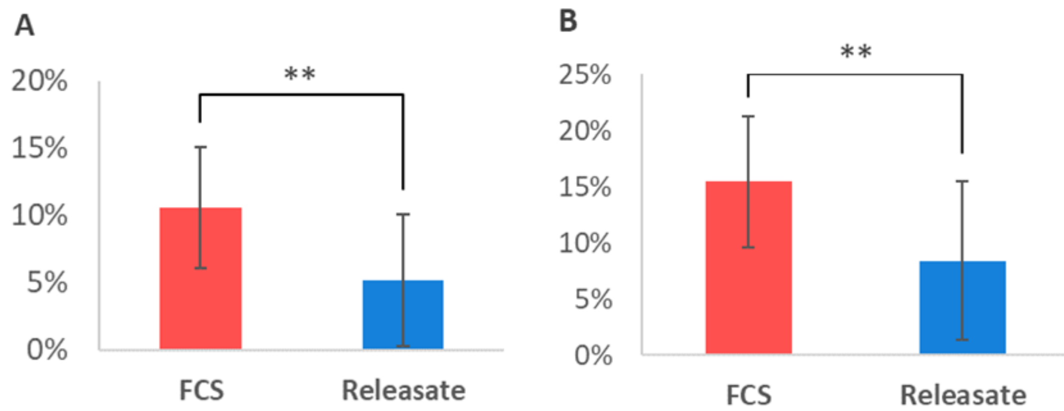

Figure 3.6. **A**. The mean blastocyst rate derived from eggs cultured with platelet releasate compared with the FCS control. Mean values were calculated from n=14 data, the error bars represent standard deviation. (**P=0.006**). **B**. The mean blastocyst rate from cleaved embryos derived from eggs cultured with platelet releasate compared with the FCS control. Mean values were calculated from n=14 data, the error bars represent standard deviation. (**P=0.002**).

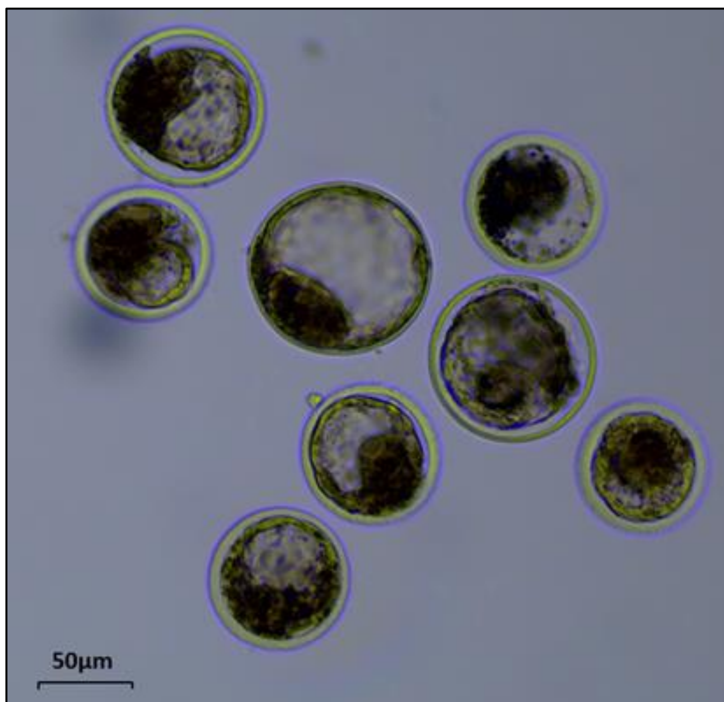

Figure 3.7. Indicative image of blastocysts 7 days post-insemination, showing different stages of maturation and blastocoel expansion, collected using demo model of Olympus EP50 microscope camera.

3.3 The Effect of Platelet Releasate on Embryo Metabolism

Having confirmed that exposing oocytes to platelet releasate reduced the number of blastocysts that form, the metabolic profile of embryos was next assessed. Embryo metabolism serves as an indicator of quality (Gardner and Harvey, 2015).

To determine this, blastocysts from the FCS control group and platelet releasate group were selected randomly on day 7 and day 8 and cultured individually for ~24 hours in 5µl droplets of culture media. After this, embryos were removed from droplets, which were then analysed to establish glucose depletion, lactate release and pyruvate depletion.

As indicated in Figure 3.8, there was no significant difference in lactate release (FCS: 22.8pmol/embryo/hr±68.7, releasate: 17.0pmol/embryo/hr±44.0; P=0.556) or pyruvate depletion (FCS: -11.79pmol/embryo/hr±38.41, releasate: -15.6pmol/embryo/hr±31.9; P=0.493) between blastocysts from the FCS control group and the platelet releasate group.

However, blastocysts derived from oocytes treated with platelet releasate depleted significantly less glucose than the FCS control (FCS: -37.96pmol/embryo/hr±44.55, releasate: -3.15pmol/embryo/hr±50.51; P=0.003).

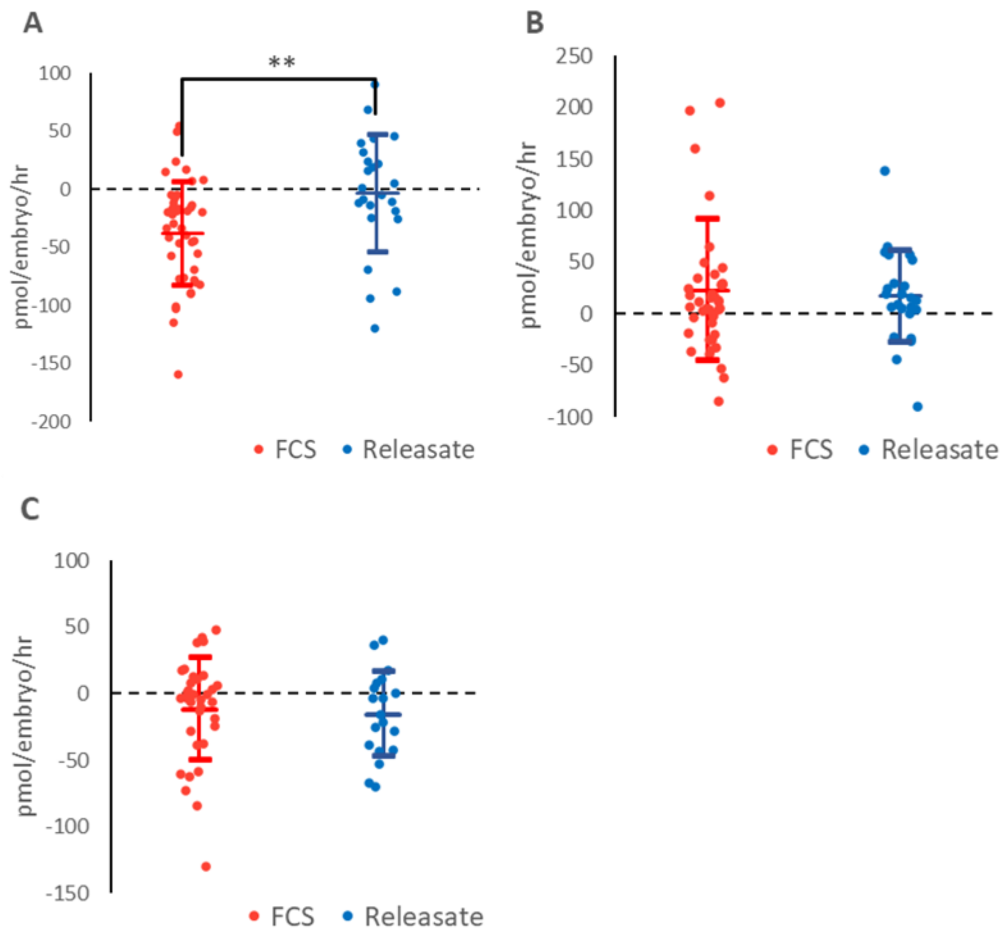

Figure 3.8. Blastocyst glucose metabolism is compromised in blastocysts derived from oocytes treated with platelet releasate. **A**. Glucose depletion (n=43 FCS, n=24 Releasate) (**P=0.003**). **B**. Lactate release (n=43 FCS, n=24 Releasate) (**P=0.556**). **C**. Pyruvate depletion (n=34 FCS, n=19 Releasate) (**P=0.493**). Plots represent mean values per oocyte collection, bars represent overall mean values, and error bars represent standard deviation.

3.3.1 The Effect of Platelet Releasate on Glycolytic Index

Measuring depletion of glucose and pyruvate, alongside release of lactate allows for the determination of glycolytic index; that is the percentage of glucose that can be accounted for by lactate release. The mean glycolytic indexes of the FCS and releasate blastocysts were 7.78%±348.66 and -153.90%±638.17 respectively (Figure 3.9). The results showed no significant difference between the groups.

The mean FCS result shows that less than 8% of glucose was converted to lactate, meaning 92% of glucose was used for other processes, potentially oxidative phosphorylation. Meanwhile, the mean glycolytic index for releasate corresponds with the glucose depletion data (Figure 3.8), whereby the level of glucose does not decrease in line with the control group, indicating glucose is not being converted to lactate.

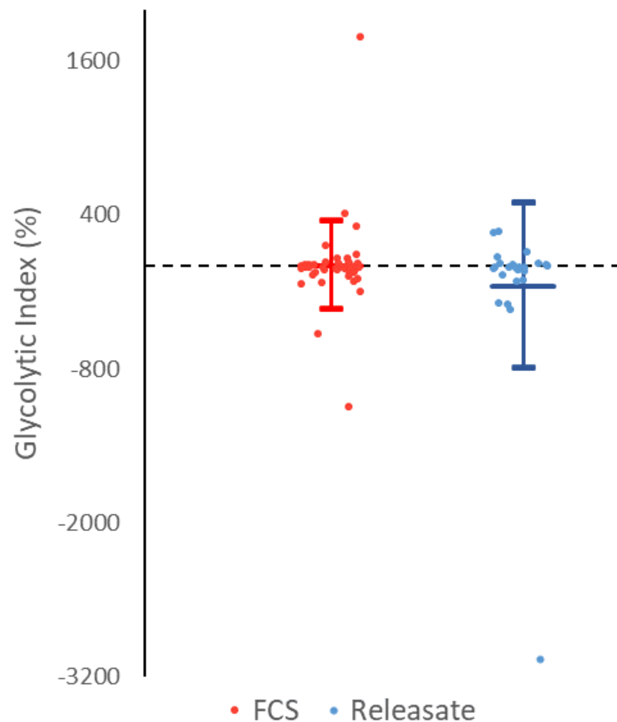

Figure 3.9. Glycolytic index (n=43 FCS, n=24 releasate) (**P=0.556**). Bars represent mean values, and error bars represent standard deviation.

3.4 The Effect of Platelet Releasate on Mitochondrial Function

Next, oxygen consumption rate (OCR) was determined. OCR allows for the estimation of mitochondrial function. A total of 28 FCS and 28 Releasate blastocysts from 4 oocyte collections were used (7 blastocysts per group, per assay).

The data showed that the mean OCR of the releasate group appeared marginally higher than the FCS control (Figure 3.10 A), although this was not statistically significant.

The use of mitochondrial inhibitors enables the quantification of components of OCR (Figure 3.10 B). Initially, oligomycin, which inhibits mitochondrial complex V was added. The oligomycin-insensitive proportion of OCR was 57.81%±33.4 of basal OCR in the FCS control, and 51.48%±14.9 of basal OCR in the releasate group.

Next, the uncoupler FCCP was added to allow estimation of maximal respiratory capacity. In controls, OCR rose by 18.95%±33.0 over basal, compared to only reaching 97.38%±58.2 of the basal level in the releasate group.

Finally, the inhibitors Antimycin and Rotenone (A/R) were added in combination to inhibit mitochondrial complex I and III respectively. The A/R-insensitive component of OCR indicates non-mitochondrial OCR. In controls, this was just 2.55%±11.2 of basal OCR, whereas non-mitochondrial OCR of the releasate group was 5.76%±15.5 of basal OCR.

Despite the observed differences, the components of oxygen consumption did not differ significantly between the Releasate and FCS groups (basal respiration P=0.686, ATP-coupled respiration P=0.686, proton leak P=0.686, maximal respiration P=1.000, non-mitochondrial respiration P=1.000, and spare capacity P=0.686).

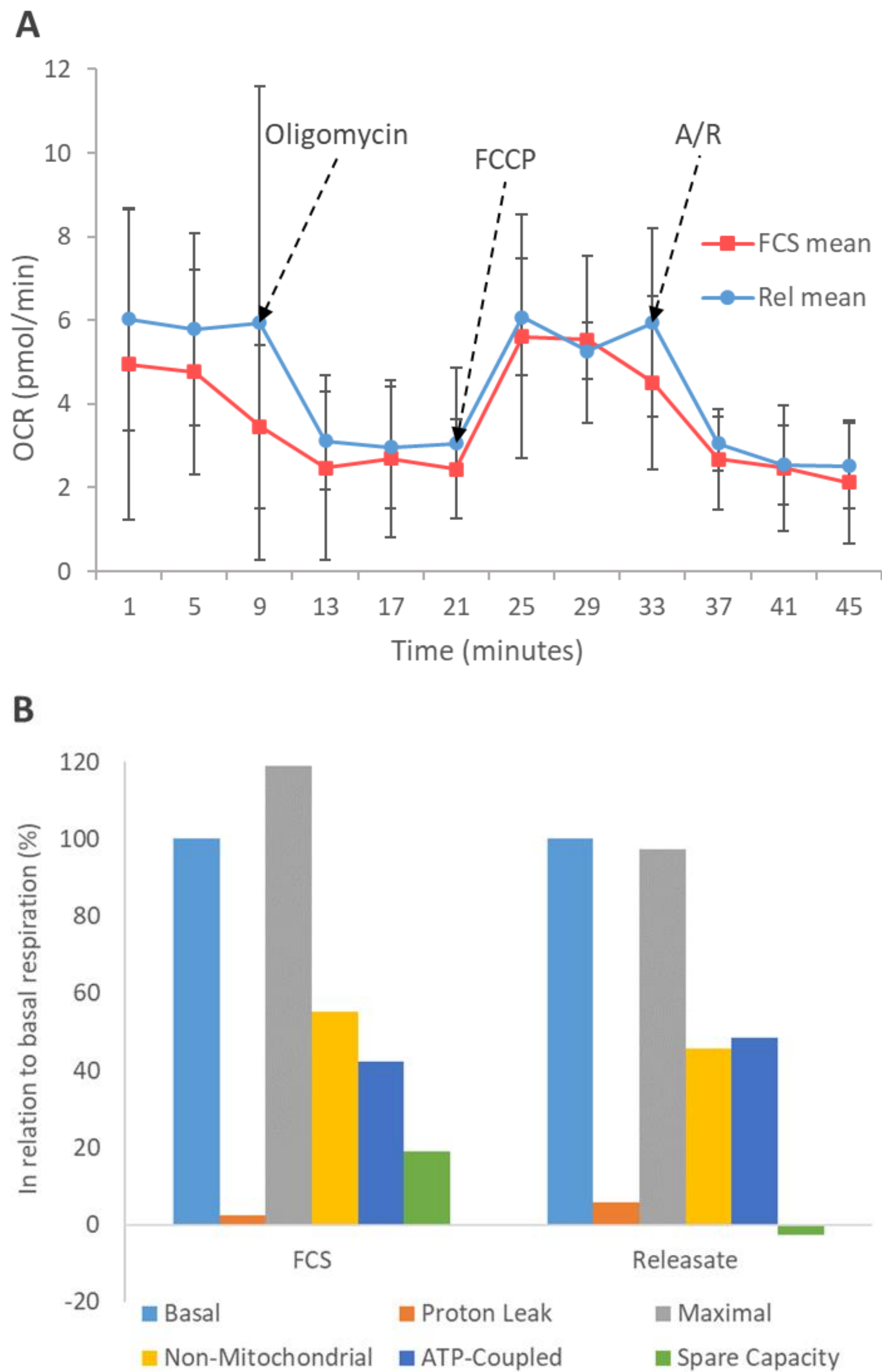

Figure 3.10. **A**. Mean oxygen consumption rate (n=4) (**P=0.089**). Dashed arrows indicate the points where inhibitors were added. Error bars represent standard deviation. OCR values are representative of OCR per 7 blastocysts. **B**. Quantification of OCR components in relation to the basal respiration level. Note: releasate's mean spare capacity was a negative value as the maximal level of respiration did not exceed the basal level.

3.5 The Effect of Platelet Releasate on Epigenetic Markers in Blastocysts

Having identified developmental impacts and metabolic alterations in blastocysts derived from oocytes treated with platelets, a series of observations were made to identify releasate-induced alterations. Initially, the effect of releasate on epigenetic processes was determined using the expression of H3K27me3, determined by immunofluorescence. A total of 10 FCS and 11 releasate derived blastocysts were used from 4 oocyte collections, with 2 from each group selected as controls (not exposed to the primary antibody). All mean fluorescence intensities were obtained using the Zeiss Axio Observer Z1 microscope and the Zeiss Zen 3.6 (blue edition) software, the exposure was 1800m/s.

The mean fluorescence intensities of the 8 FCS blastocysts were significantly higher than negative controls (P=0.044). One releasate blastocyst was excluded from the data due to failure to stain to a level equal to or greater than the releasate control. The mean fluorescence intensities of the 8 releasate blastocysts were higher than the 2 releasate control blastocysts, confirming successful staining. However, there was no statistical significance (P=0.089) in H3K27me3 levels between treatment and control blastocysts.

FCS and releasate mean fluorescence intensities were normalised against the mean intensities of the 2 control groups, giving values of intensity that are relative to the corresponding control (Figure 3.11). Representative images shown in Figure 3.12.

These data demonstrate that exposure to releasate during *in vitro* maturation does not result in a significant change in H3K27me3 expression, blastocysts from both groups expressed this modification.

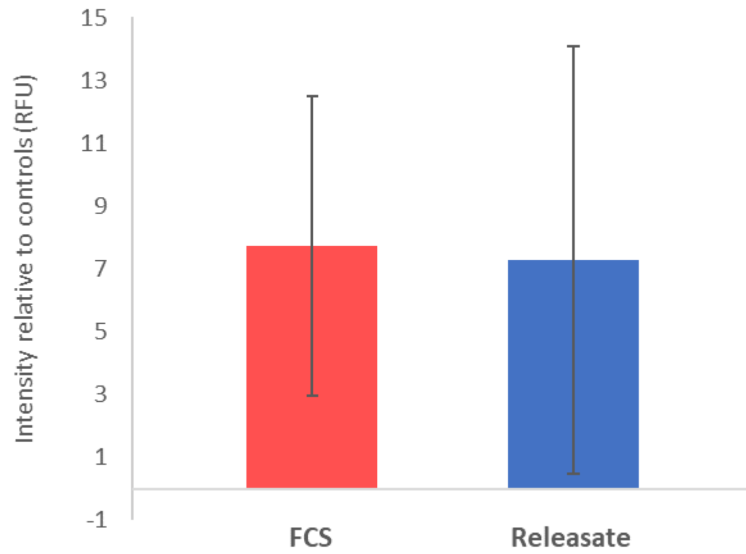

Figure 3.11. Relative mean fluorescence intensity. Mean values were calculated from n=8 FCS and n=8 releasate blastocysts, the error bars represent standard deviation. (**P=0.505**).

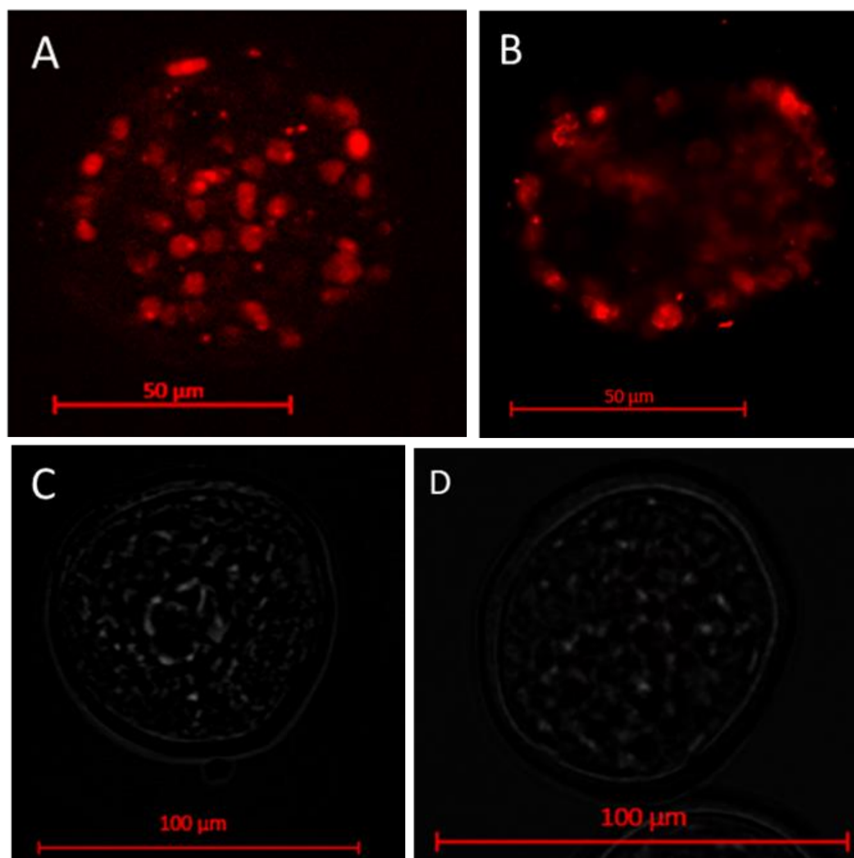

Figure 3.12. Images **A** and **B** of FCS and releasate-derived blastocysts, respectively, stained with Alexa 594, showing expression of H3K27me3. Images **C** and **D** of FCS and releasate-derived blastocysts, respectively, without Alexa 594 staining. Representative images taken using Zeiss Axio Observer Z1 microscope.

3.6 The Effect of Oocytes on Platelets

3.6.1 Dose Response

A major function of platelets is to undergo aggregation, and this can be induced by a great many stimuli. In response to aggregation, platelets release the contents of their cytoplasmic granules. While the major aim of this project was to determine the effect of platelets on oocytes, it was important to discover how platelets responded to the presence of oocytes. Prior to this, platelet response to the classical agonist TRAP-6 was confirmed. Figure 3.13 shows the consistent ability of 3µM TRAP-6 to successfully aggregate platelets to ~70% in 4 dose response replicate experiments (70.6%, 67.6%, 74.5%, and 70.6% respectively), in comparison to lower concentrations of 1µM and 0.3µM.

The data also showed that 1µM TRAP-6 elicited an aggregation of 6.9% and 15.9% respectively, in two of the experiments, although the platelets recovered quickly and returned to their original level of aggregation, but the further two repeats did not show any aggregation (Figure 3.13). Additionally, all four experiments showed no aggregation when 0.3µM TRAP-6 was used.

The level of aggregation after 1 minute for each concentration, in each repeat, was compared. The aggregation achieved when 3µM TRAP-6 was added was significantly higher than when 0.3µM was used ($P<0.007$), but was not significantly higher than that of 1µM ($P=0.492$). However, the aggregation for 1µM was still significantly higher than 0.3µM ($P<0.044$).

The level of aggregation after 3 minutes of 3µM TRAP-6 exposure was statistically significantly higher than that of 1µM ($P<0.004$), as the platelets appear to recover from their initial activation by 1µM, returning to their original level prior to exposure. This also resulted in the aggregation for 1µM no longer being significantly higher than that of 0.3µM ($P=0.279$).

These results justified the selection of 3µM TRAP-6 to act as the positive control in the aggregation experiment, as a means to confirm after the experiment that the platelets are capable of aggregating, even if they did not when exposed to the experimental substrates.

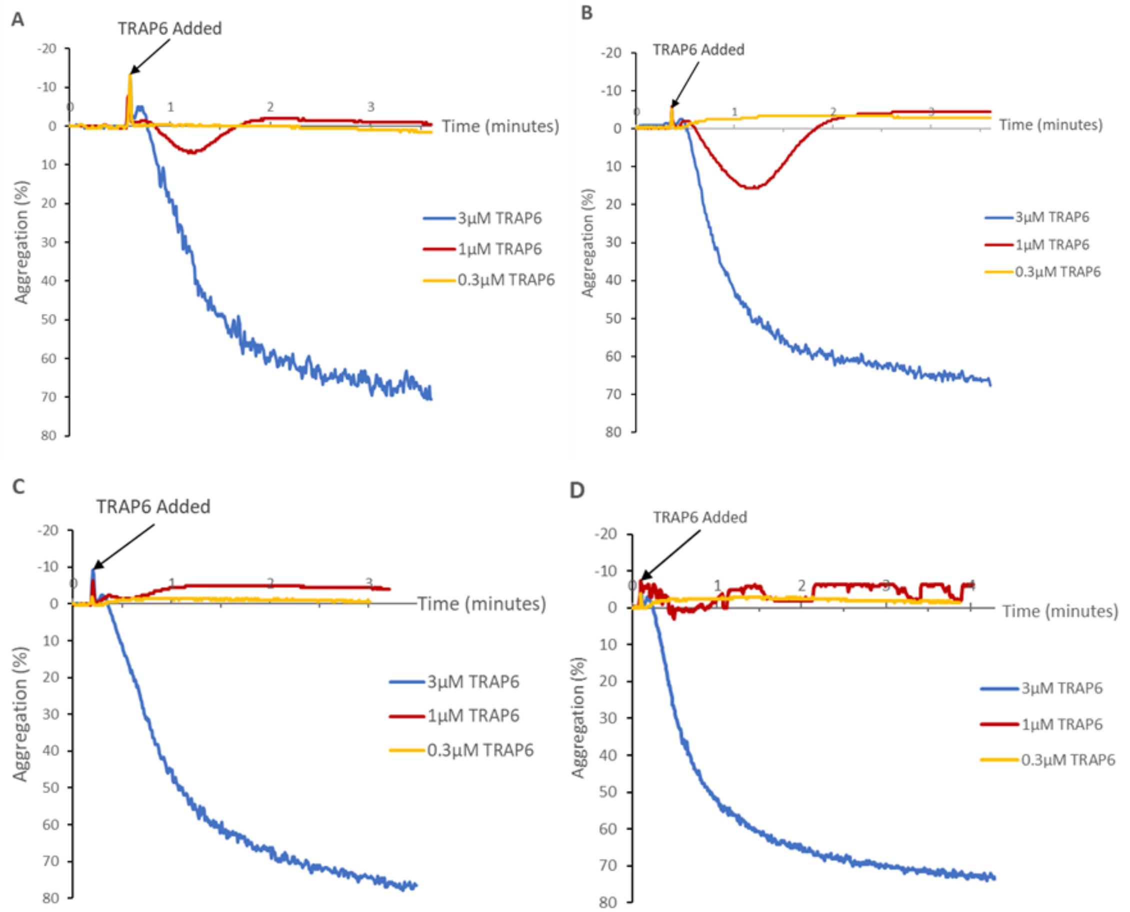

Figure 3.13. Dose response curves to justify use of 3µM TRAP-6 concentration as the positive control for the aggregation experiment. **A**. N=1, **B**. N=2, **C**. N=3, **D**. N=4.

3.6.2 Platelet Aggregation in Response to Oocytes

The ability of oocytes to induce platelet aggregation was tested on three independent occasions with platelets from three different donors. 5 mature oocytes (in 30µl media), 5 dead oocytes (in 30µl media), 30µl cumulus cells and 30µl oocyte culture media were added to 300µl PRP each, and the aggregation compared. In each case, after 18-21 minutes, the positive control (TRAP-6) was added to the suspension to elicit platelet activation to prove platelet competence.

In Figure 3.14, replicate 1 indicated that oocytes may induce aggregation. Within 9 minutes of dead oocytes being added to PRP, the maximum aggregation exceeded 70%, platelet responsiveness was confirmed by reaction to TRAP-6, where the aggregation reached 74%. A maximum aggregation of 50% was observed for mature oocytes, but this took 16 minutes to achieve, upon addition of TRAP-6 the platelets aggregated further to 69%. Cumulus cells took 16 minutes to make platelets aggregate to their highest level of 40%, which increased to 74% after addition of TRAP-6. Culture media prompted platelet aggregation of 13% at 16 minutes, and TRAP-6 was only able to push it to 34.8%.

By contrast, replicate 2 of the same experiment showed no obvious difference between the substrate groups. Dead oocytes and culture media both induced aggregations of 2.5%, meanwhile mature oocytes and cumulus cells both prompted platelet aggregations of 2% within 20 minutes of exposure. Platelet responsiveness was confirmed by reaction to TRAP-6. The dead oocytes aggregate was reduced following TRAP-6 addition to -1.5%. The mature oocytes aggregate increased to 8.3% immediately after TRAP-6 addition but stabilised to 1.5%. The cumulus cells aggregate increased to 33% after TRAP-6 addition, but stabilised at 15%. Within a minute of TRAP-6 exposure to the culture media aggregate, the aggregation rose to 9.8%, but stabilised at 1.5%.

This finding was confirmed in a third replicate; data showed no obvious difference between the substrate groups. All groups reached a stable aggregation between 13 and 20 minutes, with each group aggregating to 4.9% by 20 minutes. After the introduction of TRAP-6 at 20 minutes, each group aggregated successfully and maintained their aggregation for the 6 remaining minutes of the experiment. Culture media reached 73%, cumulus cells 71%, mature oocytes 81%, and dead oocytes 71%.

The level of aggregation immediately before TRAP-6 addition, and several minutes after addition, in each repeat, was compared. Statistically, there was no significant difference between the groups before or after the positive control was introduced (Table 3.1).

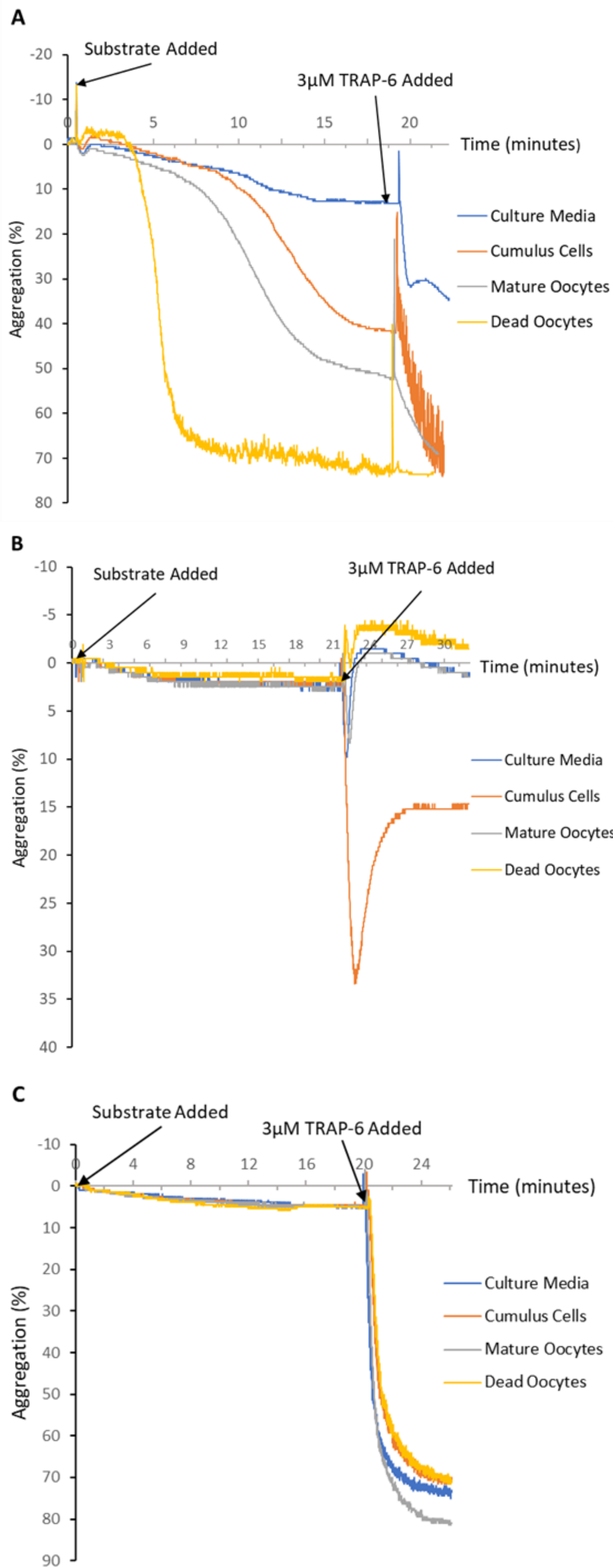

Figure 3.14. Platelet aggregations in response to substrate, followed by the addition of 3µM TRAP-6. **A**. N=1, **B**. N=2, **C**. N=3.

Table 3.1. Statistical comparisons between substrate groups after substrate-induced aggregation, and TRAP-6 induced aggregation.

Group comparison	Significance pre TRAP-6	Significance post TRAP-6
Culture media, Cumulus cells	P=0.908	P=0.647
Culture media, Mature oocytes	P=0.817	P=0.731
Culture media, Dead oocytes	P=0.908	P=0.909
Cumulus cells, Mature oocytes	P=0.729	P=0.909
Cumulus cells, Dead oocytes	P=0.817	P=0.731
Mature oocytes, Dead oocytes	P=0.908	P=0.819

Chapter 4 Discussion

Since 2016 there has been increasing interest in the use of platelet-rich plasma to rejuvenate aged or exhausted ovaries (Pantos *et al.*, 2016; Hosseini *et al.*, 2017; Sfakianoudis *et al.*, 2018; Ahmadian *et al.*, 2020; Hsu *et al.*, 2020; Ozcan *et al.*, 2020). From an initial pilot report in Abstracts of the 32nd Annual Meeting of ESHRE, by Pantos *et al.* (2016), there are 12 registered clinical trials at different stages, attempting to assess the efficacy of PRP treatment for ovarian rejuvenation. However, as these approaches have developed, there remain few reports of the effects of PRP on the female gamete. Using an *in vitro* large animal model, this project has sought to contribute to this knowledge gap. Supplementation of immature oocytes with releasate from platelets led to a significantly lower blastocyst rate and lower level of glucose depletion in blastocysts in comparison to controls. However, supplementation of platelet releasate did not have a significant effect on oocyte metaphase II rate, embryo cleavage rate, blastocyst lactate release and pyruvate depletion, oxygen consumption rate, or expression of the epigenetic modification H3K27me3.

4.1 Oocyte Metaphase II Rate

Oocyte metaphase II rate was selected as an endpoint in this study, as it signifies the final maturation of the oocyte and is prerequisite for successful fertilisation. Although the achievement of reaching metaphase II does not guarantee successful fertilisation, it does indicate that the oocyte does have the capacity for this to occur, assuming that other external factors permit it (sperm quality, appropriate temperature and concentration of culture media). Oocytes were aspirated from bovine follicles, incubated with either platelet releasate or FCS for 22-24 hours. Determining metaphase II rate gives an insight into how well the oocytes were able to mature during this incubation period. During *in vitro* maturation, it is expected that oocytes mature from metaphase I to metaphase II, ready for insemination with bull spermatozoa.

It was originally hypothesised that oocyte supplementation with platelet releasate would have a beneficial effect on oocyte development *in vitro*, giving rise to a higher metaphase II rate which could support a better rate of fertilisation. The presumed mechanism of action was via cytokine function – platelet releasate is replete with a range of cytokines at physiological levels (Amable *et al.*, 2013). Studies have reported

that cytokines have been found in ovarian follicular fluid, where they regulate cell proliferation, follicle selection or arrest, and are also considered to be biomarkers for oocyte maturation and development (Field *et al.*, 2014). A porcine study found that *in vitro* supplementation of C-X-C motif chemokine ligand 12 (CXCL12), vascular endothelial growth factor A (VEGFA), and Wingless-type MMTV integration site family member 5A (WNT5A) promoted nuclear maturation of oocytes, increasing the nuclear maturation rate from 57% in the control group, to almost 76% in the supplementation group. The study also found that the inhibition of the receptor pathways of these cytokines significantly hindered the resumption of meiosis, as well as cumulus expansion during *in vitro* maturation (Liu *et al.*, 2020).

However, this experiment found no difference in metaphase II rate between oocytes supplemented with releasate compared with oocytes supplemented with FCS. This finding means that a difference in cleavage rate or blastocyst rate of subsequent embryos, would not be a result of compromised oocyte maturation.

This data shows that releasate neither inhibits nor accelerates the maturation of oocytes *in vitro*, but that the detrimental effect of releasate supplementation became apparent during embryo development from cleavage-stage to blastocyst stage.

4.2 Cleavage and Blastocyst Rate

Upon stimulation, platelets release a large range of cytokines, including interleukin-4 (IL-4), IL-8, IL-13, IL-17, tumour necrosis factor alpha (TNF-α), and interferon alpha (IFN-α) (Amable *et al.*, 2013). Many cytokines have been investigated in terms of embryotrophic properties, such as IL-6, which is secreted by endothelial cells (Desai and Goldfarb, 1996), and Granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF has been shown to be present in the mouse preimplantation uterus at high levels and is thought to support implantation by regulating the leukocytes of the endometrium (Robertson and Seamark, 1992). Cytokines also play a role in the development of ovarian follicles from primary to secondary stage, such as oocyte-derived platelet growth factor (PDGF) which supports oocyte growth via increasing the expression of stem cell factor (SCF) of granulosa cells (Adamczak *et al.*, 2021).

On this basis, it was hypothesised that exposing oocytes to releasate would enhance onward development. Surprisingly, supplementation with platelet releasate during *in vitro* maturation neither improved nor hindered oocytes' ability to cleave after fertilisation. This further supports the metaphase II finding, as no difference in cleavage rate suggests a similar rate of fertilisation had occurred in both groups, and therefore oocyte maturity must also be similar.

However, the supplementation did reduce the number of embryos which successfully formed blastocysts. This suggests that exposure to platelet releasate at the oocyte stage can limit the developmental milestones the subsequent embryo can reach, such as the formation of an inner cell mass and trophectoderm, rendering the embryos stunted at cleavage-stage.

A recent murine study by Pock *et al.* (2022) hypothesised that GM-CSF played a role in the development of the trophectoderm and inner cells mass. The study found that supplementation of 2-5ng/ml GM-CSF into embryo culture media increased the number of cells in the inner cell mass and trophectoderm of subsequent blastocysts, however concentrations higher than 5ng/ml actually led to a substantial decrease in cell number. This suggests there is a concentration threshold which, when exceeded, results in blastocysts with fewer differentiated cells. The study also used the gene *Nanog* as a marker for the epiblast – a further differentiation of the inner cell mass, whose expression was described as increasingly ectopic with increasing GM-CSF concentrations. This finding was recorded for both mouse-derived GM-CSF and human-derived GM-CSF supplementation. The authors suggested that although 2-5ng/ml GM-CSF supplementation appeared to increase the presence of differentiated cells in blastocysts, it negatively impacted the conservation of *Nanog* gene expression as the blastocysts developed further.

It is known that GM-CSF inhibits apoptosis (Desai *et al.*, 2007), which could explain why higher GM-CSF concentrations led to blastocysts with an increasingly ectopic expression of *Nanog*, in the study by Pock *et al.* (2022). It is conceivable that although GM-CSF may be beneficial for cellular differentiation as a result of decreased apoptosis, it leads to the survival of cells which may express genetic anomalies of some form. It may then be

the case that those genetic anomalies cause embryos to be unviable, perhaps at the later blastocyst stage in mice, however this may differ between mammalian species.

This could even offer a potential explanation as to why bovine embryos in this study were terminated at the cleavage stage. It could be hypothesised that oocyte exposure to platelet releasate (which contains a variety of cytokines and growth factors) leads to reduced apoptosis of abnormal cells within the oocyte and subsequent zygote, which may have a knock-on effect on the ability of those cells to differentiate successfully to form a blastocyst (Pisko *et al.*, 2021).

Of course, this result only represents *in vitro* fertilisation, and it cannot be commented on whether cleavage-stage embryos derived from platelet releasate supplementation, would go on to form blastocysts *in vivo* if transferred on day 3, for example. It can however be said that releasate does stunt embryo development *in vitro*.

4.3 Metabolic Analysis

Due to the significant detrimental effect platelet releasate had on blastocyst rate, the metabolism of the blastocysts that did develop was compared between the releasate group and the FCS control, to discover if there were any metabolic differences that could be attributed to platelet releasate exposure during *in vitro* maturation. Embryo metabolism is associated with further developmental potential (Van Hoeck *et al.*, 2011), and so this was accepted as a suitable endpoint in this study.

For the embryos which did successfully form blastocysts, their glucose depletion was significantly lower than the control, however pyruvate depletion and lactate release did not differ to the control group. This could suggest blastocysts that originated from oocytes treated with platelet releasate were less able to consume glucose, possibly by an effect on glucose transporters, although a link between platelet releasate and glucose transporters had not yet been reported in the literature at the time of writing this thesis. A disruption in transporter activity could prevent glucose being transported into the embryo to commence its phosphorylation by hexokinase, and further conversion into pyruvate and then lactate. However, as the level of lactate secreted by releasate-derived blastocysts was found to not significantly differ from the control group, it could suggest that lactate release was maintained by an alternative pathway

which surpassed the need for glucose phosphorylation by hexokinase, the first step in the glycolytic pathway.

Some new research has suggested that minimal glucose is actually used in glycolysis, with the majority used in the pentose phosphate pathway (PPP) and hexosamine biosynthesis pathway (Chi *et al.*, 2020). The pentose phosphate pathway produces glyceraldehyde-3-phosphate, also produced during glycolysis where it is converted to pyruvate. In the study by Chi *et al.* (2020), glucose was observed to contribute considerable carbon units to the PPP, demonstrated by the reduction in PPP metabolites when embryos are cultured without glucose. They also found that when this pathway is inhibited, embryos arrest at the morula stage before they can develop to form blastocysts, but this can be restored when a ribose source (the end product of the PPP) is supplemented into the culture media. This suggests that a lack of carbon units being provided to the PPP can disrupt embryo development. It is plausible that a reduced ability to consume glucose, as found in this study, may lead to a shortage of carbon units necessary for biosynthesis and nucleotide formation, necessary for cell proliferation – crucial for blastocyst development (Krisher and Prather, 2012).

A study by Van Hoeck *et al.* (2011) found that oocyte exposure to maturation media supplemented with elevated concentrations of fatty acids led to no change in cleavage rate, but a significant reduction in blastocyst rate. The blastocysts that did develop were also observed to have reduced consumption of glucose, however they did note a reduction in pyruvate consumption and lactate release. This further supports the understanding that oocyte environment during oocyte maturation can influence embryo development beyond cleavage stage and affect the metabolism of blastocysts.

The 'normal' level of lactate released from releasate blastocysts is an important finding in itself, as blastocysts release lactate to acidify the region around the blastocyst as this aids implantation into the uterine lining, similar to tumour invasion (Smith and Sturmey, 2013; Gardner, 2015). This result suggests that the lactate-based microenvironment surrounding the blastocysts would not make them any less likely to successfully implant than FCS derived blastocysts, but the absence of glucose uptake could suggest they are unviable.

As the blastocysts consumed a significantly lower amount of glucose than the control group, it was expected that there would be a significant difference in glycolytic index (the percentage of glucose converted to lactate) between the 2 groups, but this was not the case. As there was no significant difference, this shows that very little glucose, if any at all (for some individual blastocysts) was converted to lactate, potentially leaving it available for oxidative phosphorylation.

4.4 Oxygen Consumption Rate

Measuring depletion and release of substrates is a useful marker of metabolic activity, however, the determination of oxygen consumption gives insight into specific mitochondrial function (Muller *et al.*, 2019). OCR represents mitochondrial oxidative phosphorylation, which harnesses the glucose that has not already been converted to lactate, in addition to other substrates, including endogenous fatty acids necessary for nucleotide formation (Krisher and Prather, 2012). In cleavage stage embryos, mitochondria exist in their immature form and in the numbers established in the oocyte prior to fertilisation. It is only when the embryo enters the blastocyst stage that the mitochondria become mature and replicate, as to support the blastocyst's energy requirements (Muller *et al.*, 2019).

Platelet releasate supplementation did not significantly affect OCR of blastocysts, which suggests that the blastocysts' mitochondria are neither benefitted nor limited by releasate exposure. This finding indicates that oocyte exposure to platelet releasate during *in vitro* maturation, neither affects the ability of mitochondria to replicate as the subsequent embryos enter the blastocyst stage, nor inhibit the maturation of their mitochondria into organelles which can support the embryo's energy demand.

As previously discussed, it appears that although releasate-derived blastocysts consumed less glucose, they were still capable of releasing a similar level of lactate as the control group, whose glucose consumption was significantly higher. This suggests that the releasate-derived blastocysts' lactate production is not solely, if at all, derived from the glucose they consume – potentially leaving the minimal amount of glucose consumed available for oxidative phosphorylation by mitochondria. This could offer an explanation as to why the OCR of the releasate blastocysts did not significantly differ from that of the control group.

4.5 H3K27me3 Expression

As a result of the phenotypic observations made during this study, it was hypothesised that these phenotypic changes may have been caused by an epigenetic alteration. Epigenetic modifications, such as methylation and acetylation, affect the structure of chromatin, therefore influencing gene expression – with methylation of histone 3 at lysine 27 resulting in the repression of gene transcription (Marinho *et al.*, 2017), for example. H3K27 methylation was used as an epigenetic marker in a study by Zhao *et al.* (2022), where they found that in oocytes with Ezh2 gene knockout, H3K27 dimethylation was absent, meanwhile in Ezh1/2 double knockout oocytes, H2K27 trimethylation was also absent. These knockouts also affected H3K27me in subsequent embryos, with delayed restoration of methylation. The researchers found that the double knockout led to changes in the expression of H3K27me3 in late blastocysts, as well as the disruption of normal embryo development. Such work has highlighted the benefit of using H3K27 methylation as a marker for epigenetic expression in embryos.

In this study, the expression of trimethylated H3K27 in blastocysts, was unaffected by platelet releasate supplementation. The modification is associated with repression of transcription, and therefore the downregulation of certain genes (Marinho *et al.*, 2017), and has a role in differentiation of cells (Nakamura *et al.*, 2020).

Whilst this data suggests there is no difference in H3K27me3 expression in releasate-treated and control blastocysts, it is not an unusual finding. A study by Marinho *et al.* (2017) found that expression increased as embryos cleaved from 2 to 4 cells, decreased in 8-cell embryos and morulae, but increased at blastocyst stage – although the extent of expression in blastocysts did vary within the sample. The changes in expression between embryos of different stages suggests that increases and decreases in gene repression are required during different periods of development. Genes involved with cell differentiation and embryonic development are known to be suppressed by H3K27me3 until points in time where it is necessary for them to be transcribed (Marinho *et al.*, 2017).

Further experimentation would have been beneficial to see if releasate supplementation led to differences in H3K27me3 expression during other stages of embryo development, not just blastocysts – as this perhaps could have accounted for

why releasate-derived embryos were less likely to develop into blastocysts. It is possible that H3K27me3 expression was affected in cleavage-stage embryos, and this could offer an explanation as to why cleavage-stage embryos arrested, due to some form of genetic disruption. Perhaps if H3K27me3 expression was measured at the point of zygotic genome activation, 4-8 cell stage in bovines (Zhai *et al.*, 2022), a difference may have been present. Zhai *et al.* (2022) found that when zygotic genome activation was impaired, embryos are arrested at an early stage of development – demonstrating how tightly controlled gene transcription and repression is in the developing embryo.

4.6 Platelet-Oocyte Aggregation

This work focussed on the effect of platelet releasate on female gametes. However, proposed treatment uses platelet rich plasma injected directly into the ovary. Platelets are highly reactive/responsive cells which release a plethora of compounds in response to a range of agonists (Chu *et al.*, 2021). However, whether oocytes have any effect on platelets had not been considered. The initial pilot experiment used PRP as the supplement, which caused a dense clot to form around all oocytes, making them unsuitable to inseminate. The decision was made to switch the supplement to platelet releasate, in order to remove platelets and avoid aggregation but still allowing oocyte exposure to their cytokines and growth factors.

However, this platelet observation raised the question of what exactly was causing the platelets to aggregate. To address this, an experiment was designed in which platelet function in response to culture media, cumulus cells, mature oocytes, and dead (unexpanded) oocytes was measured. Overall, there was no significant difference in aggregation between these four substrates when they were added to PRP.

Results varied between the repeats, so further repeats would be useful to confirm the finding. All three replicates used platelets derived from different donors. The first replicate showed levels of platelet aggregation that were not observed in the following two replicates. It is possible that the first donor sample contained another factor, or factors, in levels that assisted the facilitation of platelet aggregation when exposed to the substrates.

The overall finding of no significant difference suggests that in intraovarian PRP injection case studies, it is not the exposure of PRP to oocytes or their cumulus cells that causes the initial activation of the platelets, as the follicles retain their competence and the oocytes are uncompromised by aggregated platelets (nothing on the contrary was noted in studies by (Sfakianoudis *et al.*, 2018; Pantos *et al.*, 2019; Cakiroglu *et al.*, 2020; Melo *et al.*, 2020). This is not surprising as PRP injection is not intrafollicular, but intracortical (Melo *et al.*, 2020). Potentially this could explain why beneficial effects of PRP injection have been observed in embryos, as the platelet activation, and therefore the production of platelet releasate, does not occur directly around the developing oocytes. PRP and its releasate may offer beneficial support to the ovary as a whole, through increased angiogenesis and cell proliferation (Hajipour *et al.*, 2021) as previously mentioned – leading to higher numbers of pre-antral follicles and an increase in the diameter of antral follicles, indicative of a higher quality follicular pool (Dehghani *et al.*, 2018).

This study found that oocyte exposure to platelet releasate during *in vitro* maturation had a significant detrimental effect on embryos which developed following *in vitro* fertilisation (namely reduced blastocyst rate and reduced blastocyst glucose consumption). This is a stark contrast from the positive effect noted in case studies of PRP injection, but could demonstrate releasate does not enter the ovarian follicles, hence the absence of detrimental effects on IVF and successful spontaneous and assisted conception outcomes. These data could indicate the importance of intraovarian injection location, as to avoid direct exposure of oocytes to releasate.

4.7 Study Strengths and Limitations

As a result of the COVID-19 pandemic, access to the laboratory was restricted for several months, leading to delays in laboratory work and data collection. When laboratory work was permitted, restrictions at the abattoir were still in place, limiting the number of tissue collections – making it challenging to recoup the loss in experimental data or provide controls to evidence the methods worked in this study.

However, a high number of embryos were still created and were available for morphological observations and metabolic analysis. These data had a number of repeats, and statistical significance was found. This study addressed the unknown effect

of platelet releasate on female bovine gametes and the impact this exposure has on oocyte maturation and subsequent embryo development and competence.

Due to the limited time available, only a small number of repeats were conducted for OCR, H3K27me3 expression, and platelet-oocyte aggregation. Unfortunately, follow-up experiments, such as PCR and metabolic analysis of oocytes and cleaved embryos, were not able to be performed within the time constraints of this study period. There was limited means of assessing epigenetic modification, due to time and cost limitations. A more stringent approach would have been bisulfite sequencing to assess methylation (Lefèvre and Blachère, 2015).

Additionally, the PRP and platelet releasate used in this study was not autologous or homogenous. Human platelet releasate was chosen instead of bovine, as human blood samples were readily available in the laboratory, where they could be quickly processed and stored. Although this research is an indicator of how releasate affects oocytes and embryos, it cannot be ruled out that human platelet releasate elicits a different effect on bovine oocytes and embryos than autologous or homogenous platelet releasate would. Human platelet releasate may also not affect human oocytes and embryos in the way that it affects bovine oocytes and embryos, which is why further research is needed in humans. Recent work by Kim *et al.* (2022) found that human platelet-rich plasma was able to restore the uterine environment of mice with Asherman's Syndrome. Similarly to the research in this thesis, they aimed to better understand the underlying mechanism of action, so they could support future human autologous research.

4.8 Future Work

Future work would benefit from the inclusion of gene expression analysis of relevant genes. *SLC2A1* is a gene which encodes for a glucose transporter, GLUT-1 (Wu *et al.*, 2020). *SLC2A1* expression could influence glucose transport, as downregulation of this gene could result in a reduction in the expression of GLUT-1, potentially leading to a decreased capacity to consume glucose. However, if the decreased glucose consumption noted in this study is due to GLUT-1 receptors being blocked, rather than not expressed, it could cause an upregulation in *SLC2A1* expression, as the embryo may try to increase its expression of GLUT-1 to boost glucose uptake.

DNMT3A encodes a DNA methyltransferase, and is responsible for *de novo* epigenetic regulation of genes, knockout of this gene has been found to lead to embryo cleavage-stage arrest and degeneration (Uysal, Cinar and Can, 2021). It could be useful to determine if an altered expression of *DNMT3A* may be attributed to the reduced blastocyst rate/increased cleavage-stage arrest reported in this thesis.

Another relevant gene may be *BAX*, an apoptotic regulator. Under stress, *BAX* accumulates at the mitochondrial membrane instead of its normal movement into the cytosol (mediated by BCL2L1 – an antiapoptotic protein), causing an increased release of cytochrome C and ultimately apoptosis (Lindqvist and Vaux, 2014). Downregulation of *BCL2L1* and upregulation of *BAX* could potentially offer insight into why so many cleaved embryos in this study degenerated between days 2 and 7. A more holistic way to assess these, and other potential genomic impacts, would be to perform single cell RNA sequencing. This method would make it possible to analyse gene expression and quantify transcript levels in zygotes and embryos at various stages of development and with a high degree of sensitivity and resolution (Xue *et al.*, 2013).

Metabolic analysis of releasate-derived cleaved embryos on day 2 and day 4 would be advantageous as it could assist in answering how and when these embryos begin to differ metabolically from their FCS control counterparts. It would be interesting to see if there are any differences metabolically between releasate and FCS-derived cleaved embryos as well as at the blastocyst stage.

Studies into PRP's effect on human ovarian rejuvenation are ongoing, with 12 clinical trials listed globally (at the time of writing), with several recruiting or ongoing. Studies which have so far emerged have not addressed how the administration of PRP into the ovary affects oocyte maturation or embryo metabolic competence, for example. As a result of this study, it can be shown that direct exposure of oocytes to the products of activated platelets is detrimental to embryo development, and alters their ability to consume glucose at the blastocyst stage. Whilst acknowledging and appreciating the fertility benefit of ovarian rejuvenation for patients experiencing ovarian failure, this study highlights the importance of ovarian tissue being the site of activation and rejuvenation, as to preserve the developmental potential of oocytes and subsequent embryos.

Overall, this thesis has found that oocyte exposure to platelet releasate during *in vitro* maturation, has a detrimental effect on the subsequent embryos' potential to form blastocysts, as well as a limiting effect on the blastocysts' ability to consume glucose.

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