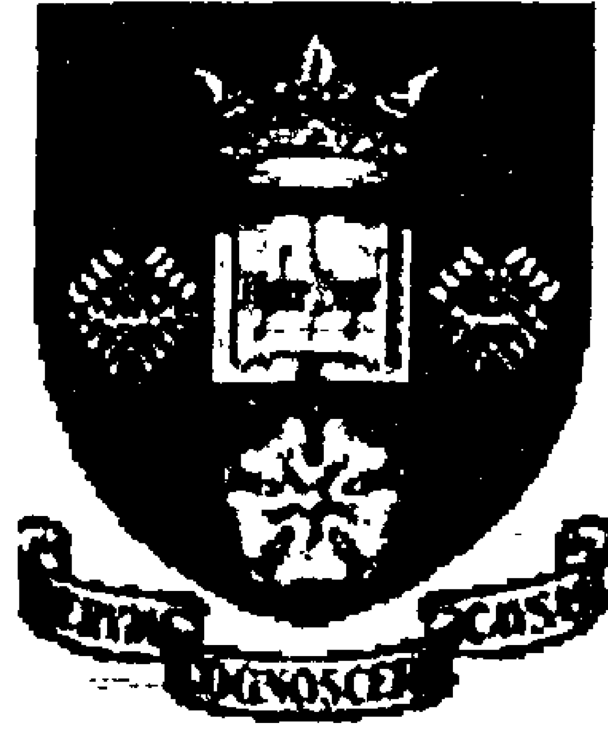

The University of Sheffield

The Interaction of Lipopolysaccharide with Human Spermatozoa: Particular Relevance to *Chlamydia trachomatis*

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Abbreviations

Ac-DEVD - N-acetyl-(Asp-Glu-Val-Asp)

AFC - 7-amino-4-trifluoromethylcoumarin

AIDS - acquired immunodeficiency syndrome

AIF - apoptosis-inducing factor

ANOVA - analysis of variance

AP-1 - activator protein-1

APAAP - alkaline phosphatase anti-alkaline phosphatase

APAF-1 - apoptosis protease-activating factor 1

AR - acrosome reaction

ASA - antisperm antibodies

ASGP-R - asialoglycoprotein receptor

BAD - Bcl-X_L/ Bcl-2-associated death promoter

BAX - Bcl-2 associated x protein

Bcl-2 - B cell leukaemia-2

BIR - baculoviral IAP repeat

CAD - caspase-activated DNase

CHX - cycloheximide

CRP - cysteine-rich proteins

DD- death domain

DED - death effector domain

DMSO - dimethyl sulfoxide

DOC - deoxycholate

EB - elementary body

EBSS - Earl's balanced salt solution

EF - endotoxin-free

ELISA - Enzyme Linked ImmunoSorbent Assay

EM - electron microscopy

EMEM - Minimum Essential Medium Eagle

EU - endotoxin unit

FADD - FAS-associated death domain

FITC - fluorescein isothiocyanate

GDW - glass distilled water

GlcN - glucosamine

GPI - glycosyl phosphotidylinositol

HBSS - Hanks' balanced salt solution

HEPES - N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]

Hib - *Haemophilus influenzae* type b

HLA-DR - human leukocyte antigen-DR

HOS - hypo-osmotic swelling test

HSP60 - heat shock protein 60

IAP- inhibitors of apoptosis

ICAD - CAD inhibitor

IF- γ - interferon-γ

IFU - inclusion forming units

IRAK - interleukin receptor-associated kinase

IVF - *in-vitro* fertilization

Kdo - 3-deoxy-D-manno-octulosonic acid

Ko - 2-keto-D-glycero-D-talo-octonic acid

LAL - Limulus Amebocyte Lysate

LAM - lipoarabinomannan

LBP - LPS-binding protein

LGV- lymphogranuloma venereum

LOS - lipooligosaccharide

LPO - lipid peroxidation

LPS - lipopolysaccharide

LTA - lipoteichoic acid

mAb - monoclonal antibody

mCD14 - membrane-bound CD14

MOMP - major outer-membrane protein

MoPn - mouse pneumonitis

MyD88 - myeloid differentiation factor 88

NF-kB - nuclear factor-kB

NGU - nongonococcal urethritis

OS - oxidative stress

PAGE - polyacrylamide gel electrophoresis

PAK2 - p21 (CDKN1A)-activated kinase 2

PAMP - pathogen-associated molecular pattern

PBS - phosphate buffer saline

PCD - programmed cell death

PCR- polymerase chain reaction

PG - peptidoglycan

PI - propidium iodide

PID - pelvic inflammatory disease

PMB - polymyxin B

PMN - polymorphonuclear

PROM - premature rupture of the membranes

PS - phosphatidylserine

PUFA - polyunsaturated fatty acid

RA - rheumatoid arthritis

RB - reticulate body

RFU - relative fluorescence unit

ROS - reactive oxygen species

RT – room temperature

RT-PCR - reverse transcription-polymerase chain reaction

sCD14 - soluble cluster of differentiation antigen 14

SDS - sodium dodecyl sulphate

SEM – standard error of mean

SFPD - Stills Fox Paster Dewhirst

SIF - spermatozoal immobilisation factor

SMAC - second mitochondria-derived activator of caspases

SOD - superoxide dismutase

2SP - 2-sucrose phosphate transport buffer

STD - sexually transmitted disease

STS - staurosporine

TB - tuberculosis

TBS - tris buffered saline

TEA - triethylamine

TEMED - N,N,N',N'-Tetramethylethylenediamine

TIR - Toll/ IL-1 receptor

TLR - Toll-like receptors

TMJ - temporomandibular joint

TNF-α - tumor necrosis factor-α

TNF-R1 - TNF-receptor 1

TNF-R2 - TNF-receptor 2

TRADD - TNFR-associated death domain

WHO- World Health Organization

ZP3 - zona pellucida glycoproteins 3

Publications and presentations from this work

Abstracts:

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Summary

Chlamydia trachomatis is an obligate intracellular bacterium which causes the most prevalent sexually transmitted bacterial infection throughout the world. According to the World Health Organization, at least 90 million chlamydial infections are detected annually worldwide. While the role of *C. trachomatis* in female infertility is well established, data revealing that this organism cause male infertility are still controversial.

In this study the effect of "home-made" *C.trachomatis* LPS and two LPS fractions, lipid A and Kdo on human spermatozoa were investigated in detail.

The effect of heated *C.trachomatis* elementary bodies (EBs) on the viability of spermatozoa and the potency of *C.trachomatis* LPS in comparison with three LPSs from the *Enterobacteriaceae* family were also studied. Treatment of spermatozoa with heated-EBs caused a significant increase in sperm death rate and revealed that the toxic component of EB was LPS which is a heat-stable complex. Incubation of spermatozoa with chlamydial LPS showed that this LPS at a concentration of 0.1 μg/ml induced significant death of spermatozoa, whereas LPSs from *E.coli*, *K.pneumoniae*, and *S. marcescens*, did so only at 50 μg/ml.

The effect of the two main LPS fractions of *C.trachomatis*, lipid A and Kdo, on spermatozoa was also investigated. A small amount of lipid A was isolated from chlamydial LPS and Kdo was purchased. Spermatozoa were treated with these fractions separately. Kdo was shown to be as spermicidal as lipid A, however, unlike lipid A, Kdo was not affected by anti-CD14 antibody or polymyxin B.

In an attempt to clarify the mechanism of interaction of chlamydial LPS with spermatozoa and the role of the TLR pathway and TNF-α production, an experimental model using HeLa and THP-1 cell lines was prepared. Because of a lack of a sufficient amount of chlamydial lipid A, lipid A like Kdo was also obtained commercially. While chlamydial LPS and commercial lipid A were able to signal via TLR2 and/or TLR4 in transfected HeLa cells and to induce TNF-α production in THP-1 cells, Kdo failed either to signal through TLRs or stimulate TNF-α secretion.

Finally, the role of chlamydial LPS, lipid A or Kdo in ROS production which could potentially induce sperm death, and the mechanism of sperm death induced by these fractions were also studied. In this investigation, spermatozoa were pre-incubated with five ROS scavengers; ascorbic acid, catalase, reduced glutathione, superoxide dismutase (SOD), and α-tocopherol, prior to treatment with the above-mentioned fractions. ROS scavengers used in this investigation neutralised the spermicidal activity of the fractions. It is suggested that these fractions possibly induce their pathogenic effect via ROS production. Results from an annexin V/PI binding assay and quantitation of caspase-3 activity in spermatozoa stimulated with chlamydial LPS, Kdo, and lipid A revealed that LPS and its fractions induced sperm apoptosis which was primarily caspase-mediated. In a hypothetical model which is shown in chapter 6, we illustrate how chlamydial LPS exerts its spermicidal activity which is via CD14, possibly TLRs, ROS production and finally apoptosis induction.

Chapter 1

General introduction

1.1 Historical aspects of chlamydial infections

The genus *Chlamydia* encompasses obligate intracellular bacteria, causing infections in a wide range of animals which contribute to sexually transmitted, ocular including trachoma, and respiratory tract infectious diseases in humans (Bavoil *et al.*, 2000). Trachoma is an ancient disease, described in ancient Chinese writing and in the Ebers papyrus of 1500 BC (Schachter, 1999). It ranks in the top three causes of blindness worldwide (Thylefors *et al.*, 1995). In 1907, the causative agent of trachoma was identified by Halberstaedter and von Prowazek who originally suggested the name Chlamydozoa, derived from the Greek chlamys meaning "mantle" describing the halo effect observed surrounding Giemsa stained inclusions. The contagious nature of the disease was recognised long before the cause was identified.

In 1910, Fritsh, and colleagues recognised the link between ocular and genital infections of *chlamydia* (Grayston and Wang, 1975). In 1911, Linder explained the similarity of lesions from the conjunctiva, trachoma cases and from conjunctival scrapings in monkeys inoculated with ocular and genital materials (Linder, 1911). Professor Tang Feifan was the first scientist who successfully isolated and cultivated *C. trachomatis* in 1946. Jones *et al.*, (1959) first recovered the organism from the cervix of an infected mother and the eyes of her infant. *C. trachomatis* was subsequently isolated from urethral secretions of men who had previous sexual contact with women with inclusion conjunctivitis, and from women diagnosed as having non-gonococcal genital tract infection (Jones, 1964; Holt *et al.*, 1967). Serotyping of *C. trachomatis* confirmed the specific association of some serovars with ocular and genital infections although the overlap for both ocular and genital infections was shown for serovars in both *C. trachomatis* biovars, LGV and trachoma (www.chlamydiae.com). In the 1970s the importance of *C. trachomatis* was established as a cause of non-gonococcal urethritis (Hilton *et al.*, 1974; Swanson *et al.*, 1975) and acute salpingitis and pelvic inflammatory disease (Mardh *et al.*, 1977; Punnomen *et al.*, 1979). It is estimated that over 600 million people are infected with *C. trachomatis* worldwide. (Gerbase *et al.*, 1998).

The recent completion of the "*Chlamydia* genome project" is one of the most important breakthroughs in chlamydial research that has provided workers with an insight to the genetic make-up of these bacteria (Rockey *et al.*, 2000).

1.2 Classification within the order *Chlamydiales*

Until recently the order *Chlamydiales* consisted of the single family *Chlamydiaceae* exclusively containing obligate intracellular bacteria. Based on comparative sequence analysis of different macromolecules, Everett *et al* (1999), reclassified these pathogens into nine species belonging to two different genera (*Chlamydophila* and *Chlamydia*) within the family *Chlamydiaceae*. The new classification provides appropriate groupings of chlamydial pathogens with distinctive underlying genetic relationships. In addition, three new families were created in the *Chlamydiales* to accommodate recently discovered chlamydia-like bacteria. Like *Chlamydiaceae*, chlamydia-like bacteria only grow in eukaryotic cells, have a similar developmental cycle, and have 16S and 23S rRNA that is at least 80% identical to that of the *Chlamydiaceae* (Everett *et al.*, 1999). The *Chlamydiales* currently comprises four families: *Chlamydiaceae, Para-chlamydiaceae, Simkaniaceae,* and *Waddliaceae* (Everett et al., 1999a; Rurangirwa *et al.*, 1999).

1.2.1 *Chlamydiaceae*:

All species are Gram negative and recognized by monoclonal antibodies (mAbs) that detect the LPS trisaccharide αKdo-(2 → 8)-αKdo-(2→4)-αKdo (Lobau *et al.*, 1995). Species within the *Chlamydiaceae* have 16S rRNA gene sequences that are > 90% identical (Pettersson *et al.*, 1997; Takahashi *et al.*, 1997). The family *Chlamydiaceae* is distinguished from *Simkaniaceae* by 16S and 23S rRNA sequence differences and by the absence of detectable *Chlamydiaceae*-specific Kdo trisaccharide antigen in '*Simkania*' using a family-specific mAb (Kahane *et al.*, 1999).

1.2.1.1 *Chlamydia trachomatis*:

Glycogen is easily detected in the human *C. trachomatis* serovars and is detectable to varying degrees in *C. muridarum* and *C. suis* (Everett *et al.*, 1999), but the absence of glycogen in inclusions is not a specific marker for *Chlamydophila* (Everett, 2000). Glycogen accumulation by *C. trachomatis* is readily detected by iodine staining of inclusions. The gene for the major outer-membrane protein (MOMP), *omp A (omp 1)*, is widely used to distinguish *C. trachomatis* strains. Many but not all, *C. trachomatis* strains have the extrachromosomal plasmid, pCT. *C. trachomatis* strains are generally sensitive to sulfadiazine and tetracyclines (Everett, 2000).

C. trachomatis has only been isolated from people and is comprised of two human biovars (18 serovars) that are transmitted by sexual or other contact: trachoma (14 serovars) and lymphogranuloma venereum (LGV, 4 serovars which can invade lymphatic tissue) (Batteiger, 1996; Moulder *et al.*, 1984). Serovars in both *C. trachomatis* biovars cause trachoma, sexually transmitted disease, some forms of arthritis, and neonatal inclusion conjunctivitis and pneumonia (www.chlamydiae.com), and are found in the temporomandibular joint (TMJ) of patients with TMJ disease (Henry *et al.*, 1999). The trachoma biovar currently has 14 serovars designated by the letters A through K, plus Ba, Da, and Ia. Infection is limited primarily to squamocolumnar cells of mucous membranes. Serovars A-C are primarily associated with endemic trachoma and serovars D-K with sexually transmitted infection. The LGV biovars consists of four serovars, L1, L2, L2a, and L3. LGV serovars are sexually transmitted and can invade lymphatic tissue. Antigenic analyses of MOMP and sequence analyses of the *ompA* gene that expresses MOMP suggest that L1 and L2 are closely related to the B- and E-trachoma serovars and that L3 is related to the A-, C- and H-trachoma serovars (Fitch *et al.*, 1993).

1.2.1.2 *Chlamydia suis*:

C. suis strains were previously referred to as *C. trachomatis* because of *ompA* DNA sequence homology. They have only been isolated from swine, where they cause conjunctivitis, enteritis, pneumonia, and a high incidence of apparently

asymptomatic infection. Some strains are resistant to sulfadiazine and/or tetracycline. Several strains of *C. suis* have an extrachromosomal plasmid, pCS (Everett, 2000).

1.2.1.3 *Chlamydia muridarum*:

Two strains of *C. muridarum*, mouse pneumonitis (MoPn) and hamster proliferative ileitis, also known as Stills Fox Paster Dewhirst (SFPD), have been isolated from mice and hamsters, respectively, and previously belonged to *C. trachomatis* (Everett, 2000). MoPn infection may be asymptomatic or produce pneumonia in mice. MoPn is sensitive to sulfadiazine, and its glycogen production is difficult to detect. The MoPn genome has been sequenced (Read *et al.*, 2000) and this strain has an extrachromosomal plasmid, pMoPn.

1.2.1.4 *Chlamydophila psittaci*:

C. psittaci primarily infects birds. *C. psittaci* has eight known serovars using serovar-specific monoclonal antibodies (Andersen, 1991; Varompay *et al,*. 1993) and *ompA* sequence differences (Everett *et al.*, 1999). All should be considered to be transmissible to humans. Serovar A is endemic among psittacine birds and has caused sporadic zoonotic disease in humans, other mammals, and tortoises. Serovar B is endemic among pigeons, has been isolated from turkeys, and has also been identified as the cause of abortion in a dairy herd. Serovars C and D are occupational hazards for slaughterhouse workers and for people in contact with birds. Serovar E isolates have been obtained from a variety of human or avian hosts worldwide. Two other serovars were isolated during outbreaks in mammals (Everett, 2000). *C. psittaci* strains did not accumulate glycogen and were usually resistant to sulfadiazine. Several *C. psittaci* strains have an extrachromosomal plasmid (Everett *et al.*, 1999).

1.2.1.5 *Chlamydophila pneumoniae*:

C. pneumoniae has three biovars, TWAR which infects humans, Koala, and Equine. The species is responsible for approximately one in ten cases of atypical pneumonia in developed countries (Grayston, 1999). The name TWAR was formed

by a combination of the first two letters of isolates TW-183 and AR-39 (Grayston *et al.*, 1989). TWAR is primarily a respiratory pathogen, and has been associated with obstructive pulmonary disease, and other acute and chronic respiratory diseases. In addition it has also been associated with non-respiratory diseases such as atherosclerosis, and Alzheimer's (Everett, 2000).

1.2.1.6 *Chlamydophila pecorum*:

C. pecorum strains are serologically and pathogenically diverse, having been isolated only from mammals: cattle, sheep and goats (ruminants), koalas (marsupials), and swine. They are generally non-invasive in a mouse model of virulence. In koalas, *C. pecorum* causes reproductive disease, infertility and urinary tract disease. In other animals, *C. pecorum* has been associated with abortion, conjunctivitis, encephalomyelitis, enteritis, pneumonia and polyarthritis (Everett, 2000).

1.2.1.7 *Chlamydophila felis*:

C. felis is endemic among house cats worldwide, primarily causing conjunctival inflammation, rhinitis, and respiratory problems. It can be recovered from the stomach and reproductive tract. Zoonotic infection of humans with *C. felis* has been reported. Strains differ in pathogenesis and, although some have an extrachromosomal plasmid, the causes of pathogenic differences are not known (Everett, 2000).

1.2.1.8 *Chlamydophila caviae*:

C. caviae can be recovered from the conjunctiva of guinea pigs suffering from ocular inflammation and eye discharge. All known isolates have identical *ompA* sequences. The strain GPIC (Guinea Pig Inclusion Conjunctivitis) contains an extrachromosomal plasmid. *C. caviae* infects primarily the mucosal epithelium and is not invasive. *C. caviae* is markedly specific for guinea pigs (Everett, 2000).

1.2.1.9 *Chlamydophila abortus*:

C. abortus strains efficiently colonize the placenta and are primarily associated with cases of abortion and weak neonates. By producing spontaneous abortion, stillbirth, or delivery of weak lambs, it is a major cause of reproductive failure in most sheep-rearing countries and, consequently, a serious economic problem (Schlossberg, 1995). *C. abortus* is endemic among ruminants and has been isolated from sheep, cattle, and goats worldwide as well as in association with cases of abortion in a horse, a rabbit, guinea pigs, mice, and pigs. It was first isolated from products of a septic human abortion in 1967 (Roberts *et al.*, 1967). Women who work with sheep have suffered sporadic, documented cases of zoonotic abortion due to *C. abortus* (Everett, 2000).

1.2.2 *Parachlamydiaceae*:

The *Parachlamydiaceae* naturally infect amoebae, specifically *Acanthamoeba* and *Hartmanella*, and one isolate will grow in *Dictyostelium*. This family comprises two genera, of which the type strains are *Parachlamydia acanthamoebae* (Amann *et al*, 1997) and *Neochlamydia hartmanellae* (Horn *et al*, 2000). EBs of *Parachlamydia acanthamoebae* have a Gram positive cell wall structure using electron microscopy (EM). Isolates have been obtained from clinical and environmental sources, including nasal swabs, humidifier water, and water in a dental unit. Trophozoites of *Acanthamoeba* hosting *Parachlamydiaceae* strains were isolated from humans in an outbreak of humidifier fever in Vermont, USA ('Hall's coccus') and also from asymptomatic women in Germany (Everett, 2000). The *Parachlamydiaceae* are not recognized by monoclonal antibodies specific for the antigenic LPS trisaccharide αKdo-(2 → 8)-αKdo-(2→4)αKdo of the *Chlamydiaceae* (Everett *et al.*, 1999).

1.2.3 *Simkaniaceae*:

Simkaniaceae currently includes only the type strain *Simkania negevensis Z*. Originally found as a bacterial contaminant in cell cultures, PCR and serology indicate that *S. negevensis* is associated with pneumonia in humans. Strain Z does not contain an extrachromosomal plasmid and has an unusually slow developmental

cycle (12-14 days) in cultured Vero cells (African green monkey kidney cell line), compared to other *Chlamydiales* (Everett, 2000). The *S. negevensis* full-length 16S and 23S rRNA sequences are each 80–87% identical to those of members of the *Chlamydiaceae*, whereas all members of the *Chlamydiaceae* have > 90% identity with each other (Friedman *et al.*, 2003). *S. negevensis* does not contain the LPS antigen common to all members of the *Chlamydiaceae*, neither is it recognized by monoclonal antibodies to the chlamydial major outer-membrane proteins nor by primers to the *ompA* gene (Kahane *et al.*, 1999).

1.2.4 *Waddliaceae*:

The species *Waddlia chondrophila* currently includes only the type strain, *W. chondrophila* WSU 86-1044 (Everett, 2000). WSU 86-1044 was isolated from pooled lung and liver tissues of an aborted bovine foetus (Dilbeck *et al.*, 1990). No case of human infection by *W. chondrophila* has been reported, but *Waddlia* - related phylotypes have been identified by PCR in human samples (Corsaro *et al.*, 2002).

1.3 Developmental cycle of *Chlamydiae*

The chlamydial developmental cycle consists of infectious and non-infectious stages that exhibit unique morphological, biochemical, and biological properties (Belland *et al.*, 2003) (Figure 1. 1). The infectious elementary bodies (EB) are metabolically inactive particles and resistant to a variety of physical insults, *e.g.*, sonication, and variation of osmotic pressure. They are 0.2 to 0.5 μm in diameter, with a rigid, disulfide cross-linked outer membrane (OM) (Bavoil *et al.*, 1984; Hatch *et al.*, 1986) that enables the EB to attach to and enter host cells (Stephens *et al.*, 2001). Attachment of EBs to the mucosal epithelial surfaces of the host cells is the first step in the infectious process. At the very early stage of infection (1-3 h) the parasite exerts profound effects on the host. Through an unknown mechanism, dependent on both bacterial transcription and translation (Scidmore *et al.*, 1996a), *Chlamydia* modifies the properties of the phagosome and prevent its entry into the lysosomal pathway (Scidmore *et al.*, 1996b). After host cell entry, the EB is localized

to a phagosome, and the primary differentiation process is initiated. This developmental process involves the commencement of bacterial metabolism and the conversion of the EB to the intracellular replicating form of the organism, termed the reticulate body (RB) (Belland *et al.*, 2003). RBs are metabolically active, sensitive to environmental variations (Bavoil *et al.*, 1984), 0.5 to 1.5 μm in diameter, take up nutrients from the host cell, and undergo multiple rounds of binary division within the inclusion membrane for a period of 24-36 h (Everett, 2000). After division, the RB undergoes a secondary differentiation process back to an infectious EB. Entry of infective forms (EBs) into host cells and their differentiation into reproductive forms (RBs) is completed by 4 to 12 h postinfection. By 20 h postinfection, some of the RBs cease to divide and commence transformation to EBs; other RBs in the population continue to divide for up to 72 h after infection. Most of the infected cells lyse by 48 to 72 h, releasing a population of *Chlamydia* consisting mainly of EBs. The conversion of EBs to RBs and RBs to EBs is gradual and continuous (Hatch *et al.*, 1986). The cycle is accomplished in 36 to 96 h, depending on the species (Corsaro *et al.*, 2003). Some exceptions are *Simkania negevensis*, which has *in vitro* a life cycle of about 2 weeks with a stationary phase and with RBs that seemly retain infectious ability, and *Neochlamydia hartmannellae*, which has been described as living free in the ameobal cytoplasm (Kahane *et al.*, 1999, and 2002). EBs and RBs are generally Gram negative and have inner and outer membranes with a variable periplasmic space (Everett, 2000). In contrast to the envelopes of Gram-negative bacteria, the envelopes of both forms of *Chlamydia* lack or contain very little muramic acid (Barbour *et al.*, 1982; Garrett *et al.*, 1974). Nonetheless, the possibility that *Chlamydia* possess peptidoglycan cannot be dismissed in that penicillin alters chlamydial growth by inducing the formation of swollen, abnormal RB forms (Barbour *et al.*, 1982) and also bioinformatic analysis of the *C. trachomatis* genomes reveals a complete set of genes whose products are apparently capable of synthesizing peptidoglycan in these organism (Chopra *et al.*, 1998; Kalman *et al.*, 1999). Tamura and Manire (1967) reported that EB envelopes have a higher phospholipid content than do RB envelopes and that RB envelopes lack both cysteine and methionine. Failing to detect muramic acid in *Chlamydophila psittaci* cell walls and noting that osmotically fragile RB envelopes lack cysteine, these workers

suggested that S-S bonding might play a role in the formation of rigid membranes of EBs. Observations based on metabolic studies independently support the view that formation of cysteine-containing structure is essential in RB-EB transformation (Hatch *et al.*, 1983).

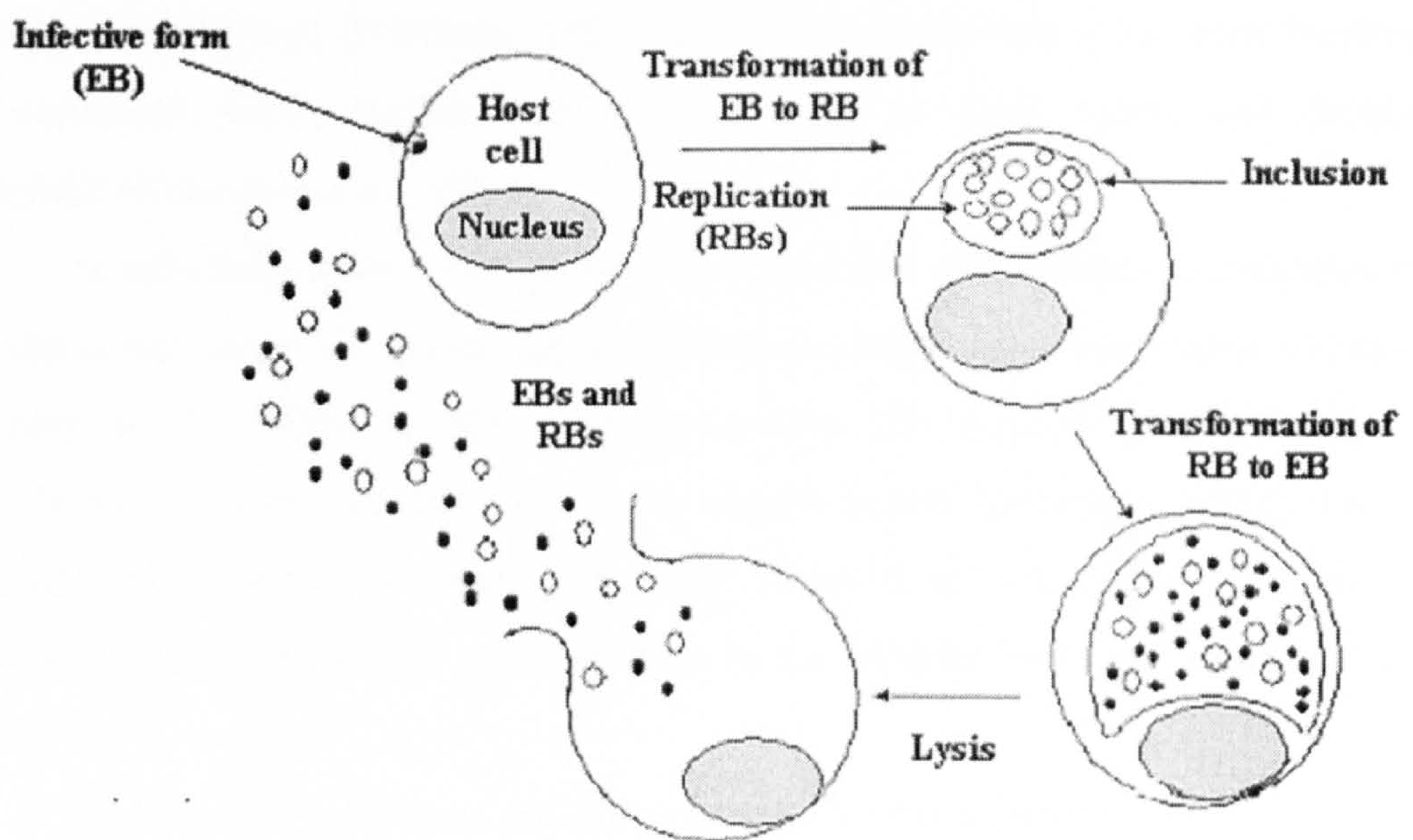


Figure 1. 1. The *Chlamydiales* developmental cycle of infection and replication.

1.4 *C. trachomatis* infections

1.4.1 Epidemiology

The role of *C. trachomatis* as an important sexually transmitted disease (STD) agent is well recognized. Infection with this organism is the most prevalent sexually transmitted bacterial disease in the world (Gonzales *et al.*, 2004). The World Health Organization estimated that 92 million new cases of *C. trachomatis* infection occurred throughout the world in 1999 (WHO, 2001). Every year, an estimated four million new cases occur in the United States and three million in Europe (Peeling and Brunham, 1996). Moreover, infections with these bacteria are an important public health problem, especially in third world and developing countries (Gonzales *et al.*, 2004).

In addition to the STDs caused by *C. trachomatis,* the ocular serovars of this organism can cause trachoma that is the most common infectious cause of blindness (Mabey *et al.*, 2003). It affects approximately 150 million people living in the world's poorest and rural communities (Kumaresan and Mecaskey, 2003). The WHO estimates that trachoma remains endemic in 48 countries including many of the countries of Africa as well as focal areas in the Middle East, and south and central Asia.

Chlamydial infections are also acquired by the newborn during delivery and might cause conjunctivitis and pneumonia in these children. Considering that, there is a high need for correct, quick and cost-effective diagnosis and treatment of these oculourogenital infections.

Chlamydia are transmitted by sexual intercourse of an infected partner of a sexually active couple (Eggert-Kruse et al., 1997), mother to child at delivery (Fenton, 2000), family cluster infections (Thompson *et al.*, 2001) and by extragenital infection (mechanical deposition, flies, dirty fingers, towels and bad hygienic conditions) (Emerson *et al.*, 1999). Therefore, people with a higher number of sexual partners, direct nonsexual family contamination, offspring of infected couples, personnel who deal with patients at hospitals and clinics, and clinical laboratory technicians would be at greater risk of infection (Gonzales *et al.*, 2004).

1.4.2 Trachoma

Trachoma is a chronic keratoconjunctivitis caused by repeated reinfection with the ocular serovars A, B, Ba, and C of *C. trachomatis*. After an incubation period of 5–10 days, infection with these ocular serovars causes a mild mucopurulent conjunctivitis, which is generally self-limiting and heals without permanent sequelae (Mabey *et al.*, 2003). Repeated infections with the ocular strains result in chronic inflammation of the tarsal conjunctiva of the upper lid. The chronic condition results in scarring, shortening of the upper lid with in-turning of the eyelashes—ie, trichiasis. The painful abrading of the cornea, if not corrected, results in corneal scarring, opacity, and blindness. Women are blinded two to three times as often as men probably due to increased exposure and infection as a result of child rearing (Congdon *et al.*, 1993).

Trachoma is spread from eye to eye in endemic communities by fingers, shared clothing or towels, and probably by fluid-seeking flies in the poor, dry, and dusty areas where it is so often found. Transmission of the infection seems to require close personal contact and therefore the disease commonly clusters in families and villages (West *et al.*, 1991). The prevalence of active trachoma is highest in children, peaking between 2 and 5 years of age. Prevalence rates of 20% in children are common in endemic areas and may rise to 90% in severely affected communities (Mecaskey *et al.*, 2003).

1.4.3 Adult inclusion conjunctivitis

In both the male and the female, *C. trachomatis* causes inclusion conjunctivitis, characterized by conjunctival hyperemia, mucopurulent discharge, and lymphoid follicle formation (Garland *et al.*, 1995). Infection is thought to occur from infected genital tract secretions via hand-to-eye contact. The illness is self-limiting, although treatment will shorten the clinical course. Progression to trachoma has not been documented, but pannus and conjunctival scarring may result from reinfection (Schachter, 1999).

1.4.4 Chlamydial infections in perinatal and neonatal periods

Maternal *C.trachomatis* infections during pregnancy may cause a variety of perinatal and neonatal complications. *C.trachomatis* may lead to abortion, perinatal or neonatal death, and low birth weight (Witkin, 1995; Andrews *et al.*, 2000).

Although transmission of the organism from mothers to their infants generally occurs at the time of delivery with passage of the infant through the infected endocervix, the possibility of intrauterine infection at late pregnancy has also been reported (Nnumazaki and Niida, 2000). An infant born to a mother with active infection has a risk of acquiring infection at any anatomical site of 50 to 75%. Approximately 30 to 50% of infants born to chlamydia-positive mothers will have conjunctivitis, and at least 50% of infants with chlamydia conjunctivitis will also have nasopharyngeal infection. Chlamydial pneumonia develops in about 30% of infants with nasopharyngeal infection (Stamm, 1999). Pneumonia due to *C.trachomatis* is a disease limited for the most part, to infants less than 6 months of age (Numazaki *et al.*, 2003).

1.4.5 Urogenital infections in men and women

Urogenital infections with *C.trachomatis* in women have a clinical course varying from asymptomatic infections to ascending infections leading to pelvic inflammatory disease (PID) associated with late ectopic pregnancy and tubal infertility (Lan *et al.*, 1995). As many as 70 to 80% and 50% of *C.trachomatis* infections in women and men are asymptomatic respectively (Gonzales *et al.*, 2004) and these infections can persist for several months. Despite the frequent absence of symptoms, at least one third of women have local signs of infection on examination (Stamm, 1999). The two most commonly reported signs are mucopurulant discharge from the cervix and hypertrophic cervical ectopy.

Clinical manifestations of *C.trachomatis* infections in women include acute urethral syndrome, urethritis, bartholinitis, cervicitis, upper genital tract infection [endometritis, salpingo-oophoritis, or pelvic inflammatory disease (PID)], perihepatitis (Fitz-Hugh-Curtis syndrome), and reactive arthritis (Stamm, 1999). Infection of the urethra and lower genital tract may cause dysuria, abnormal vaginal

discharge, or postcoital bleeding, whereas infection of the upper genital tract may be manifested as irregular uterine bleeding and abdominal or pelvic discomfort.

McGregor *et al,* (1995) suggested an association between colonization of the genital tract by *C.trachomatis* and an increased risk of premature rupture of the membranes (PROM). Ectopic pregnancy, stillbirth, early pregnancy loss or recurrent pregnancy loss may be among consequences caused by asymptomatic *C.trachomatis* infections (Gravett *et al.*, 1986; Vigil *et al.*, 2002a).

Clinical signs and symptoms of genital chlamydial infections in men include urethral discharge of mucopurulent or purulent material, dysuria, or urethral pruritus (Peipert, 2003). Nongonococcal urethritis (NGU) is the most common clinical manifestation of *C. trachomatis* infection in men. In fact, *C. trachomatis* causes approximately 35 to 50% of all cases of NGU in heterosexual men. Symptoms of NGU may develop after an incubation period of 7 to 21 days and include dysuria and mild-to-moderate whitish or clear urethral discharge. In most cases, physical examination reveals no abnormalities other than the discharge. Using clinical and pathological evidence, Gonzales *et al.*, (2004) suggested that there is a relationship between *C. trachomatis* and urethritis, urethritis and epididymo-orchitis, and epididymo-orchitis and infertility.

In homosexual men, the rectum is the common site of infection by *C. trachomatis.* These infections are generally asymptomatic too (Gonzales *et al.*, 2004). Chlamydial infections are also responsible for 70-80% of post-gononococcal urethritis. Other clinical syndromes in men include acute epididymitis, acute proctitis, acute proctocolitis, conjunctivitis, and Reiter's syndrome. Male infertility, chronic prostatitis, and urethral strictures are possible results of infection. Both Reiter's syndrome (urethritis, conjunctivitis, arthritis, and mucocutaneous lesions) and reactive tenosynovitis or arthritis (without the other components of Reiter's syndrome) have been associated with genital *C. trachomatis* infection (Stamm, 1999).

1.4.6 *C.trachomatis* and its effect on female and male reproduction

In women, untreated chlamydial infection can lead to severe reproductive complications. *C. trachomatis* is an important causal agent in PID, with sequelae

including infertility (Westrom *et al.*, 1992). Up to two thirds of cases of tubal-factor infertility may be attributed to *C. trachomatis* infection (Paavonen and Eggert-Kruse 1999). Women with previous chlamydial infections are four times more likely to have obstructed Fallopian tubes compared with women who had no infection. Besides the damage to tubal mucosa and smooth muscle, chlamydial infections cause tubal adhesion and obstruction, and therefore impair the function of the fimbriae and lead to infertility (Nalbanski *et al.*, 1999; Barlow *et al.*, 2001).

Data revealing that *C. trachomatis* cause male infertility are still conflicting. Some reports suggest that chlamydial infections probably do not significantly contribute to male infertility. Bjercke and Purvis, (1992) found no clear association between the presence of *C. trachomatis*-IgA antibodies in the seminal plasma of infertile patients and sperm quality. Eggert-Kruse *et al*, (1997) also showed that *C. trachomatis* antibodies in semen did not influence male fertility in terms of sperm parameters and pregnancy rate in asymptomatic infertile couples and Rezacova *et al.*, (1999) were unable to demonstrate the presence of chlamydia in the ejaculate by a DNA amplification method. However, some workers found that chlamydial infections are related to male infertility. Witkin *et al.*, (1995) showed that men with seminal antichlamydial IgA had a lower median sperm count than those without antichlamydial IgA. They also suggested that antisperm antibodies (ASA) were more associated to antichlamydial IgA in semen than seminal IgG antibodies to *C.trachomatis,* or circulating antichlamydial IgA and IgG antibodies in asymptomatic male partners of infertile couples with no history of exposure to *C. trachomatis.* The presence of heat shock protein 60 (HSP60) in semen correlated with the occurrence of antichlamydial antibodies and ASA (Munoz *et al.*, 1996). Antibody to HSP60 may indicate long-standing infection with persistent chlamydial antigen (Paavonen and Eggert-Kruse, 1999). In addition, Radouani *et al.*, (1996) observed that azoospermia was more frequently seen in patients who had antichlamydial antibodies and Penna Videau *et al.*, (2001) found a strong association between antichlamydial antibodies in semen and a past history of STD.

Galdiero *et al.*, (1994) suggested that *C. trachomatis* probably influences sperm function. They showed that *C. trachomatis* LPS can decrease the percentage of vital spermatozoa *in vitro.* Similar experiments were reported by Hosseinzadeh *et*

al., (2001). *In vitro*, they observed a significant decline of sperm motility during incubation with EBs of serovar E. Furthermore, using electron microscopy, Erbengi, (1993) demonstrated the presence of EB and RB forms of *C. trachomatis* in spermatozoa. EBs of *Chlamydia* were also demonstrated in connective tissue from testes and in Leydig cells (Villegas et al., 1991).

In spite of these controversial data, it is of tremendous importance to remember that the female partners carry the main risk of male chlamydial infection of the genital organs, as *C. trachomatis* infection of the female genital organs may be deleterious to female fertility. Purvis and Christiansen (1993) pointed out that an important aspect of *C.trachomatis* infection in men may be that the male accessory sex glands function as reservoirs for the organism, increasing the probability of infection in the female. Therefore, it is necessary to determine markers for the function of the prostate, i.e. zinc and citric acid. Eggert- Kruse *et al.*, (2002) demonstrated that the zinc level in seminal fluid is neither associated with silent male genital tract infections, nor related to semen cultures in asymptomatic individuals however, Wolff *et al.*, (1991) showed that citric acid is decreased in male infertility patients indicating possible inflammatory damage of the prostate induced by *C.trachomatis.*

1.4.7 Lymphogranuloma venereum (LGV)

LGV is a rare form of the STD caused by serovars L1, L2, L2a and L3 of *C. trachomatis* (Lynch *et al.*, 1999). This disease is rare in industrialised countries, but is endemic in parts of Africa, Asia, South America, and the Caribbean. The LGV serovars infect predominantly monocytes and macrophages, pass through the epithelial surface to regional lymph nodes, and may cause disseminated infection. The clinical course of LGV can be divided into three stages. The first stage corresponds to a primary genital lesion at the site of inoculation (usually the prepuce or glans in men, and the vulva, vaginal wall or, occasionally, cervix in women), usually a small papule or a herpetiform ulcer, which often remains unnoticed. LGV is most often recognized at the second stage, which corresponds to a local lymphadenopathy (most often inguinal), which may evolve to a local abscess (or bubo), which ruptures in approximately 30% of cases, and fistulizes to the skin. This

stage may include systemic manifestations, such as fever, headaches, and myalgia. The third stage corresponds to late fistula and stricture formation (Maurin and Raoult, 2000).

1.5 Chemical structure of the cell envelope of *C.trachomatis*

1.5.1 General characteristics of the chlamydial envelope

The cell envelopes of both the EB and RB forms resemble those of Gram-negative bacteria, consisting of an outer membrane and an inner membrane (Tamura *et al.,* 1971). While the chlamydiae share a common structure with other Gram-negative bacteria, there are some unique features. Perhaps the most striking feature of the chlamydial envelope is its apparent lack of peptidoglycan (PG), although chlamydiae contain genes that appear to encode products with PG biosynthetic activity (Hesse *et al.,* 2003), possess three penicillin-binding proteins (Barbour *et al.,* 1982) and are sensitive to drugs that inhibit PG synthesis, such as penicillin G and D-cycloserine. These drugs interrupt normal development, giving rise to abnormally large RBs that are unable to divide by binary fission (Raulston, 1995). The conclusion that chlamydiae lack PG is based largely on the failure to detect muramic acid. Supporting observations include the failure to detect a PG layer by electron microscopy and the failure of antibodies directed against PG to react with chlamydiae (Barbour *et al.,* 1982). Secondly, a predominance of cysteine-rich envelope proteins, which are cross-linked by disulphide bonding in EBs provide a supramolecular structure that may be responsible for the osmotic stability of EBs. This structure is not found in osmotically fragile RBs (Hatch, 1996). Hackstadt *et al.,* (1985) showed that EBs are lysed when incubated for 30 min in the presence of dithiothreitol (that quantitatively reduces disulphides) and possibly this observation is the best evidence that disulphide bonds play a role in maintaining the structural integrity of EBs. Lastly, there are unique surface appendages, termed 'projections'.

The chlamydiae have neither pili nor flagella but do possess unique macromolecular surface projections. These straight protrusions, approximately 18-22 per EB, are clustered hexagonally on the surface and a radical nine component 'rosette' surrounds each projection in the outer membrane (Matsumoto, 1982). These

structures are also present on RBs, but in a less-ordered array, and have been shown by electron microscopy to be in contact with the inclusion membrane (Raulston, 1995).

1.5.2 The structural proteins in the chlamydial envelope

The envelope of EBs consists of disulphide-bond-cross-linked MOMP in the outer membrane and cross-linked cysteine-rich proteins (CRPs) in the periplasmic space (Tanzer and Hatch, 2001) (Figure 1. 2). MOMP (40 kDa protein) that is encoded by *omp1*, constitutes nearly 60% of the total outer membrane protein, exhibits potential porin function (Bavoil *et al.,* 1984), and may play a role in the attachment of *C.trachomatis* to host cells (Su and Caldwell, 1991). Although, the overall quantity of MOMP in the RB is similar to that found in EBs, the MOMP of RBs is predominantly in a monomeric form. Dimeric, trimeric and multimeric complexes of MOMP have been identified in EBs (Newhall and Jones, 1983).

Large CRP (60 kDa, omcB) and small CRP (12 kDa, omcA) are encoded by *omp2* and *omp3* respectively (Raulston, 1995). Studies by Newhall, (1987) and Hatch *et al.,* (1986) showed that RBs are deficient in CRPs and that they are found in the cell envelope of intracellular chlamydiae only late in the cycle, starting at 18 to 24 h postinfection, when RB multiplication has decreased and they have begun to turn back to EBs. The property of being resistant to mechanical and osmotic stress is attributed to inter-and intramolecular disulphide cross-linking of the EB CRPs (Raulston, 1995).

After EBs have entered the phagosome, one of the initial steps leading to the formation of RBs could be the cleavage of disulphide bonds in the reducing environment of the phagosome (Brade *et al.,* 1997).

Miyashita *et al.,* (1993) reported that the periplasmic protein layer (P-layer) is in close association with the outer membrane in chlamydiae. The P-layer was observed by these workers like a regular array of hexagonally packed subunits on the inner surface of the outer membrane of EBs. As the P-layer may consist entirely of cross-linked large CRP, Matsumoto and Manire, (1970) suggested that RBs lack this layer.

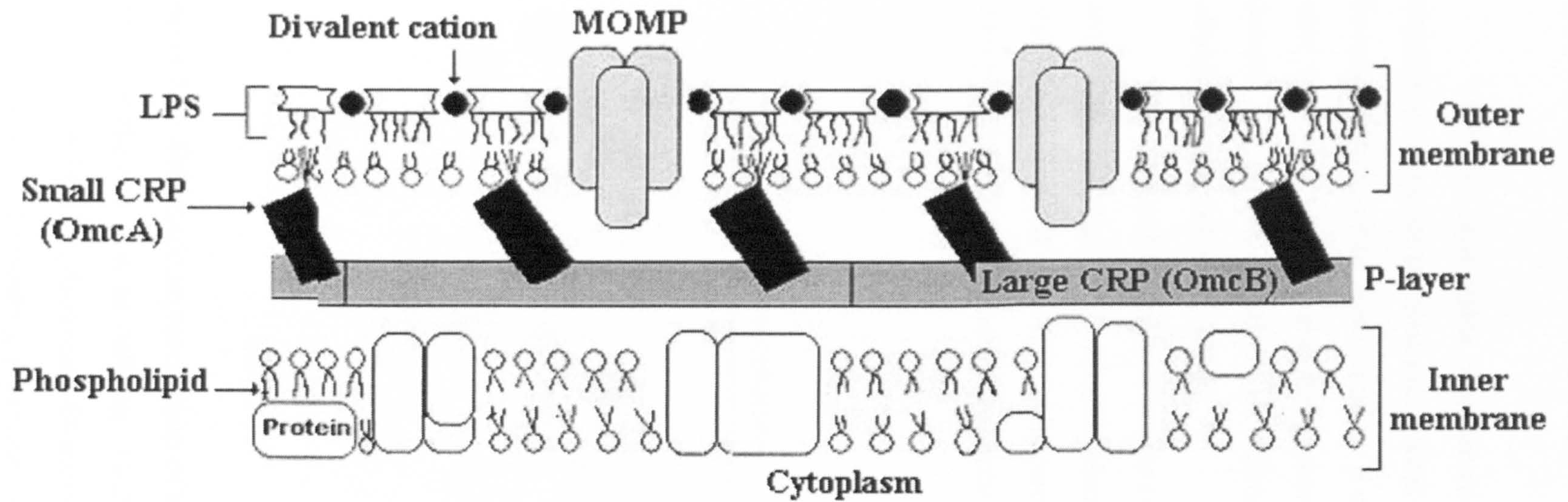


Figure 1. 2. Hypothetical model of the envelope of chlamydial EBs.

1.5.3 Chemical structure of LPS

LPS is a heat-stable complex and unique glycolipid that seems to be present in all Gram-negative bacteria and is vital to both structural and functional integrity of the outer membrane of these bacteria. It covers nearly 75% of the outer surface of the outer membrane. LPS is also the immunodominant antigen of most Gram-negative bacteria and is considered to be intimately associated with the virulence of the pathogen (Rietschel *et al.*, 1994).

The LPS of *Enterobacteriaceae* consists of three general parts (Figure 1. 3): a variable region called the O-specific chain (repeating oligosaccharides, O-antigen), a relatively conserved core region (heterooligosaccharide), and a conserved lipid A (highly acylated and phsphorylated glucosamine disaccharide) (Erridge *et al.*, 2002).

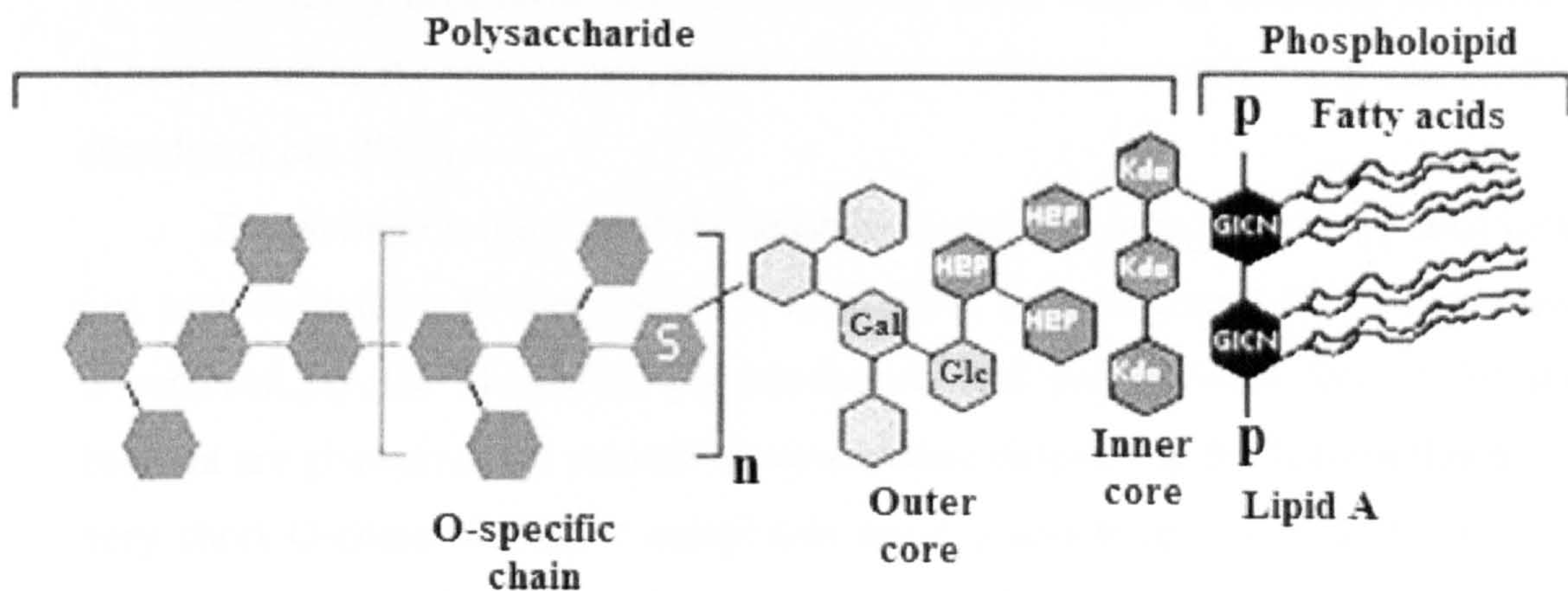


Figure 1. 3. Schematic representation of the general structure of LPS (Smooth form). The abbreviations are as follows: GlcN, glucosamine; Kdo, 3-deoxy-D-manno-octulosonic acid; Hep, L-glycero-D-manno-heptose, Glc, glucose; Gal, galactose; S, sugar; n, number of repeating sugar units within the O-antigen.

1.5.3.1 O-polysaccharide

A polymer of repeating saccharide subunits in the outermost part of the LPS called O-polysaccharide, or O-chain, is typically composed of common hexoses and is therefore the major antigen targeted by host antibody responses. These responses can be highly O-chain specific, and for this reason the O-chain is often also referred to as the O-antigen.

Not all LPSs have a long repeating O-chain structure. Some bacteria express only saccharide joined to a short non-repeating polysaccharide unit. These LPSs, such as those from *Neisseria meningitidis* and *N.gonorrhoeae* have been termed 'lipo-oligosaccharide' (LOS) to differentiate them. The repeating units of the O-polysaccharide region consist of between one and eight glycosyl residues and differ between strains by the sugars, sequence, chemical linkage, substitution, and ring forms utilized. This leads to an almost limitless diversity of O-chain structure. In addition, the number of subunits used to complete the chain varies between 0 and ~50 (Erridge *et al.,* 2002).

For many organisms (*e.g., Salmonella*), the O-chain is essential for survival in host serum as it prevents penetration of the complement membrane attack complex (Erridge *et al.*, 2002).

Depending on the size of the saccharide portion, there are two types of LPSs, i.e., smooth- and rough-form (S-and R-form) LPS. In the S-form LPS the core region is replaced by the O-specific polysaccharide and the colonies formed by such bacteria are glistening and smooth however, these colonies in the R-form that have a very short O-chain or lack it completely are dry and rough. Both LPS forms are found in wild-type Gram-negative bacteria: the S-form, for example, in *Escherichia coli, Vibrio cholerae*, or *Klebsiella pneumoniae*, and the R-form in *Neisseria meningitidis, N.gonorrhoeae*, and *C.trachomatis.*

1.5.3.2 Core polysaccharide

Although the O-chain region is extremely variable, the oligosaccharide structures in the core part are much more limited, with some regions being highly conserved between different strains and species. The outer core consists of common

hexose sugars such as glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine and is generally more variable than the inner core.

The inner core is characterized by more unusual sugars, particularly Kdo and heptose. Kdo (Figure1. 4) is found in almost every LPS and is attached to the carbohydrate backbone of lipid A. The only exceptions yet seen to this rule are *Acinetobacter* and *Burkholderia cepacia* which employ the alternative 2-keto-D-glycero-D-talo-octonic acid (Ko) in its place. Although the O-chain and the majority of the core can be dispensed with in some viable mutants, Kdo is always required for bacterial viability and along with lipid A is the minimal structure compatible with bacterial viability. Helander *et al.,* (1988) and Brade *et al.,* (1987) respectively showed that the smallest saccharide component seen in the LPS of any organism is in that of a deep rough mutant of *Haemophilus influenzae* which has only one Kdo residue attached to its lipid A, and the *Chlamydia* spp LPS with the smallest core yet seen which consists of only a triplet of Kdo units. These findings confirm the essential role of Kdo in bacterial viability. Kdo has also been reported to act as the immunodominant sugar for the enterobacterial Re specificity (Luderitz *et al.,* 1966). Both inner and outer core sugar residues can be substituted with a charged group like phosphate, pyrophosphate, 2-aminoethylphosphate and 2-aminoethylpyrophosphate. Rietschel *et al.,* (1994) reported that these substituents in the inner core maintain a close association with the Ca^{2+} and Mg^{2+} ions that are required for membrane structure and function. While the endotoxically active part of LPS is lipid A, the nature and number of attached saccharide residues and substituents do have considerable impact on modulation of this activity (Erridge *et al.,* 2002).

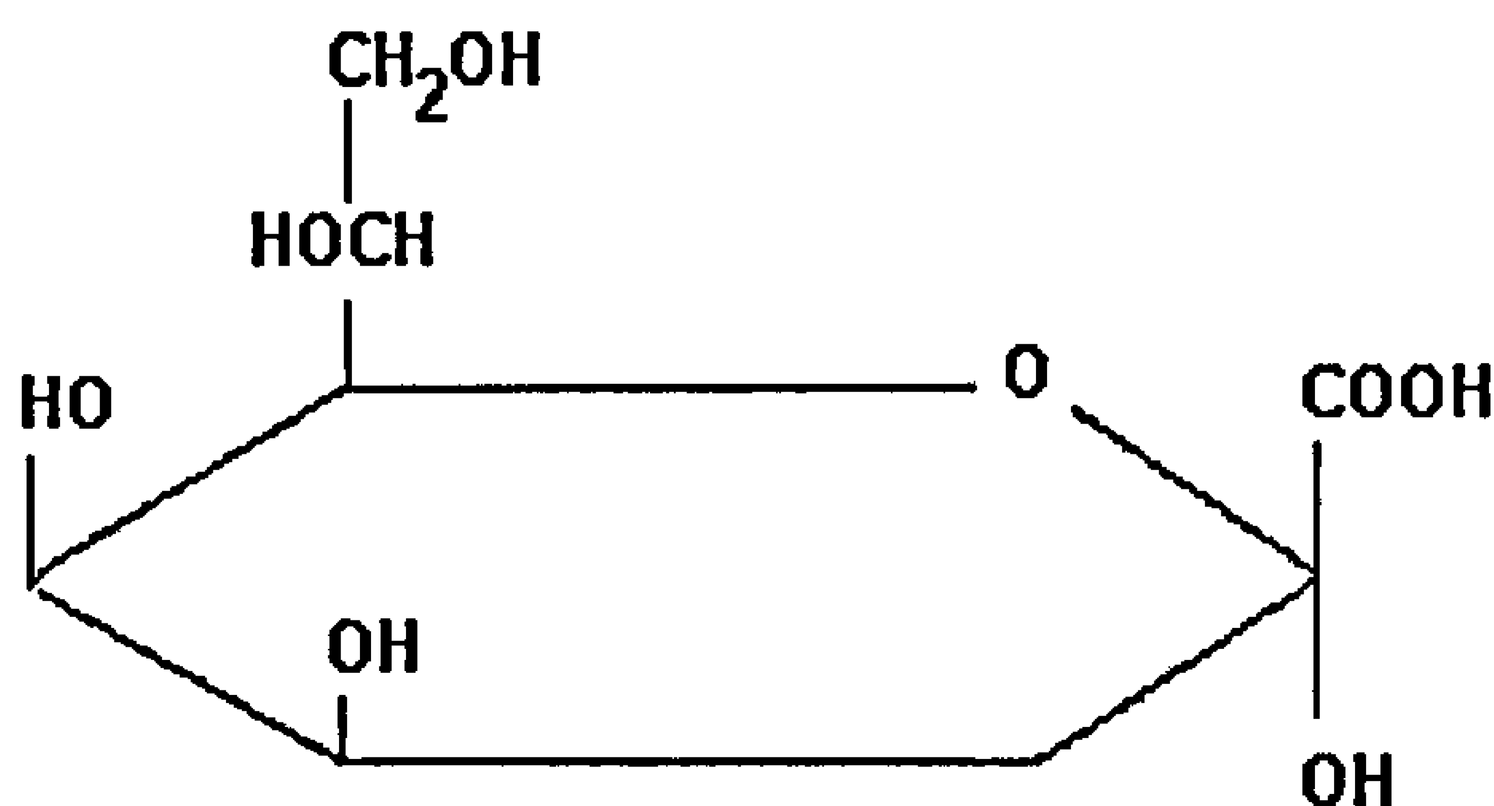


Figure 1. 4 3-deoxy-D-manno-octulosonic acid (Kdo).

1.5.3.3 Lipid A structure

Lipid A is composed of a β-D-GlcN-(1-6)-α-D-GlcN disaccharide carrying two phosphoryl groups (Figure 1. 5). Both phosphates can be further substituted with groups such as, ethanolamine, ethanolamine phosphate, ethanolamine diphosphate, and glucosamine (GlcN). Up to four acyl chains are attached to this structure and these chains can also in turn be substituted by further fatty acids to provide LPS molecules with up to seven acyl substituents, which vary quite considerably between species in nature, number, length, order and saturation. Erridge *et al.*, (2002) reported that the major contributing factors to endotoxicity are the number and lengths of acyl chains present and the phosphorylation state of the disaccharide backbone.

Netea *et al.*, (2002) suggested that the shape of the lipid A component determines the bioactivity of LPS, with lipid A that adopts a conical conformation (*E. coli*) being more active than lipid A that adopts a cylindrical shape (*Porphyromonas gingivalis*).

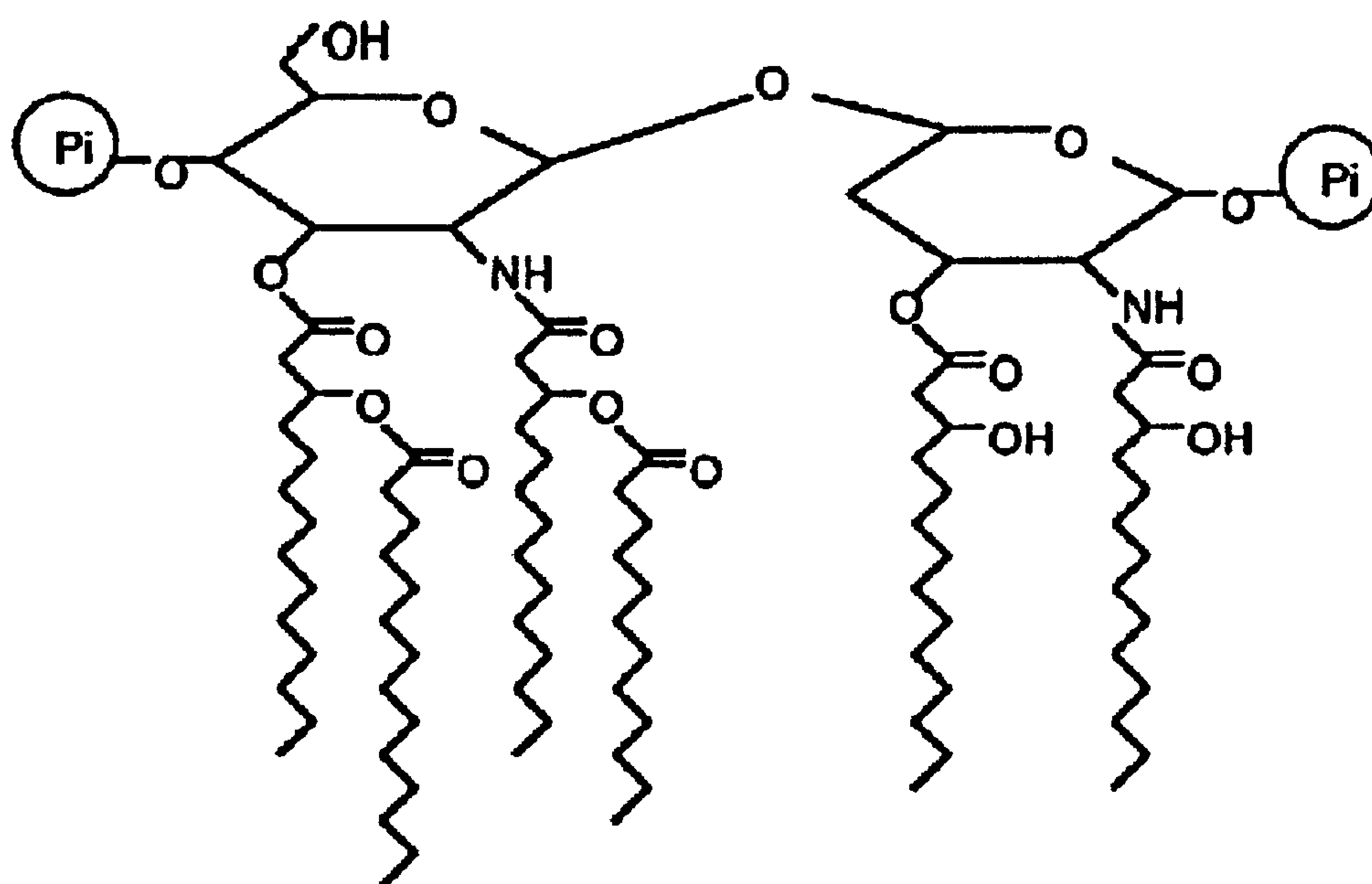


Figure 1. 5. Chemical structure of lipid A (*E.coli*).

1.5.4 The characteristics of *C.trachomatis* LPS

Chlamydial LPS or, more correctly, (LOS) (http://www.chlamydiae.com) is similar to the rough forms of enterobacterial LPS. It consists of the lipid A and core regions (Figure 1.6) that contain D-glucosamine, fatty acids, Kdo, and phosphate in a molar ratio of ~2, 5, 3, 2.6, and a concentration of 480, 1185, 715, 625 nmol/mg respectively (Nurminen *et al.,* 1985). The major lipid A component of chlamydial LPS is a glucosamine disaccharide that contains five fatty acids with up to 22 carbon atoms (pentaacyl) and a phosphate group in the distal segment. There is a complex mixture of long-chain, normal, 3-hydroxylated, and branched fatty acids. The most prominent fatty acids are tetradecanoic, iso- and anteiso-branched tetradecanoic, hexadecanoic, and 3-hydroxyeicosanoic acids (Qureshi *et al.,* 1997).

Fatty acids in LPS of *Chlamydiae* have acyl chains with up to 22 carbon atoms and 3-hydroxy fatty acids occur only with amide-linkage. All hydroxyl fatty acids are amide-linked (–(C=O)NH–) and all non-hydroxylated fatty acids are ester-linked (–COO–) (Nurminen *et al.*, 1985).

The core region of chlamydial LPS is composed of a unique trisaccharide of αKdo(2→8)α Kdo(2→4)αKdo (Brade *et al.*, 1990; Holst *et al.*, 1991). Since Kdo has been found to be a constituent of chlamydial LPS, Brade *et al.,* (1986) suggested that the Kdo is probably involved in the Re specificity of chlamydial LPS.

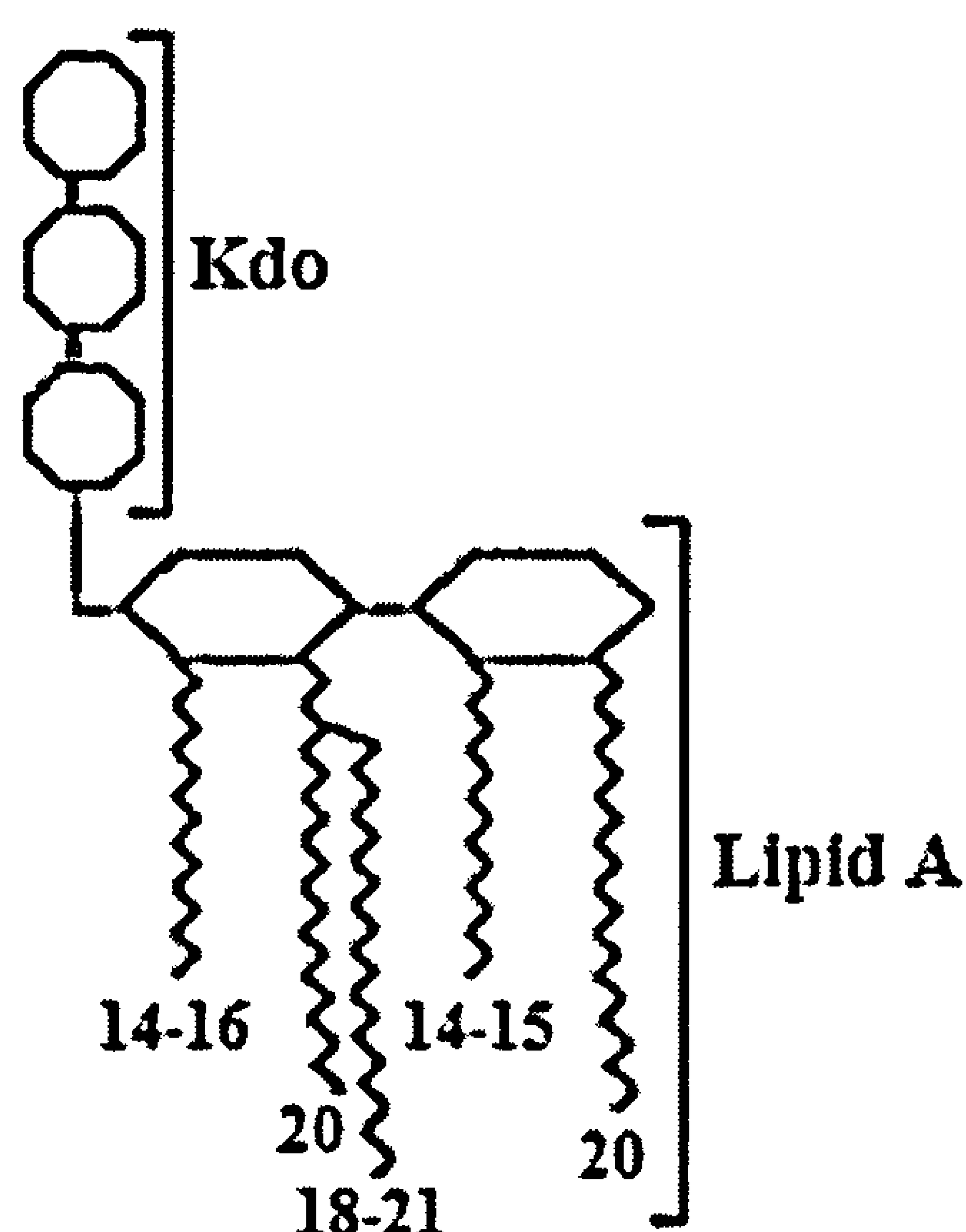


Figure 1. 6. Chemical structure of *C.trachomatis* LPS.

1.6 LPS recognition mechanisms by host cells

The immune system of vertebrates has two components: adaptive immunity and innate immunity. Adaptive immunity is mediated by T and B lymphocytes that proliferate clonally in response to a specific pathogen or antigen. The generation of adaptive immune responses requires a number of days but is anamnestic through the generation of memory T and B lymphocytes. By contrast, the goals of the innate immune system are to provide protection in the first minutes to hours after an infectious challenge (Abreu and Arditi, 2004). The innate immune system as the first line of defence against microbial pathogens, is composed of macrophages, natural killer cells, neutrophils, mucosal epithelial cells, and endothelial cells. This system plays two crucial roles. First, it contains the infection prior to the induction of adaptive immune responses. Second, the innate immune system controls the activation of adaptive immunity and determines the type of effector responses that are appropriate for the infecting pathogen. Both functions critically depend on the ability of the innate immune system to detect the presence of infectious microorganisms and to induce a set of endogenous signals, such as inflammatory cytokines and chemokines (Medzhitov and Janeway, 2000).

A wide variety of bacterial components or products, pathogen-associated molecular patterns (PAMPs), are capable of stimulating the innate immune system. These include LPS; peptidoglycan (PG) and lipoteichoic acid (LTA) from Gram-positive bacteria; formylated peptides from both Gram-negative and Gram-positive bacteria (Pan, 2004). LPS is a principal component of Gram-negative bacteria that potently activates the innate immune system (Miyake, 2004).

LPS-induced cellular responses are the result of the interaction of LPS with various plasma components such as the soluble cluster of differentiation antigen 14 (sCD14), LPS-binding protein (LBP) and membrane receptors such as membrane-bound CD14 (mCD14) and Toll-like receptors (TLRs). This initiation of cellular responses is essential for the host defense against bacterial infections. However, if large amounts of endotoxin are present in the circulation, an excessive cellular response can be deleterious for the host (Vreugdenhil *et al.*, 2001).

LBP, a 452-aa protein with a molecular mass of 60 kDa, is an acute-phase reactant produced during Gram-negative bacterial infections (Tobias *et al.*, 1988). It is synthesized mainly in the liver and also in pulmonary and gastrointestinal epithelial cells. LBP (Jack *et al.*, 1997) and CD14 (Read *et al.*, 1993) play key roles in the recognition of LPS. The main role of LBP is to bind and deliver LPS to CD14 (Hailman *et al.*, 1994). LBP also binds to glycolipids of mycobacteria (Savedra *et al.*, 1996) and lipoteichoic acid of Gram-positive bacteria (Schroder *et al.*, 2003).

LBP facilitates the interaction of LPS with CD14 by two basic processes. First, LBP acts as an opsonin for LPS-bearing particulates enhancing the interaction with CD14 (Wright *et al.*, 1989), and secondly, LBP enables cells to respond to extremely low concentrations of LPS via a CD14-dependent pathway (Schumann *et al.*, 1990).

CD14 is a 55 kDa glycosyl phosphotidylinositol (GPI)-anchored protein that belongs to a family of receptors, termed pattern recognition receptors, which are involved in innate immune recognition of pathogen-associated molecular patterns (Wright, 1995). Membrane forms of CD14 are expressed on the surface of monocytes, macrophages, neutrophils (Haziot *et al.*, 1993), and spermatozoa (Harris *et al.*, 2001). Soluble forms of CD14 have been reported in plasma, cerebrospinal fluid, amniotic fluid, breast milk, and synovial fluid. This form of CD14 is also present in seminal plasma at levels comparable to those in serum (Harris *et al.*, 2001). sCD14 is reported to facilitate the LPS signal at low concentrations, but suppresses it at high concentrations (Bazil and Strominger, 1991). CD14 is critical for an efficient recognition not only of LPS but also of a number of other bacterial cell wall components from diverse organisms, heat shock proteins and some viruses (Aderem and Ulevitch, 2000).

In the presence of LBP, CD14 has been implicated as a high-affinity LPS receptor, facilitating LPS-induced macrophage activation in response to endotoxin. CD14 is not capable of initiating a transmembrane activation signal because it is a (GPI)-anchored protein and thus, lacks transmembrane and intracellular domains. The main function of CD14 is to catalyze the transfer of LPS from extracellular space to the membrane where it associates with a complex of receptors including TLR4-MD2 (Triantafilou and Triantafilou, 2002). CD14 also enhances the responses

of phagocytes to bacterial products that activates cells via TLR2 (Moore *et al.*, 2000).

Toll receptors were first discovered in the fruit-fly, Drosophila. TLRs are expressed by myelomonocytic cells, endothelial and epithelial cells as well as cells from various organ systems (Zarember and Godowski, 2002). Toll-like receptors are transmembrane proteins, all of which have a common extracellular leucine-rich domain and an intracellular region belonging to the IL-1R family called the Toll/ IL-1 receptor (TIR) (Beutler and Rehli, 2002). Both the IL-1 receptor and the TLRs share similar downstream affects, such as the activation of immune response genes, which are initiated by the activation of myeloid differentiation factor 88 (MyD88) by the intracellular domains of the IL-1 and Toll-like receptors (Abreu and Arditi, 2004).

The primary role of TLRs is to recognize bacterial, viral, fungal, and other pathogens that may be deleterious to the host (Abreu and Arditi, 2004). Ten human TLRs have been characterized so far (Underhill and Ozinsky, 2002). TLR2 is essential for the recognition of a variety of PAMPs, including bacterial lipoproteins, lipoteichoic acids and peptidoglycan, and TLR4 is predominantly activated by LPS (Jurk *et al.*, 2002). TLR4 alone is not sufficient to confer LPS responsiveness. TLR4 requires MD-2, a secreted molecule, to functionally interact with LPS. MD-2 is thus a link between TLR4 and LPS (Shimazu *et al.*, 1999). MD2 also increases TLR2 responsiveness to TLR2 ligands (Dziarski *et al.*, 2001).

Stimulation of TLRs by PAMPs initiates a signaling cascade (Figure 1. 7) that involves a number of proteins, such as MyD88 and interleukin receptor-associated kinase (IRAK). This signaling cascade leads to the activation of two transcription factors, activator protein-1 (AP-1) and nuclear factor-kB (NF-kB) which in turn induce the secretion of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), IL-1, IL-6, IL-8 (Medzhitov *et al.*, 1997), IL-12, and interferon-γ (IF- γ) (Dentener *et al.*, 1993).

TNF-α, one of the major mediators of inflammatory responses in mammals, is a multifunctional cytokine with effects not only in the pro-inflammatory response (Fiers, 1991) but in immuno-regulatory responses (Beutler, 1995), and apoptosis (Baker and Reddy, 1998). It also plays a prominent role in bridging the innate and

adaptive phases of immunity (Ma, 2001). These functions are mediated by the binding of TNF to at least two receptors, TNF-receptor 1 (TNF-R1) and TNF-receptor 2 (TNF-R2), which are expressed by most nucleated cells and are members of a family of related receptors (Beutler and van Huffel, 1994). Stimulation of TNF-R1 induces an increase in reactive oxygen species (ROS) production via mitochondria as a major source of TNF-α-induced ROS production (Schulze-Osthoff *et al.*, 1993). Also it has been shown that TNF-induced apoptosis is mediated primarily through the activation of TNF-R1 (Rath and Aggarwal, 1999). ROS overproduction induced by TNF-α is accompanied by a cytotoxic or pro-apoptotic effect (Garcia-Ruiz *et al.*, 1997).

The principal cells producing TNF-α are monocytes and macrophages with additional producers including B and T lymphocytes. TNF-α is a double-edged sword with both pro-inflammatory and anti-inflammatory propensities. Natural induction of TNF-α is protective and important for the efficient control of growth and dissemination of invading pathogens, but its overproduction may be detrimental and even lethal to the host (Ma, 2001).

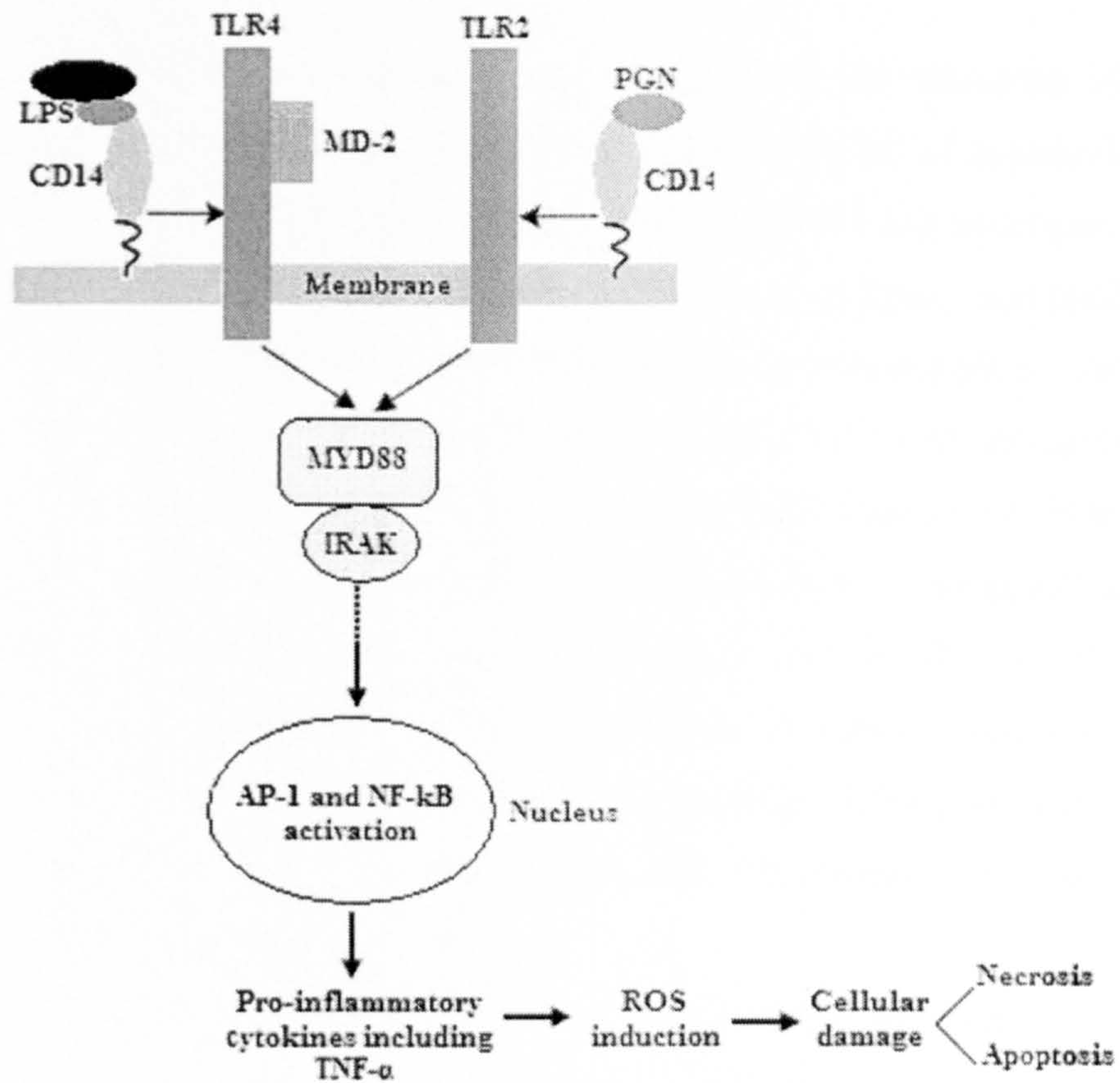


Figure 1. 7. A model of TLR2 and TLR4 signaling pathway and cytokines and ROS induction. Upon recognition of pathogen by TLRs a signaling cascade is initiated. This signaling cascade leads to the activation of AP-1 and NF-kB which induces the secretion of pro-inflammatory cytokines. The cellular damage induced by ROS may be lead to cell death via apoptosis or necrosis.

1.7 Cellular damage and defence mechanisms in ROS production

Aerobic organisms, which derive their energy from the reduction of oxygen, are susceptible to the damaging actions of the small amounts of superoxide anion radicals ($O_2^{\bullet-}$, • = unpaired electron), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) that inevitably form during the metabolism of oxygen, especially in the reduction of oxygen by the electron transfer system of mitochondria. These three species, with other free radicals such as nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) are referred to as reactive oxygen species. Other sources of ROS include cigarette smoke, radiation (*e.g.*, UV light), toxic chemicals (*e.g.*, paraquat) and drugs (*e.g.*, adriamycin, bleomycin). ROS with free radicals such as $O_2^{\bullet-}$ or $OH^{\bullet}$ contain unpaired electrons and have a lifetime in the range of nano-to milliseconds and therefore react with molecules in their direct surrounding. Others, such as H_2O_2, are not radicals. They are long-lived and, as they are not charged, can pass through plasma membranes (Ochsendorf, 1998).

Free radicals are not only produced as an unwanted product, they are also formed deliberately in the body for useful purpose and have important physiological functions (Rice-Evans and Burdon, 1993; Halliwell *et al.*, 1995). Activated phgocytic cells (neutrophils, monocytes, or macrophages) for example, produce $O_2^{\bullet-}$ and $OH^{\bullet}$ as a defence mechanism to kill bacteria and fungi and to inactivate viruses (Curnutte and Babior, 1987).

ROS, in particular the hydroxyl radical, can react with all biological macromolecules (lipids, proteins, nucleic acids and carbohydrates). The initial reaction generates a second radical, which in turn can react with a second macromolecule to continue the chain reaction. Oxidative damage to DNA, proteins, and lipids can ultimately lead to outcomes such as disorganisation, dysfunction and destruction of membranes, enzymes and proteins (Halliwell, 1997). Among the susceptible targets, polyunsaturated fatty acids (PUFAs) are more susceptible to react with ROS (Gutteridge, 1995). Removing of a hydrogen atom from a PUFA initiates the process of lipid peroxidation. Peroxidation of membrane lipids may cause impairment of membrane function, decreased fluidity, inactivation of membrane-

bound receptors and enzymes, increased permeability to ions and possibly, eventually membrane rupture (Gutteridge, 1995). If the oxidative stress (disturbance in the balance between antioxidants and free radicals and other ROS) is severe, it can produce cell death (Halliwell, 1997). Death can occur by necrosis, however a mild oxidative stress can trigger the process of apoptosis (Hampton and Orrenius, 1997).

1.7.1 Defence mechanisms

There are a number of antioxidants present in the body and derived from the diet. Superoxide dismutase (SOD), catalase and glutathione peroxidase are intracellular antioxidant enzymes that convert potential substrates ($O_2^{\bullet-}$ and H_2O_2) to less reactive forms in the body and thus eliminate their damaging effects from the cells (Gutteridge, 1995). For example, SOD catalysis of the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 and the conversion of H_2O_2 to $2H_2O$ by glutathione peroxidase or to O_2 + H_2O by catalase is a metabolic reaction against ROS.

Antioxidants from dietary sources include lipid soluble vitamins such as vitamin E and carotenoids as well as the water-soluble vitamin C. Vitamin E is one of the most important free radical-scavenging antioxidants within membranes and lipoproteins. It is an effective chain-breaking antioxidant that protects PUFAs from peroxidation by scavenging peroxyl radicals ($ROO^{\bullet}$) (Halliwell, 1997).

1.8 Cellular death, necrosis and apoptosis

Cells may die in either of two distinct processes: necrosis (pathological or accidental cell death) or apoptosis [physiological or programmed cell death (PCD)] (Vermes and Haanen, 1994). Necrosis is a pathological process which occurs when cells are exposed to a serious physical or chemical assault including toxins, radiation, heat, trauma, lack of oxygen due the blockage of blood flow, and also various viruses, bacteria, and protozoa etc. Necrosis begins with an impairment of the cell's ability to maintain homeostasis including ion homeostasis resulting in excess intracellular calcium and/or sodium. Excess influx of magnesium and zinc as well as acidosis can also induce necrosis (Trump and Berezesky, 1995). Intracellular organelles, especially mitochondria, within the whole cell swell and rupture and

finally the whole cell disintegrate. Following disruption of the cell membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid (Proskuryakov *et al.*, 2003). Necrosis is often associated with extensive tissue damage resulting in an intense inflammatory response (Kelly *et al.*, 2001).

Apoptosis is a mode of cell death that usually occurs under normal physiological conditions. The term 'apoptosis' from the Greek, meaning "to fall away from" and conjuring notions of the falling of leaves in the autumn from deciduous trees, defined as a controlled type of cell death that can be induced by a variety of physiologic and pharmacological agents, was first suggested by Kerr *et al.,* (1972). Cells undergoing apoptosis show characteristic morphological and biochemical features. These features include plasma membrane blebbing, cellular shrinkage, condensation and margination of the nuclear chromatin, DNA fragmentation, and cytoplasmic vacuolisation (Kanduc *et al.*, 2002). Eventually, the cells breaks into small membrane-surrounded fragments (apoptotic bodies), which are cleared by phagocytosis without inducing an inflammatory response (Reed, 2000). In the absence of phagocytosis, apoptotic cells may undergo secondary necrosis, a process associated with additional proteolytic degradation of specific autoantigens. Secondary necrosis may occur *in vivo* in autoimmune disorders associated with impaired clearance of apoptotic cells and serve as a source of modified forms of specific autoantigens that might stimulate autoantibody responses under proinflammatory conditions (Wu *et al.*, 2001). Differences between necrosis and apoptosis regarding morphological, biochemical, and physiological features have been shown in Table. 1 and Figure 1. 8.

Necrosis	Apoptosis
• Loss of membrane integrity	• Membrane blebbing, but no loss of integrity
	• Aggregation of chromatin at the nuclear membrane
• Begins with swelling of cytoplasm and mitochondria	• Begins with shrinking of cytoplasm and condensation of nucleus
• Ends with total cell lysis	• Ends with fragmentation of cell into smaller bodies
• No vesicle formation, complete lysis	• Formation of membrane bound vesicles (apoptotic bodies)
• Disintegration (swelling) of organelles	• Mitochondria become leaky due to pore formation involving proteins of the Bcl-2 family
• Loss of regulation of ion homeostasis	• Tightly regulated process involving activation and enzymatic steps
• No energy requirement	• Energy (ATP)-dependent
	• Release of various factors (cytochrome *c*, AIF) into cytoplasm by mitochondria
	• Activation of caspase cascade
• Affects groups of contiguous cells	• Affects individual cells
• Evoked by non-physiological disturbances (hypothermia, hypoxia, ischemica, metabolic poisons)	• Occur in both physiologic and pathologic conditions
• Phagocytosis by macrophages	• Phagocytosis by adjacent cells or macrophages
• Significant inflammatory response	• No inflammatory response

Table 1. 1. Necrosis versus apoptosis (http:// www.roche-applied-science.com)

The process of apoptosis is fundamental during normal developmental and physiological conditions such as cell turnover and tissue homeostasis, embryogenesis (Danial and Korsmeyer, 2004), maintenance of immunological tolerance (Lamhamedi-Cherradi *et al.*, 2003), and developmental of the nervous system (Martin, 2001). However, dysregulation of the apoptotic pathway has been implicated in a number of diseases. For example, in cancer and autoimmune disorders, cells fail to undergo apoptosis. Conversely, excessive apoptosis has been implicated in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. Inappropriate apoptosis is also associated with AIDS, ischemic heart damage or viral infections (Harvey and Kumar, 1998).

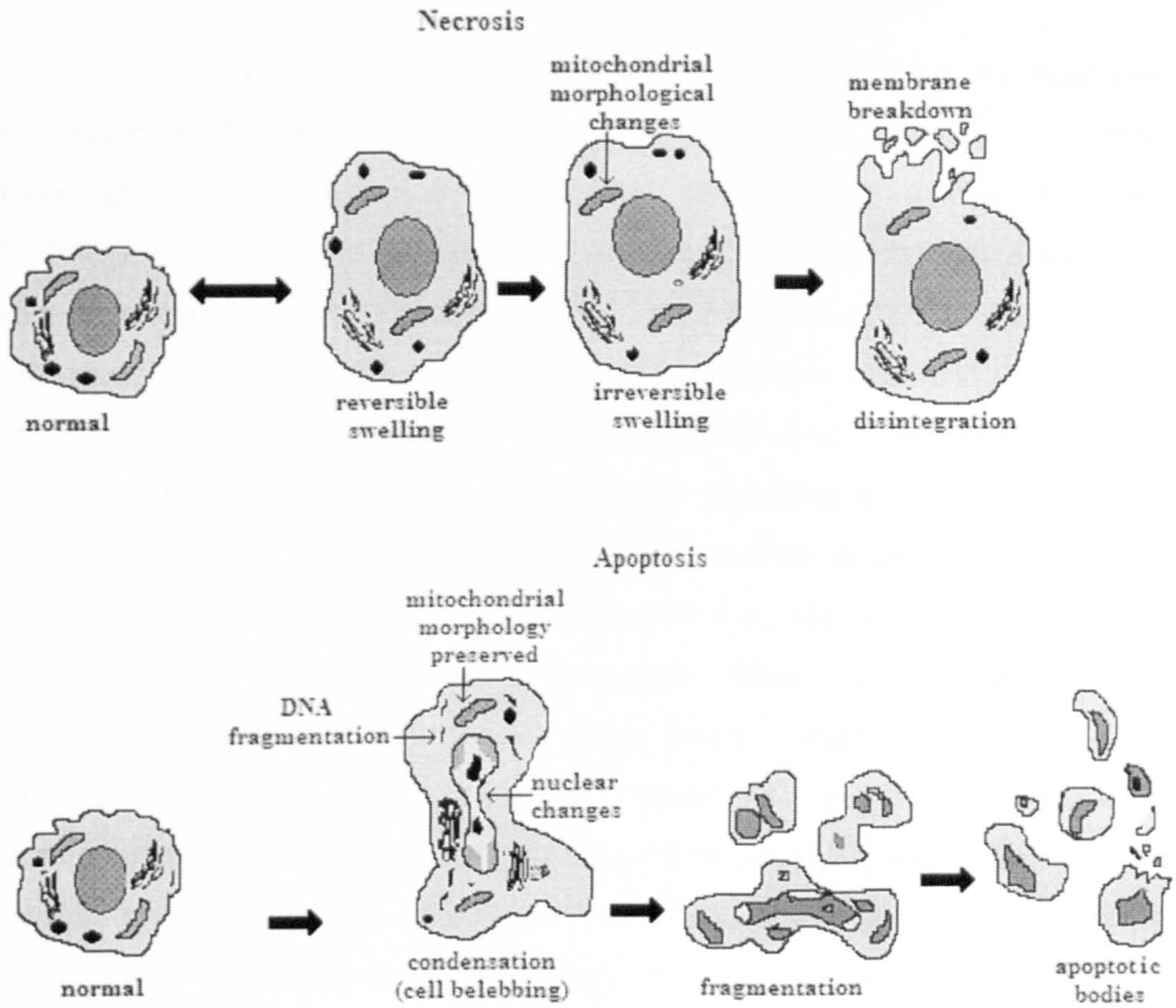


Figure 1. 8. Schematic model of the morpholiogical features of necrosis and apoptosis

1.8.1 Mechanisms of apoptosis and caspase activation

Apoptosis can be broken down into four stages; stimuli, signals, regulations, and executors (Morita and Tilly, 1999). The first stage comprises of different physiological and pathological stimuli that interact with a cell. In normal physiological cell turnover the apoptotic stimuli can be represented by cytokines, or death factors, such as FAS ligand (FASL, also referred to as CD95 or APO-1 ligand) (Ashkenazi and Dixit, 1998). Pathological insults include chemical and radiation stimuli, environmental toxicants or hormone withdrawal. The second stage of apoptosis is the recruitment of early intracellular signalling molecules (*e.g.*, TNFR and FAS) that relay the plethora of external information received by the cell to a central regulatory checkpoint for interpretation (i.e., the third stage) governed primarily by the Bcl-2 (B cell leukaemia-2) family proteins on the outer mitochondrial membrane. The signals at the second stage are processed by a regulatory mechanism, which evaluates the strength of the apoptosis inducing signal against anti-apoptotic signals in the third stage. If the death inducers prevail, the cell commits to apoptosis and enters the fourth and final stage (at the level of mitochondria) where specific executor proteins are responsible for the organised destruction of the cell (Green and Reed, 1998).

There are at least two major pathways for caspase activation (Figure 1. 9). The death receptor (extrinsic) pathway and mitochondrial (intrinsic) pathway (Reed, 2000). The extrinsic pathway can be induced by members of the TNF family of cytokine receptors, such as TNFR1 and FAS. These proteins recruit adapter proteins to their cytosolic DDs (death domains), including FADD (FAS-associated death domain), which then binds DED-containing pro-caspases (death effector domain), particularly pro-caspase-8. FADD functions as a bridge between FAS and downstream signal transduction termed DED. FADD links TNF family death receptors to pro-caspase-8, using its DD to bind TRADD (TNFR-associated death domain) or interacts directly with the cytosolic DD of the TNFR family member FAS, and employing its DED to bind DED-containing caspases (Reed, 2000).

The intrinsic pathway can be induced by release of cytochrome *c* from mitochondria, induced by various stimuli, including elevations in the levels of pore-

forming pro-apoptotic Bcl-2 family proteins such as BAX (Bcl-2 associated x protein) and BAD (Bcl-X_L/ Bcl-2-associated death promoter). In the cytosol, cytochrome *c* binds and activates APAF-1 (apoptosis protease-activating factor 1), allowing it to bind and activate pro-caspase-9.

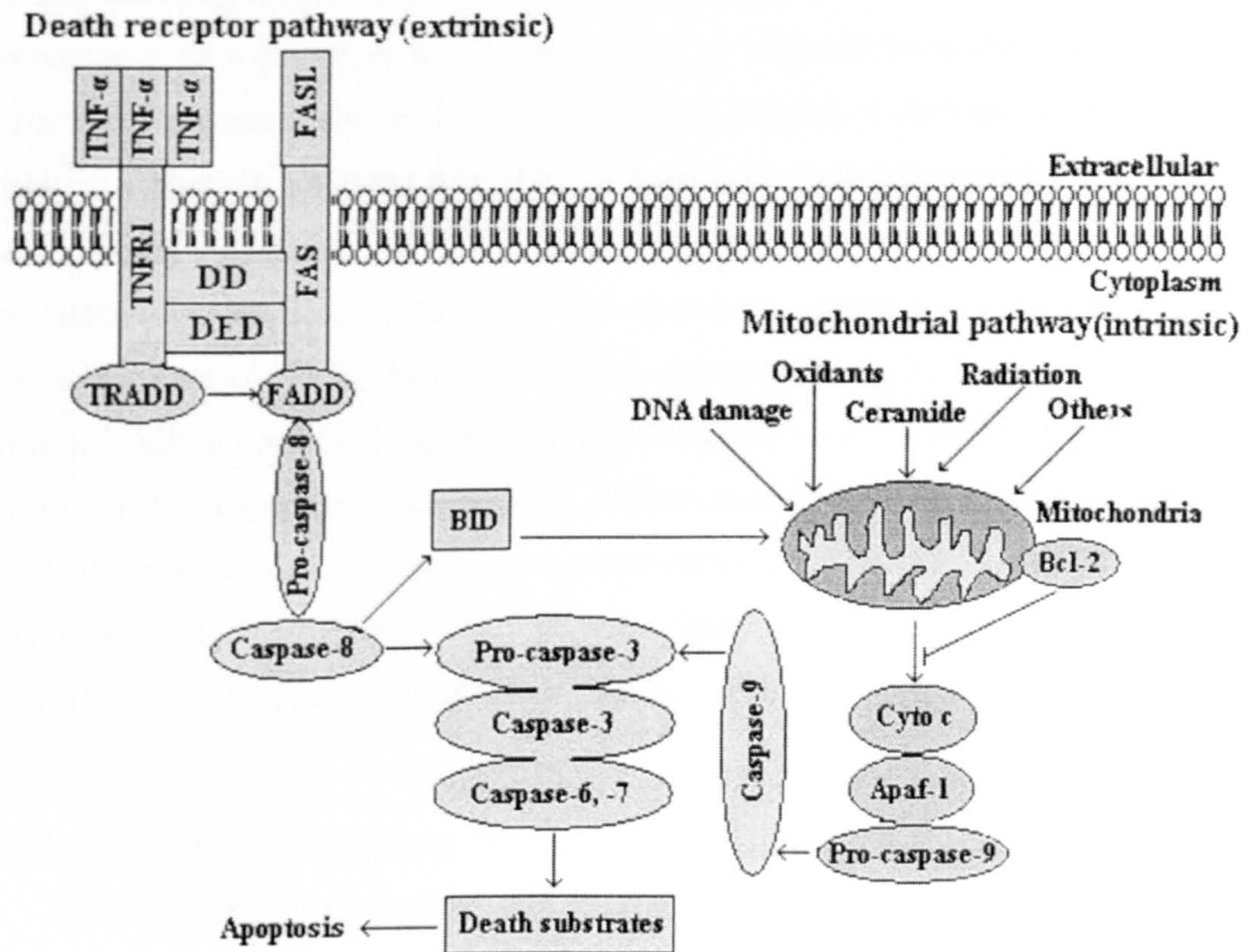


Figure 1. 9. Major apoptotic pathways in mammalian cells and regulation of death receptor signaling. Activated FAS recruits an adaptor protein called FADD, through interaction with DDs. The TNFR1 mediated pathway utilizes TRADD protein in recruitment of FADD. Bcl-2 prevents apoptosis by blocking the release of cytochrome *c* from the mitochndria. Activated caspase-8 is able to induce apoptosis through a mitochondrial pathway by cleaving BID, or directly by activating downstream effector caspases.

1.8.1.1 Bcl-2 family proteins

In humans, 20 members of the Bcl-2 family with pro- (*e.g.*, BAX, BAD, BID) and anti-apoptotic [(*e.g.*, Bcl-2 itself, Bcl-X_L, and Mcl-1 (myeloid cell leukaemia-1)] functions have been described to date. Anti-apoptotic members of this family can inhibit not only apoptotic, but also necrotic death (Proskuryakov *et al.*, 2003). The relative ratios of anti- and pro-apoptotic Bcl-2 family proteins dictate the ultimate sensitivity or resistance of cells to various apoptotic stimuli (Reed, 2000). If the apoptotic-inducing fraction of Bcl-2 family outbalances their anti-apoptotic relatives, cytochrome *c* as a principal apoptosis inducer is released from the mitochondrial compartment and associates with APAF-1 and pro-caspase-9. Release of cytochrome *c* from mitochondria, an event directly regulated by Bcl- family members, produced conformational changes in APAF-1, leading to caspase-9 activation. Together these three factors, cytochrome *c*, APAF-1, and caspase-9, create a holoenzyme termed apoptosome, which is a key connection between mitochondria and caspase activation. Active caspase-8 (extrinsic) and the apoptosome (intrinsic) proceeds to activate the executioner caspases 3 and 7. Active caspase-3, in turn, cleaves caspases 2, 6, 8, 10, and perhaps 9, providing a tremendous amplification loop (Liou *et al.*, 2003). Caspase-8 cleaves BID, which releases cytochrome c, bridging the "extrinsic" and "intrinsic" pathways (Henshall *et al.*, 2001).

1.8.1.2 The family of caspases

Most of the morphological and biochemical changes that are recognised as apoptosis are the results of protease activity. Especially, activation of a family of intracellular cysteine proteases which cleave their substrates at aspartic acid residues, are known as caspases for Cysteine Aspartyl-specific Proteases (Alnemri *et al.*, 1996). Caspases are synthesized as precursor molecules (zymogens), which require proteolytic processing into two subunits for their activation (Earnshaw *et al.*, 1999). Many caspases have two potential cleavage sites at the junction of the large and small subunits. These sites are separated by a few amino acids and cleavage may occur at one or both sites. Dimerization and proteolysis generates an active caspase composed of two large (~20 kDa) and two small (~10 kDa) subunits.

In mammals, approximately 14 caspases have been identified so far (Said *et al.*, 2004). Of the 14 caspases currently known, not all seem to play a role in apoptosis. For example, caspase-1 (ICE) and its close relatives caspase-4 (TX, ICH2), -5 (TY, ICErel-III) and -11 (ICH3) appear to be primarily involved in cytokine processing (Kumar, 1999). The subgroup of caspases involved in apoptosis has been referred to as either initiators (upstream) or effectors (downstream). Downstream caspases are largely dependent on upstream caspases for their proteolytic processing and activation (Reed, 2000). Caspases-8 (FLICE, MACH, MCH5), -9 (ICE-LAP6, MCH6), -10 (MCH4, FLICE2) and possibly, -2 (ICH-1) can initiate the caspase activation cascade and are therefore called initiators. The effector caspases-3 (CPP32, Yama, Apopain), -6 (MCH2), and -7 (MCH3, ICE-LAP3, CMH-1) propagate the cascade and are activated by proteolytic cleavage by other caspases (Slee *et al.*, 1999).

1.8.1.3 Cellular targets of caspases

Approximately 40 proteins are now known to be cleaved by effector caspases in cells undergoing apoptosis (Kumar, 1999) of which three are mentioned here. One of the end points in apoptosis is fragmentation of DNA into multiples of approximately 180 bp. The enzyme responsible for this action is termed caspase-activated DNase (CAD). CAD is an inactive complex that is normally bound to an inhibitory subunit, known as CAD inhibitor (ICAD), which is degraded by caspase-3 in apoptotic cells. The free CAD then enters the nucleus and acts on chromatin (Enari *et al.*, 1998). p21 (CDKN1A)-activated kinase 2 (PAK2), a serine/threonine kinase, is another substrate activated by caspase cleavage. Because PAK2 is involved in the regulation of the actin cytoskeleton, its activation seems to help formation of apoptotic bodies (Rudel and Bokoch, 1997). Bcl-2 and Bcl-X_L proteins are also cleaved during apoptosis and the fragments generated by the cleavage appear to enhance apoptotic changes in dying cells (Clem *et al.*, 1998).

1.8.1.4 Inhibitors of apoptosis proteins

A family of proteins named inhibitors-of-apoptosis (IAP), have the ability to bind procaspases and activated caspases, blocking their processing and activity. IAPs

were first found in baculoviruses as proteins that suppress apoptosis, so allowing the virus to replicate in infected cells (Birnbaum *et al.*, 1994). IAPs are found in the genomes of mammals, insects, and certain animal viruses and characterised by a novel domain of 70 amino acids, termed the baculoviral IAP repeat (BIR) domain, which is essential for suppression of apoptosis (Reed, 2000). Human IAPs can arrest apoptosis induced via either the intrinsic or extrinsic pathways, probably because they target effector caspases common to both pathways (Zhou *et al.*, 1998). Interestingly, IAPs also have a controlling factor termed second mitochondria-derived activator of caspases (SMAC) or Diablo that can prevent IAPs from binding caspases, allowing them to be activated and perform their part in the apoptotic process (Verhagen *et al.*, 2000). SMAC is normally confined to the mitochondria, but once released, it binds to IAPs and removes this block in the cell death pathway.

1.9 Structural and functional characteristics of human spermatozoa

The spermatozoa originates from spermatogonial stem cells and are the end product of spermatogenesis (Abou-Haila and Tulsiani, 2000). A spermatozoon is a highly polarised and motile cell with a haploid nucleus that fuses with haploid egg nucleus forming 2n zygote nucleus during a process known as fertilisation. Fertilisation generally consists of four major events; contact and recognition between sperm and egg, regulation of sperm entry into the egg, fusion of the genetic material of sperm and egg, and activation of egg metabolism to start development. Successive stages in the process of spermatogenesis are in the following order; primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa.

There are three main regions in the sperm cells; head, middle piece, and flagellum (Chavarria *et al.*, 1997) (Figure 1. 10). The head (4-5 μm in length) consists of; sperm cell membrane that covers the anterior portion of the head and contains receptors for recognising egg, acrosome with digestive enzymes for penetration of the material surrounding the egg that facilitates the fertilisation process, and highly condensed haploid nucleus that fuses with haploid egg nucleus. The acrosome itself consists of an anterior cap and a posterior region called

equatorial segment. The middle piece (3.5 μm) of the sperm mainly dominated by a spiral complex of mitochondria that are fused together generate energy for the flagellar movement. The flagellum (55 μm) has the same basic structure of other eukaryotic cilia or flagella, but also has particular characteristics due to the presence of the outer dense fibres and the fibrous sheath. The flagellum consists of 9 + 2 microtubules (9 doublets + a pair of complete microtubules) which form the axoneme. Sliding of the microtubules generates motive force.

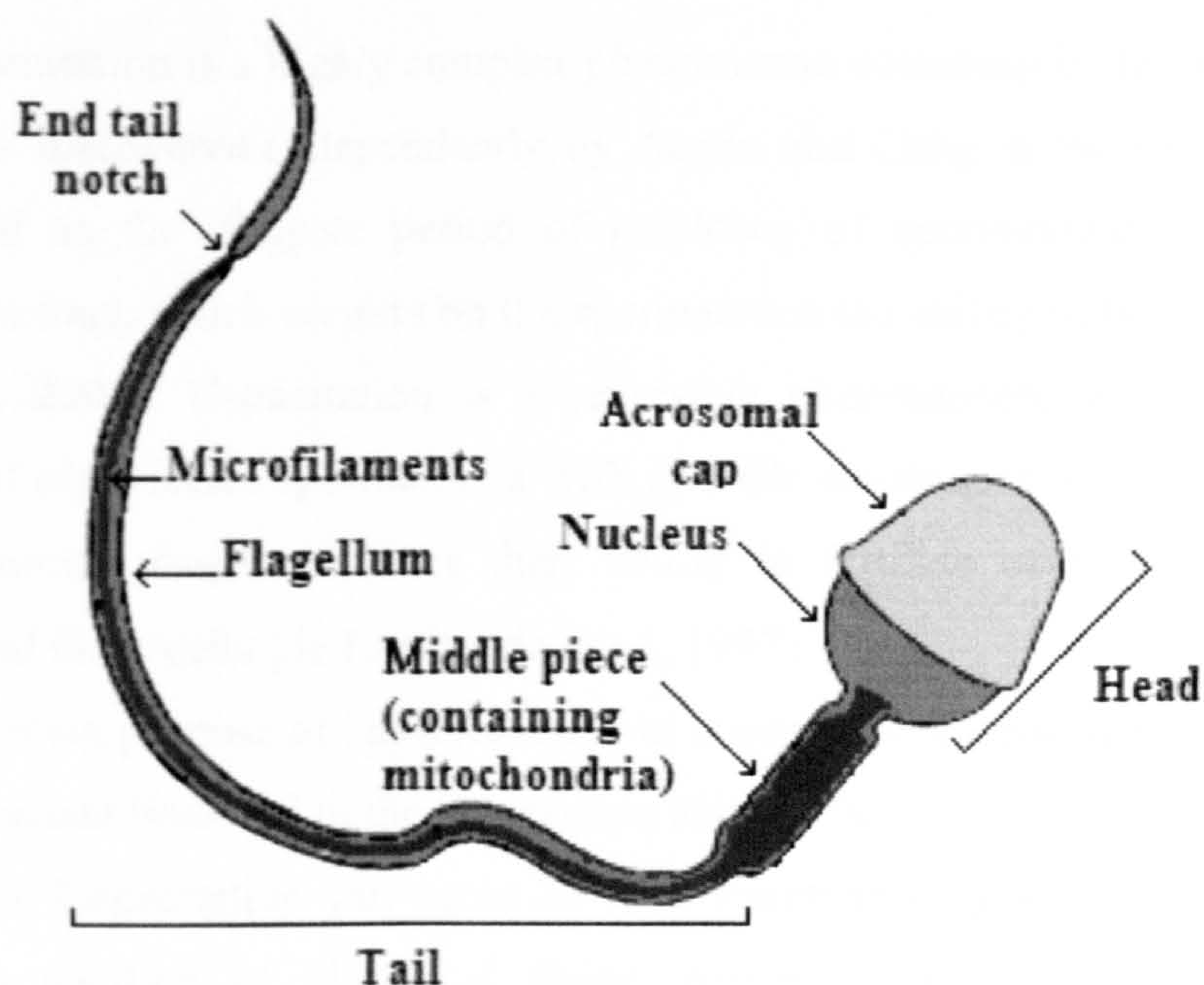


Figure 1. 10. Basic structure of human spermatozoon

Spermatozoa undergo several important maturation steps throughout their life, resulting from their interaction with the different environments through which they migrate and the various functions that they are expected to accomplish. In the epididymis the immotile and incompetent spermatozoa acquire the potential for motility and fertility by the action of epithelial cell secretions and constituents of the luminal fluids (epididymal maturation). At ejaculation, they become motile and are

developed by seminal fluid constituents although they are not yet able to fertilize oocytes. During their migration through the female genital tract, spermatozoa undergo a second phase of maturation termed capacitation, that enables them to reach and bind to the zona pellucida (de Lamirande *et al.*, 1997). The most important change in spermatozoa after capacitation is its ability to undergo the acrosome reaction (AR) in response to zona pellucida glycoproteins 3 (ZP3), progesterone (Yanagimachi, 1994), and calcium ionphores (Russell *et al.*, 1979).

1.9.1 Capacitation

Capacitation is a highly complex phenomenon occurring in the female genital tract. It was discovered independently by Austin and Cang in the early 1950s and was defined as the obligate period of residency of spermatozoa in the female reproductive tract, which confers on the spermatozoa the ability to fertilise an oocyte (Jha *et al.*, 2003). Capacitation is a reversible phenomenon, which means that treatment of capacitated spermatozoa with specific substances or biological fluids, such as seminal plasma, reduces their ability to fertilise oocytes and therefore decapacitated these cells (de Lamirande *et al.*, 1997).

The main purpose of capacitation is to ensure that spermatozoa reach the egg at the appropriate time and in the appropriate state to fertilise this egg (de Lamirande *et al.*, 1997). Capacitation can occur *in vitro* spontaneously in a defined medium without the addition of biological fluids, which suggests that this process is intrinsically modulated by the spermatozoa themselves such that these cells are preprogrammed to undergo capacitation when they are incubated in the appropriate medium (Yanagimachi, 1994).

During capacitation various biochemical and biophysical changes occur in the spermatozoa and the spermatozoal membrane. These alterations in metabolism, intracellular ion concentration, membrane fluidity, intracellular pH, cAMP concentration and concentration of reactive oxygen species, ultimately make the spermatozoa fertilization-competent. In spite of the importance of capacitation, the

precise mechanisms of these cellular, biochemical and molecular events are still obscure (Jha *et al.*, 2003).

Modulation of a variety of ion channels of the spermatozoa is a characteristic event associated with capacitation and AR of mammalian spermatozoa. The major mobilisation of ions are the influx of calcium, potassium, chloride, bicarbonate and the efflux of zinc (Purohit *et al.*, 1999). An increase in the intracellular pH has been observed during capacitation. This increase in pH is attributed to the bicarbonate anion (Cross and Razy-Faulkner, 1997). Bicarbonate levels are low in the epididymis and high in seminal plasma and the oviduct. Changes in the concentration of bicarbonate in the male and female reproductive tracts could play an important role in the suppression of capacitation in the epididymis and the promotion of this process *in vivo* in the female reproductive tract (Purohit *et al.*, 1999).

A very low and controlled concentration of superoxidase anion, hydrogen peroxide, and nitric oxide are involved in sperm acquisition of fertilizing ability (Purohit *et al.*, 1999). The ROS produced during the capacitation and AR are known to activate several enzymes including protein kinase C (Gopalakrishna and Anderson, 1989) and tyrosine phosphorylation (Aitken *et al.*, 1995), leading to changes in the fluidity of the membrane. ROS also regulates the fluidity of the membrane by lipid peroxidation in a controlled manner (Jain *et al.*, 1994). Excess ROS generation is associated with a decrease in the fusion rates between sperm and oocytes (Aitken *et al.*, 1991) and inhibition of the AR, motility and fertilizing potential of spermatozoa (Kodama *et al.*, 1996). Accordingly, the needs for a fine balance between ROS production and scavenging, as well as the right timing for ROS production, are important for capacitation and AR (de Lamirande *et al.*, 1997). During *in vivo* capacitation, spermatozoa may not be the only source of ROS. Fluids and cells from the female reproductive tract may also produce ROS or promote the formation of ROS by spermatozoa. Indeed, the concentration of oxygen in these fluids rises sharply at the time of ovulation (Maas *et al.*, 1976).

The other change which takes place during capacitation is the removal of cholesterol from the sperm plasma membrane. The efflux of cholesterol during the process of capacitation alters the permeability of the membrane to ions and generates areas which are prone to fusion and vesiculation process during the AR. The most

important consequences of the cholesterol efflux are the massive influx of extracellular calcium, a prerequisite for the AR. (Purohit *et al.*, 1999). There are cholesterol rich and cholesterol deficient regions on the head of the spermatozoa and during *in vitro* capacitation, loss of cholesterol takes place to form cholesterol-free patches and these patches are the sites of fusion at the time of AR (Tesarik and Flechon, 1986). The cholesterol/phospholipids (C/P) ratio of the sperm determines the capacitation state of the sperm (Hoshi *et al.*, 1990). A freshly ejaculated spermatozoon has a high C/P ratio and during capacitation this ratio falls (de Lamirande *et al.*, 1997).

Another key intracellular event that occurs during capacitation is protein tyrosine phosphorylation. Protein tyrosine phosphorylation mediates a variety of cellular functions such as growth regulation, cell cycle control, cytoskeleton assembly, ionic current regulation, and receptor regulation (Hunter, 1996). Phosphorylation increases significantly with capacitation and is localized mainly to the principal piece of the flagellum. Following binding to the zona pellucida, the percentage of spermatozoa with phosphotyrosine residues is significantly higher in bound spermatozoa than in capacitaed spermatozoa in suspension (Sakkas *et al.*, 2003).

1.9.2 Hyperactivation and capacitation

Hyperactivated movement of spermatozoa was first reported by Yanagimachi (1969), who observed that, as spermatozoa gained the ability to fertilize oocytes in vitro, their flagella began beating more vigorously than before. Hyperactivated motility is defined as the swimming pattern shown by most spermatozoa retrieved from the oviductal ampulla at fertilization. The flagella of hyperactivated spermatozoa are thrown into deeper bends and their beating is usually less symmetrical than that of flagella of activated spermatozoa (Ho and Suarez, 2001). Sperm hyperactivation occurs at the site and time of fertilisation and it appears as an essential event of capacitation. It is associated with an increase velocity, a decreased linearity, an increased amplitude of lateral head displacement, and whiplash movement of the flagellum (de Lamirande *et al.*, 1997). A number of physiological

factors, such as Ca^{2+}, cAMP, bicarbonate and metabolic substrates, are essential for the initiation and maintenance of hyperactivated motility *in vitro*. Human cervical mucus also induces sperm capacitation and hyperactivation (Zhu *et al.*, 1994). Ca^{2+} and cAMP not only induce hyperactivation they might also modulate protein phosphorylation (Tash, 1989). Tyrosine phosphorylation has been associated with heat-induced hyperactivation in human spermatozoa (Chan *et al.*, 1998).

1.9.3 Acrosome reaction

The process of the AR is of fundamental importance in the fertilisation of oocytes by spermatozoa. The AR is defined as the fusion of sperm plasma membrane and the outer acrosomal membrane. Physiological AR occurs upon interaction of the spermatozoon with the ZP3. AR as an exocytotic process in spermatozoa involves the fusion, vesiculation (fenestration) and loss of outer acrosomal membrane and its overlying sperm plasma membrane (Purohit *et al.*, 1999). This is followed by liberation of several acrosomal enzymes including hyaluronidase, acrosin, esterases, and neuraminidase that facilitate penetration of the zona and expose molecules on the sperm equatorial segment that allows fusion of sperm membrane with the oocyte (Baldi *et al.*, 2000). AR involves the anterior region of the sperm head and is not extended beyond the equatorial segment (Yanagimachi, 1994).

Under physiological and *in vitro* conditions, ZP3 stimulates AR in mammalian sperm. Progesterone and its analogue 17α-OH-progesterone, a major component of follicular fluid, has also been found to rapidly induce AR (Yanagimachi, 1994). AR can be induced in vitro by ionophores which exchange Ca^{2+} for other ions such as H^{+} and Na^{+} (Russell *et al.*, 1979). Fluxes of ions like sodium, chloride, hydrogen, and bicarbonate occur during the AR, suggesting that besides calcium, other ions also play a role in the process of calcium-dependent fusion of acrosomal membrane and the sperm plasma membrane (Purohit *et al.*, 1999).

While superoxide anion production is shown to be a part of ionophore induced AR (Griveau *et al.*, 1995), hydrogen peroxide is known to induce

hyperactivation and promote capacitation, but is not involved in the acrosome reaction of the spermatozoa (Griveau *et al.*, 1994).

1.10 Aims

The aims of the thesis were to investigate:

- The potency of chlamydial LPS compared with enterobacterial LPS against human spermatozoa.
- The role of two main fractions of LPS, lipid A and Kdo, on human spermatozoa.
- The role of chlamydial LPS, lipid A, and Kdo in stimulation of the TLR pathway and TNF-α production.
- The effect of LPS and its fractions in ROS production.
- The mechanism(s) of endotoxin-induced sperm death via necrosis, apoptosis, or both.

Chapter 2

Comparison between the spermicidal activities of several LPSs including that from *C.trachomatis*

2.1 Introduction

Bacterial infections have long been recognised as having an association with infertility. Bacteria are also capable of agglutinating and immobilising spermatozoa (del Porto *et al.*, 1975). In Gram-negative bacteria, LPS as a biologically active constituent of endotoxin is one of the major structural and immunodominant molecules of the outer membrane. LPS plays several roles in the pathogenesis of Gram-negative bacterial infections. First, it is a permeability barrier that is permeable only to low molecular weight, hydrophilic molecules. Secondly, it impedes destruction of the bacterial cells by serum components and phagocytic cells. Thirdly, LPS plays an important role as a surface structure in the interaction of the pathogen with its host.

LPS consists of three components or regions, lipid A, core region and an O antigen (more details in chapter 1). Both lipid A (the toxic component of LPS) and the polysaccharide side chains (the immunogenic portion of LPS) act as determinants of virulence in Gram-negative bacteria. The involvement of the polysaccharide chain in virulence is shown by the fact that small changes in the sugar sequences in the side chains of LPS result in major changes in virulence. The chemical composition of LPS varies widely among bacterial species. Regarding this diversity in the chemical structure of LPS, in this chapter the effect of four different kinds of LPS including, *C. trachomatis, E. coli, Klebsiella pneumoniae,* and *Serratia marcescens,* were investigated for their effect on human spermatozoa. Enterobacterial LPS were chosen as they would be derived from bacteria which could be found in the male urogenital tract and also the chemical structure of LPS from a great number of enterobacterial species have revealed common structural features (Haeffner-Cavaillon *et al.*, 1989).

Hosseinzadeh *et al.*, (2000 and 2001) showed that chlamydial EBs can have a direct and detrimental effect on sperm physiology. Also, Galdiero *et al.*, (1994) and Hosseinzadeh *et al.*, (2003) demonstrated that *C.trachomatis* LPS can kill spermatozoa.

E. coli is the most common cause of urinary tract infections and is also responsible for many different gastrointestinal diseases including travellers' and infantile diarrhoea.

K. pneumoniae is an opportunistic pathogen causing bacteraemia, pneumonia and urinary tract infections in humans. Often associated with nosocomial infections, it is second only to *E. coli* as the culprit behind Gram-negative sepsis syndrome (Hansen *et al.*, 1998) and is a major cause of mortality in hospital-acquired infections.

The human opportunistic pathogen *S. marcescens* is a bacterium with a broad host range, and represents a growing problem for public health. *S. marcescens* is a recognized nosocomial pathogen that causes pneumonia, septicaemia, meningitis, and urinary tract infections (Bollmann *et al.*, 1989).

Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins are heat stable (boiling for 30 minutes does not destabilize endotoxin), but certain powerful oxidizing agents such as superoxide, peroxide and hypochlorite, degrade them (http://www.smtl.co.uk/MDRC/Gloves/Endotoxin).

2.2 Aims

The association of pathogenic bacteria with male infertility has been noted by researchers. The purpose of the present study was to compare the potency of the LPS of *C.trachomatis*, *E.coli*, *K. pneumoniae,* and *S. marcescens* against spermatozoa and also to investigate the possible effect of heat-treated chlamydia EBs on spermatozoa.

2.3 Materials and methods

2.3.1 Cell culture technique

2.3.1.1 Growth and maintenance of the McCoy cell line

For the growth of *C.trachomatis*, McCoy cells were used. These cells are a mouse fibroblast cell line that is widely used for the propagation of *C.trachomatis* (Schachter and Wyrick, 1994). The McCoy cells were grown at 37°C in 5% CO_2 in 25 cm^3 (for the growth of serovar E), 75 cm^3 (for the growth of serovar LGV) plastic disposable culture flasks (Sterilin Ltd., Felthan, UK), and 1ml Trac bottles with coverslips (for the quantification of EBs) in McCoy growth medium [Minimum Essential Medium Eagle ([EMEM], Cambrex Biosciences, Wokingham, UK)], which contained 10% foetal calf serum (Bioclear, Mile Elm, UK). The cells were subjected to passage as follows:

A laminar flow cabinet was switched on for 15 min before use and all surfaces in the hood were disinfected with methyl alcohol. After checking the confluency of the cells by using a light microscope, the growth medium was discarded, the cells were washed with 1 x phosphate buffer saline [(PBS), 1 x working solution containing 0.14M sodium chloride, 0.003M potassium chloride, 0.002M potassium phosphate, and 0.01M sodium phosphate] twice and they were then treated with sufficient volume of Trypsin-Versene mixture (Cambrex, UK) to coat the cell sheet. The cells were then incubated at 37°C for 5 min to detach from the surface of culture flasks. The detached cells were resuspended in 10-20 ml growth medium and the flasks were then incubated at 37°C in 5% CO_2 for 24-48h until they were found to be confluent.

2.3.1.2 Culture of *C. trachomatis* serovars E and LGV on the confluent McCoy cells

The growth medium from tissue culture flasks was replaced by maintenance medium [EMEM in 10% foetal calf serum and 2μg/ml cycloheximide (Sigma, UK)], and from fresh or frozen stocks of *C. trachomatis* EB 50-100 μl (~10^7 EBs/ml) were added to the flasks separately. The flasks containing serovar E were centrifuged at 4000 x g for 1h and then incubated for 48-72h. The flasks containing serovar LGV

were incubated directly. The tissue culture flasks were then checked under a light microscope to look for chlamydial inclusions.

2.3.2 Purification of chlamydial EBs

The EBs were isolated from McCoy cells by density gradient centrifugation as described by Caldwell *et al.*, (1981). Infected McCoy cells were detached from the tissue culture using a cell scraper (Corning Costar Co, Boston, USA) and the resulting suspension disrupted by three bursts of sonication, 30 sec at amplitude of 12 microns interspersed by 1 min intervals to allow the dissipation of the heat generated during sonication, using a sonicator (MSE, Liverpool, UK). The suspension was then centrifuged at 500 x g for 15 min and then transferred to a plastic centrifuge tube (Beckman, High Wycombe, UK) before centrifuging it again at 30, 000 x g for 1h at 4°C. The resulting pellet was then resuspended in 1 ml PBS and sonicated as before and was then made up to a volume of 8ml with PBS. The chlamydial suspension was placed on the top of 8ml urografin® 150 (Schering HealthCare Ltd, Burgess Hill, UK) in a Beckman centrifuge tube and the suspension centrifuged again as above. The final pellet was then gently washed by adding 1ml of PBS without disturbing the pellet, before being resuspended in 2–3 ml of 2-sucrose phosphate transport buffer [(2SP); 74.62 g/l sucrose, 0.512 g/l KH_2PO_4, 1.23 g/l K_2HPO_4 in one litre of glass distilled water (GDW) and filter sterilised (0.2 nm filter pore size)]. The solution was then vortexed and sonicated as above and 200 µl aliquots were kept at –70°C for further use.

2.3.3 Quantification of infectious forming units in EB preparations

McCoy cell monolayers were prepared in Trac bottles. Prior to infection, the growth medium was replaced with maintenance medium and the Trac bottles were subsequently infected with a dilution series of purified EBs (10^{-2} to 10^{-6}). Following incubation for 48h (37°C, 5% CO_2), monolayers were fixed using 70% (v/v) ethanol for 5-10 min. The ethanol was decanted and the cells were then washed with PBS. The coverslips were removed using forceps and placed on microscope slides with the

cell monolayer facing upward. 20 μl of IMAGEN chlamydia fluorescent stain (DAKO, Ely, UK) was added onto the monolayer and slides were incubated at 37°C for 30 min in a moist box. Coverslips were gently washed with PBS to remove any excess reagent and mounted using glycerol with 0.1 M Tris, pH 8 (1:1, v/v). Slides were viewed using a fluorescent microscope with the FITC filter and the number of inclusions [(represented by apple green vacuoles within red counterstained cells) (Figure 2. 1)] were counted for 25 fields at x 400 magnification. The number of inclusion forming units per coverslips for each dilution was determined and used to calculate an approximate number of IFUs/ml.

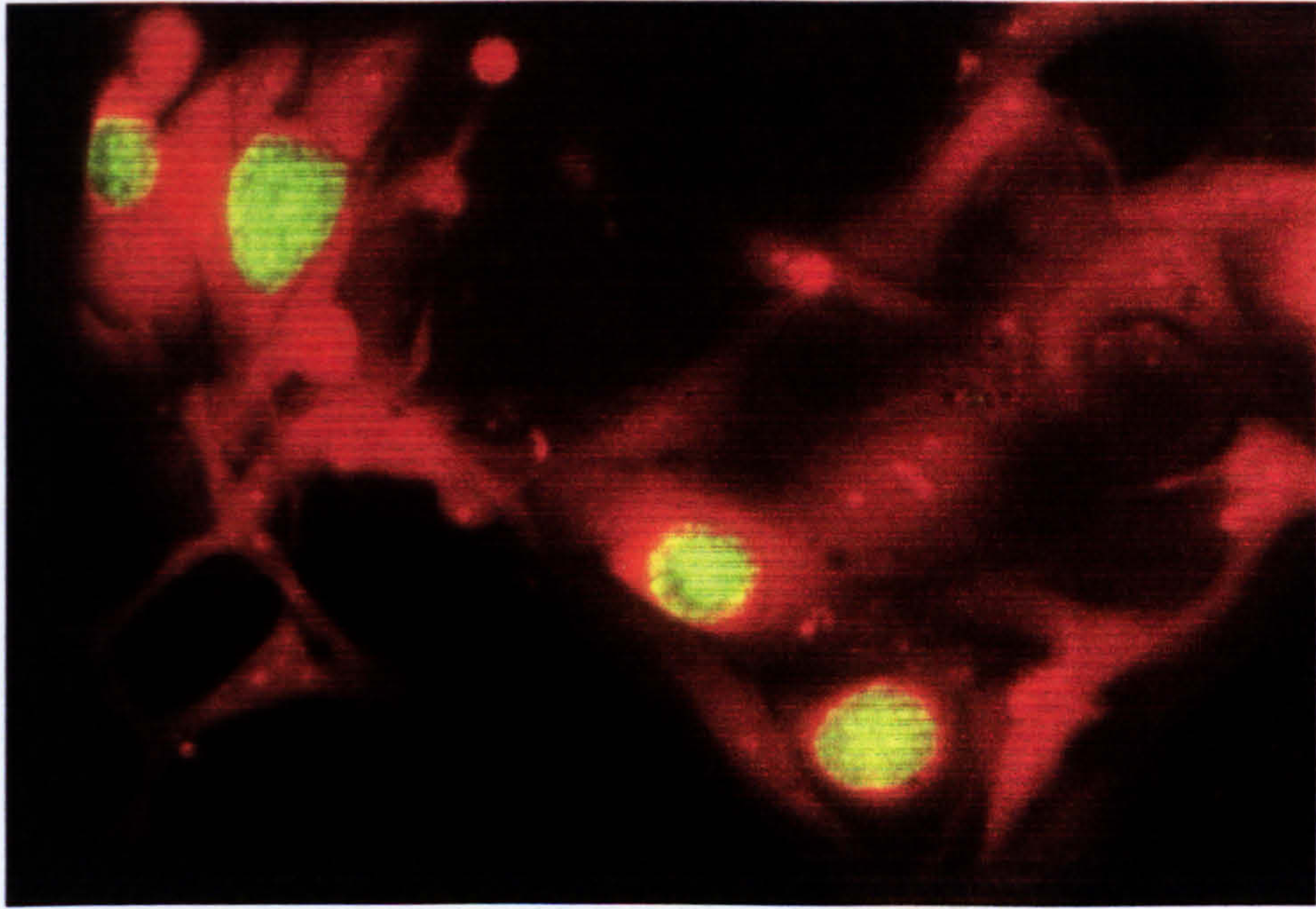


Figure 2. 1. Immunofluorescence staining of *C.trachomatis* inclusion bodies in McCoy cells. Four inclusions (apple green vacuoles) are shown.

2.3.4 LPS extraction from purified *C. trachomatis* EBs

C. trachomatis LPS was extracted as previously described by Nurminen *et al.* (1985) with a few modifications. The purified EBs from serovars E and LGV were suspended in 2.5 ml of 90% aqueous phenol and homogenised for 5 min followed by magnetic stirring at 50°C for 30 min to dissolve and partition the EB proteins from the LPS. 4 ml petroleum ether (b.p. 90 to 100° C) and 2.5 ml of chloroform were then added to the mixture and stirring was continued for 1h at room temperature (RT). After centrifugation at 10000 x g for 30 min, the supernatant was freed from organic solvents under reduced pressure using a rotary evaporator, and the remaining phenol phase was precipitated with 10 volumes of acetone at -20°C overnight followed by centrifugation at 10000 x g for 30 min. The precipitate (LPS) was washed twice with cold acetone, dried under vacuum and kept at -20°C after resuspension in sufficient volume of 2SP with 0.1% triethylamine.

2.3.4.1 Detection of extracted LPS by polyacrylamide gel electrophoresis (PAGE)

To confirm the successful extraction of LPS, 20 µl of the LPS was then run on a 14% polyacrylamide gel at 150 V according to the following method:
Before preparing polyacrylamide gels, glass plates, dividers, and the comb were washed, dried and cleaned with 70% (v/v) methanol. The recipe for making a 14 % resolving gel and a 1 cm 5% stacking gel is as follows (volume shown was sufficient for two gels).

2.3.4.2 14 % Resolving gel

30 % Acrylamide[1]	4.66 ml
1.5 M Tris-HCl (pH 8.8)[2]	2.5 ml
Distilled water	2.64 ml
TEMED[3]	5 µl
10 % (w/v) Ammonium persulphate[4]	0.35 ml

1- A 30% solution (electrophoresis reagent) of acrylamide/bis-acrylamide was purchased from Sigma and stored at 4° C.

2- pH was adjusted with 1 M HCl.

3- N,N,N',N'-Tetramethylethylenediamine.

4- A fresh 10% (w/v) solution of ammonium persulphate was made up prior to gel preparation.

2.3.4.3 5% Stacking gel

30 % (w/v) Acrylamide	1.3 ml
0.5 M Tris-HCl (pH 6.8)	2.1 ml
Distilled water	4.4 ml
TEMED	17 μl
10 % (w/v) Ammonium persulphate	100 μl

2.3.4.4 Running buffer

A 5 x stock solution of running buffer was prepared as follows; 24g Tris, 115g glycine, 8g sodium dodecyl sulphate (SDS), and 2 litres GDW. The pH of the buffer was adjusted to 8.3 and stored at 4°C. Prior to use, a 1 x solution of running buffer was prepared with GDW.

2.3.4.5 Sample loading buffer

A stock solution of sample loading buffer (2 x) was prepared containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.125 M Tris-HCl (pH 6.8) and 0.005% bromophenol blue made up in GDW. 200 μl aliquots were stored at -20°C.

The PAGE apparatus and polyacrylamide gels were assembled and prepared according to the manufacturer's instructions (Bio-Rad, Hemel Hempstead, UK). To prepare polyacrylamide gels, GDW, acrylamide, Tris buffer, and TEMED were first mixed, and just before pouring the gel, ammonium persulphate was added. After

pouring the resolving gel, water-saturated butanol was overlaid on the surface of the gel to ensure a flat interface between the resolving and stacking gel. Once the resolving gel was set, butanol was removed using blotting paper, the stacking gel was poured and a suitable comb was placed into position. The gel was left to polymerise, the comb removed and excess liquid was removed from wells using fine strips of filter paper. Gels were then inserted into the gel running apparatus, samples were loaded and electrophoresed at a constant 150 V for approximately 1h.

2.3.4.6 Silver staining

The Bio-Rad silver stain kit was used for detection of LPS extracts. After LPS extracts were run on a 14 % PAGE gel, the gel was fixed either overnight statically or for 1 h shaking in 50 ml fixing solution (40 % (v/v) methanol, 10 % (v/v) glacial acetic acid). The gel was oxidised for 5 min in 50 ml of 0.7 % (v/v) periodic acid prepared in fixing solution and subsequently was washed 5 times in distilled water for 5 min. The gels were then washed twice in 20 ml of 10 % (v/v) ethanol, and then soaked in 50 ml of silver stain oxidiser (5 ml stock solution / 45 ml water) for 5 min. The gel (orange coloured) was washed repeatedly in water until the gel became clear, and the gel was incubated in 50 ml of silver reagent (5 ml stock solution / 45 ml water) for 20 min with gentle agitation. The silver stain was discarded and the gel was washed for 1 min in distilled water, this was followed by addition of 50 ml developer solution (3.2 g/ 50 ml water, pre-warmed at 37° C for 1 h prior to use) to wash off brown/orange colour residues after the addition of silver stain. LPS bands were developed by the addition of another 50 ml of developer and the reaction was stopped by addition of 5 % (v/v) glacial acetic acid when the bands appeared.

2.3.4.7 LPS quantification

A Limulus Amebocyte Lysate (LAL) (Cambrex Biosciences, UK) kit was used to quantify chlamydial LPS. The method was performed as recommended by the manufacturer. A micro-plate method with four standard endotoxin solutions was used as follows:

A solution containing 1 EU/ml (standard 1) endotoxin was prepared by diluting 0.1 ml of the endotoxin stock solution with (X-1)/10 ml of LAL water (X= endotoxin concentration of the vial). This solution was vigorously vortexed for at least 1 min before proceeding. 0.5 ml of standard 1 was transferred into 0.5 ml of LAL reagent water, giving a concentration of 0.5 EU/ml (standard 2). Another 0.5 ml of standard 1 was added to 1.5 ml of LAL reagent water, giving a concentration of 0.25 EU/ml (standard 3). Finally 0.1 ml of standard 1 was transferred into 0.9 ml of reagent water, giving a concentration of 0.1 EU/ml (standard 4). All standard solutions were vigorously vortexed for 1 min before use.

The micro-plate was pre-warmed at 37°C in a heating block. 50μl of samples or standards were dispensed into the appropriate micro-plate well. Each series of samples included a blank plus the four endotoxin standards run in duplicate. The blank wells contained 50μl of LAL reagent water instead of samples. All reagent additions and incubation times were identical. At time 0, 50μl of LAL was added to all micro-plate wells. The wells were then pipetted gently to facilitate mixing. At time 10 min, 100 μl of substrate solution (pre-warmed to 37°C) was added to each well and pipetted as before. At time 16 min, 100 μl stop reagent was added to stop the reaction. The absorbance of each microplate well was read using the UV-visible spectrophotometer (Shimadzu, Tokyo, Japan) at 405 nm. All assays were performed in duplicate. The Table 2. 1 and Figure 2. 2 show a dilution scheme for the construction of standards from the endotoxin supplied in the kit and the graphic method to calculate the concentration of LPS respectively.

Endotoxin stock solution	Endotoxin std. solution 1 EU/ml	LAL reagent water	Endotoxin concentration (EU/ml)
0.1 ml	-	(X-1)/10 ml	1.0
-	0.5 ml	0.5 ml	0.5
-	0.5 ml	1.5 ml	0.25
-	0.1 ml	0.9 ml	0.1

Table 2. 1. Dilution scheme for the preparation of standards from the endotoxin supplied in the kit. EU= Endotoxin unit, 10 EU= 1 ng, X= endotoxin concentration of the vial. The initial dilution from the endotoxin stock was 1/X. This yielded an endotoxin solution containing 1.0 EU/ml. In this study for example, the potency of endotoxin was 23 EU/ml. Accordingly, the initial dilution was 1/23 or 0.1 ml of endotoxin stock into 2.2 ml of LAL reagent water.

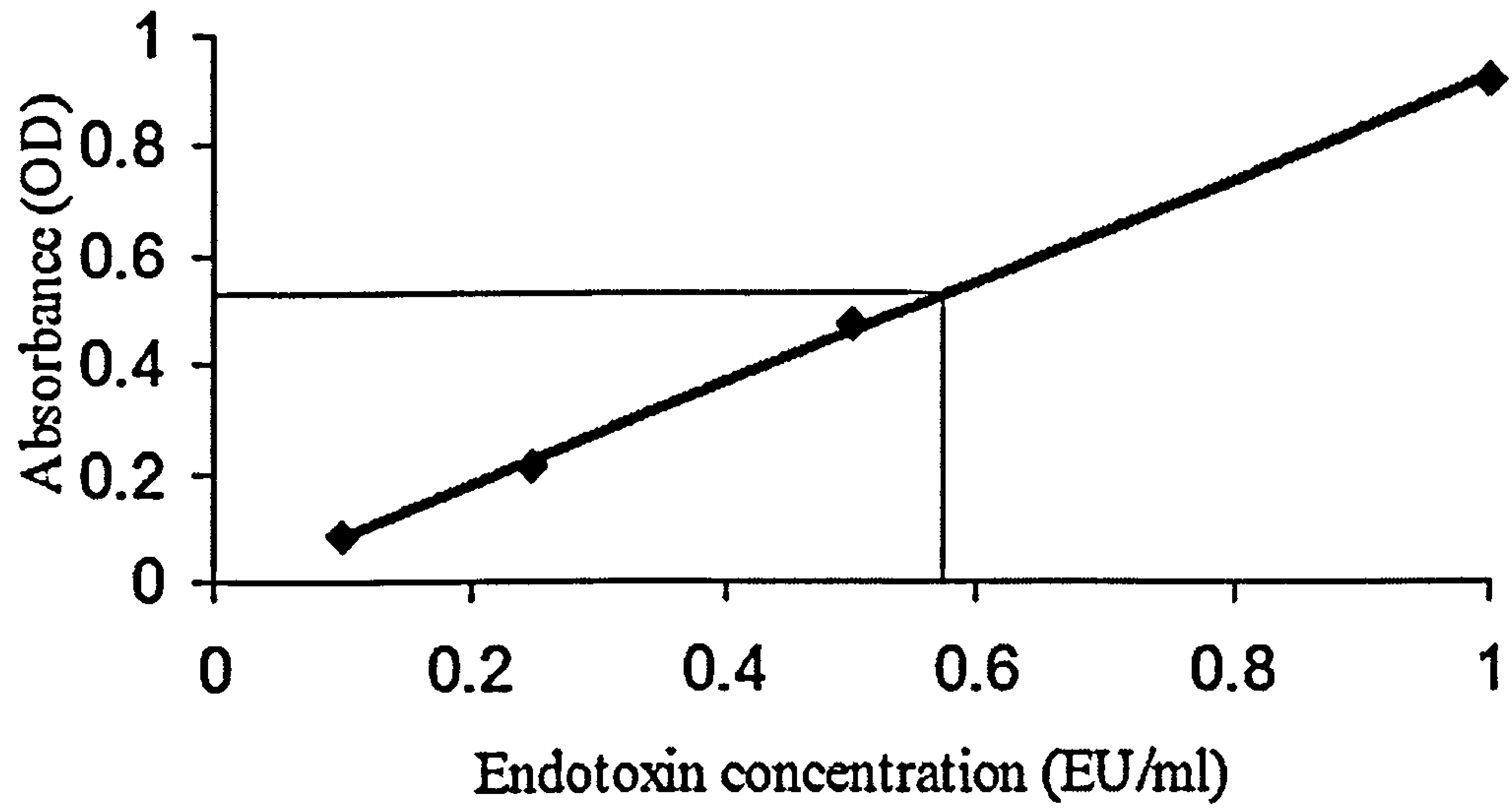


Figure 2. 2. Graphic method to determine the endotoxin concentration of samples. The absorbance at 405 nm was linear in the concentration range of 0.1 to 1 EU/ml (standard 4 to standard 1). The corresponding endotoxin concentration of the samples was determined from their absorbance.

2.3.5 Human sperm preparation

The human ejaculate is comprised of a mixture of seminal plasma, mature and immature spermatozoa, non-reproductive cells, various micro-organisms and non-specific debris. There are several sperm separation methods available. These include simple washing, sperm migration into culture medium (swim-up), Sephadex and glass wool columns and density gradient centrifugation [(single- (90%) or two-layers (40/80%) Percoll)]. All of these techniques are capable of effectively separating sperm from the seminal plasma, but to varying degrees (Centola *et al.*, 1998; Adiga and Kumar, 2001). Discontinuous (two-layer) Percoll gradients were shown to select sperm with better motion characteristics, increased hyperactivation and improved longevity compared with swim-up (Saad and Guerin, 1992; Moohan and Lindsay, 1995). In all experiments in this study the two-layer Percoll gradient method was used as follows:

Semen samples were collected from normozoospermic male obtained from the Infertility Clinic at the Jessop Wing, Royal Hallamshire Hospital, Sheffield. Ethical approval for the use of semen samples in this study was granted by the South Research Ethics Committee (project number 02/337). The samples were obtained by masturbation and allowed to liquefy for 30 min at 37°C. All samples had a concentration of > 20 x 10^6 with >30% ideal morphological forms by the World Health Organization (WHO) 2000 criteria (WHO, 2000). A summary of the reference values of semen variables are shown in Appendix 1.

Firstly, a 100% Percoll solution was prepared and diluted to 80% (v/v) and 40 % (v/v) percoll fractions using 1 x Earl's balanced salt solution (EBSS) (Invitrogen Ltd, Paisley, UK) containing 0.3% (w/v) Bovine serum albumin (BSA). The consistuents of 10 x EBSS, 40%, 80%, and 100% Percoll are as follows:

2.3.5.1 Constituents of 10 X Earl's balanced salt solution (EBSS)

KCl	0.08g
NaCl	1.36g
$NaH_2PO_4.H_2O$	0.0242g
D-Glucose	0.2g
GDW	20ml

(Sterile-filtered by 0.2 nm filter pore size)

2.3.5.2 Constituents of 100% Percoll

10 x EBSS	1 ml
Percoll (Sigma, UK)	9 ml
Bovine serum albumin (BSA)	30 mg
Sodium pyruvate	0.3 mg
Sodium lactate (60% syrup)	0.037 ml
Sodium bicarbonate	20 mg

2.3.5.3 Constituents of 80% Percoll

100% percoll	6 ml
1 x EBSS (contains 0.3% BSA)	1.5 ml

2.3.5.4 Constituents of 40% Percoll

100% Percoll	4 ml
1 x EBSS (contains 0.3% BSA)	6 ml

Carefully, 1ml of 80% Percoll was pipetted into a 15 ml sterile conical-bottomed tube (BD Biosciences, San Jose, USA). Gently, 1 ml of 40% Percoll was added to the top of the 80% Percoll layer. The interface was clearly visible between

the two layers. One ml of semen was deposited gently onto the top of the Percoll gradient and centrifuged at 500g for 20 min. After centrifugation, all supernatant was removed and the pellet with highly motile sperm was resuspended in pre-warmed 1 x EBSS (containing 0.3% BSA) and adjusted to 5 x 10^6 sperm/ml.

2.3.6 Comparison of spermicidal activities of four different LPSs

15 x 180 µl aliquots of sperm suspension were prepared. To two aliquots, 20 µl of *C.trachomatis* LPS from serovars E and LGV were added, giving a final LPS concentration of 0.1 µg/ml. To 12 further aliquots, 20 µl of *E. coli* 055: B5 (Sigma, UK), *K. pneumoniae* (Sigma, UK), and *S. marcescens* (Sigma, UK) LPS were added, giving a final concentration of 0.1, 10, 25, and 50 µg/ml for each LPS. Finally, to the last aliquot, 20 µl of 1 x EBSS was added as a negative control. All aliquots were maintained at 37°C in 5% CO_2 for 6h. After 6h incubation the sperm viability was measured using the HOS test described on page 64.

2.3.7 The spermicidal activity of heated *C.trachomatis* EBs

The purified EBs from serovars E and LGV were heated at 37°C, 56°C, and 100° C separately for 30 min using a heated-block. 9 x 180 µl aliquots of 5 x 10^6 sperm suspension were prepared. To 6 aliquots, 20 µl of heat-treated purified EBs from both serovars at the above-mentioned temperature were added, giving a final concentration of 6 x 10^5 EBs/ml. To another two aliquots, 20 µl of non-heated purified EBs from both serovars were added at a similar concentration. 20 µl of 1 x EBSS was added to the last aliquot as a negative control. The aliquots were kept at 37°C in 5 % CO_2 for 6 h and the sperm viability was assessed by the HOS test.

2.3.8 Inhibitory effects of polymyxin B on the chlamydial EB

12 x 180 µl aliquots of 5 x 10^6 sperm suspension were prepared as described earlier. To 4 aliquots, 20 µl of heat-treated purified EBs at 56°C and 100°C from serovars E and LGV was added at a final concentration of 6 x 10^5 EBs/ml. To another 4 aliquots of heat-treated purified EBs at 56°C and 100°C from both serovars, polymyxin B (PMB) was added to give a final concentration of 100 µg/ml. In the

control aliquots, 20 µl of non-heated purified EBs from both serovars at a similar concentration, 20 µl of 1 x EBSS and 100 µl/ml of PMB were added separately. The aliquots were kept at 37°C in 5 % CO_2 for 6h before removing 20 µl of each aliquot for the evaluation of sperm viability.

2.3.9 Measurement of sperm viability by the Hypo-Osmotic Swelling test (HOS)

The viability of spermatozoa was determined using the HOS test (WHO, 2000). This test is based on the semi-permeability of the intact sperm cell. Live spermatozoa will swell when placed in a hypo-osmotic solution which causes curling in its tail due to bulging of its plasma membrane, non-viable spermatozoa do not swell. 20 µl of each incubate was transferred to 200 µl of pre-warmed HOS solution and incubated at 37° C for 30 min. Each incubate was then spotted onto a microscope slide and allowed to air dry overnight followed by fixing in absolute methanol for 45 min. For each incubate or control, 100 spermatozoa were counted to establish the viability of the samples. The spermatozoa with swollen, curled-up tails were considered viable with intact membrane integrity and represented as percentage HOS positive (WHO, 2000).

2.3.9.1 Constituents of HOS solution

Sodium citrate dehydrate	0.735g
Fructose	1.351g
GDW	100ml

The aliquots of this solution was stored at -20°C, thawed and mixed well before use.

2.3.10 Statistical analysis

The results of all experiments in this study were analysed using GraphPad InStat software version 3.0 (GraphPad Software, Inc. USA). The statistical significance of differences between two means was evaluated by Student's *t* test,

and p values of > 0.05, < 0.05, < 0.01, and < 0.001 were considered not significant (NS), significant (*), very significant (**), and extremely significant (***) respectively.

2.4 Results

2.4.1 Detection of extracted LPS on a 14% PAGE gel

The LPS of *C.trachomatis* serovars LGV and E was extracted from purified EBs. The LPS was then run on a 14 % PAGE gel followed by staining with silver reagent (Figure 2. 3). LPS from *E.coli* O55: B5 was used as a control. Because *E.coli* LPS has polysaccharide side chains it produced a longer smear on the gel.

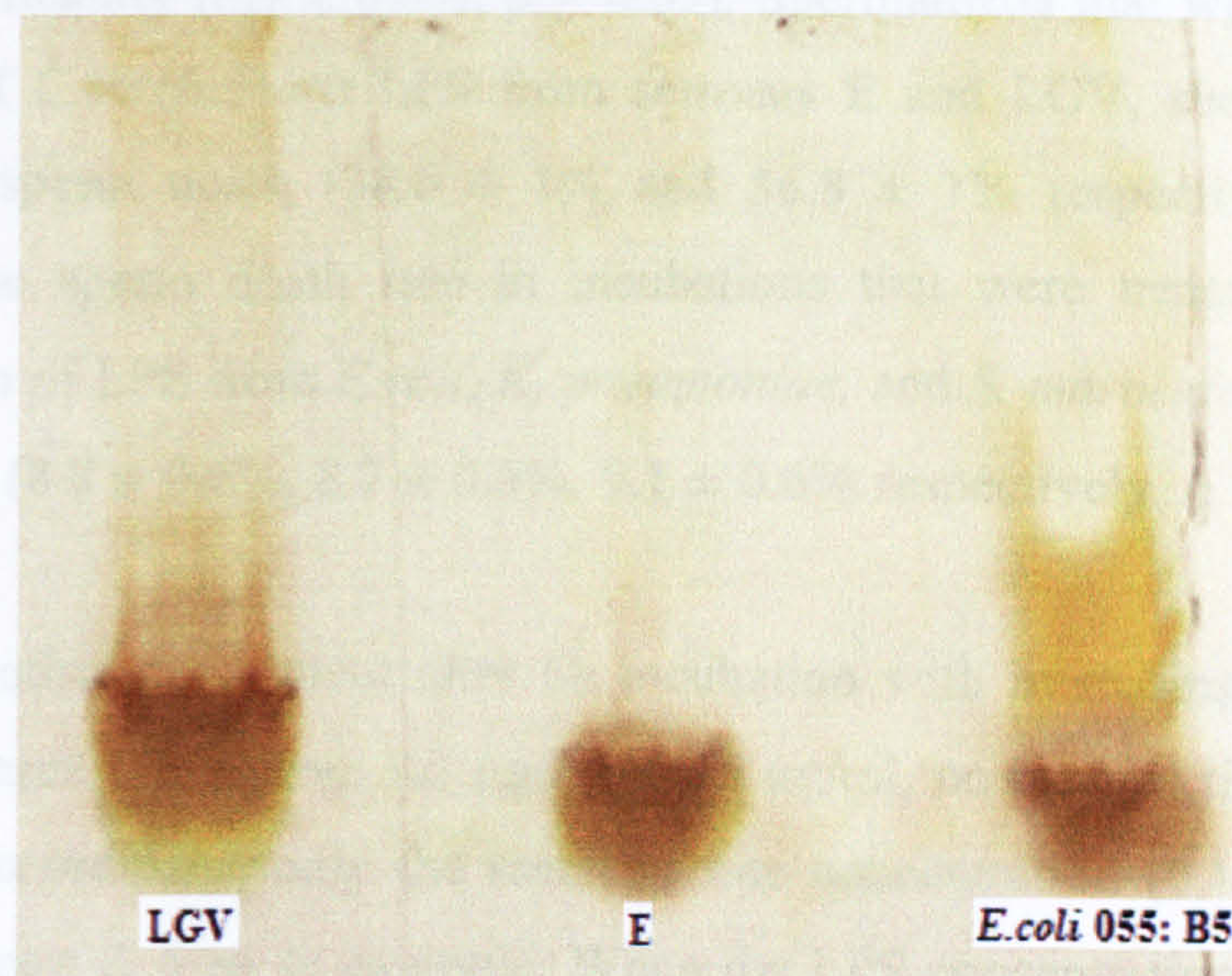


Figure 2. 3. The LPS of *C.trachomatis* serovars LGV and E and *E.coli* 055: B5 were electrophoresed through a 14% acrylamide gel and stained using the Bio-Rad silver stain kit.

2.4.2 *C.trachomatis* LPS quantification

To calculate the concentration of LPS extracted from *C.trachomatis*, a standard curve was drawn and the concentration of LPS (EU/ml) was calculated according to the standard curve (Figure 2. 2). The final concentration of LPS was recorded in μg/ml (10 EU = 1 ng). Depending on the concentration of purified EBs used for the LPS extraction, the final concentration of LPS ranged from 55 μg/ml to 345 μg/ml.

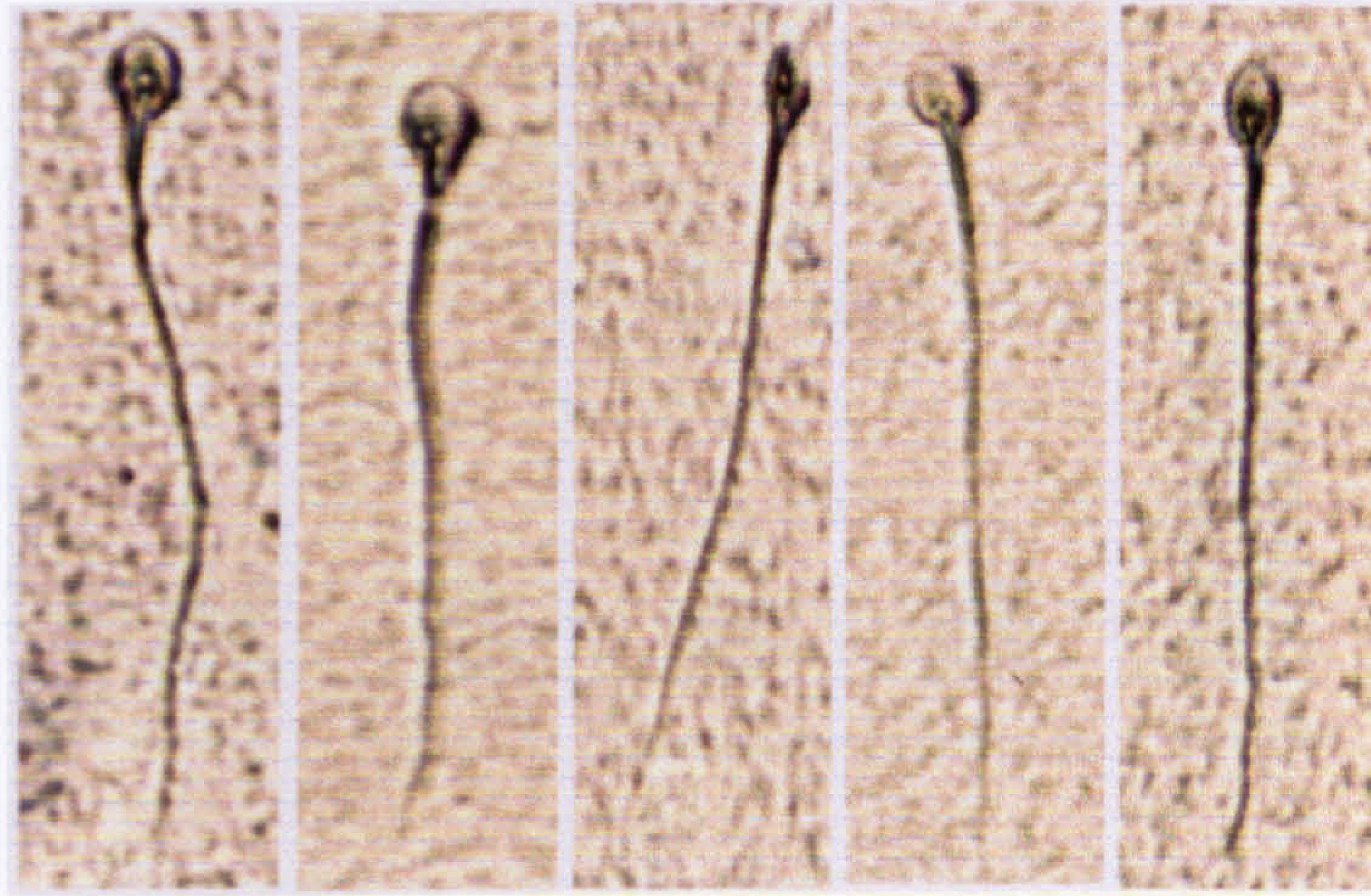
2.4.3 Interaction between spermatozoa and four LPSs

The sperm preparations after density gradient centrifugation had a mean sperm death of 8.7 ± 0.9%. Examples of viable and non-viable sperm using the HOS test are shown in Figure 2. 4.

After 6h incubation, the mortality rate in the control population did not change significantly (8.9 ± 0.6%, $p > 0.05$). Spermatozoa that were incubated with 0.1 μg/ml of *C.trachomatis* LPS from serovars E and LGV, showed a significant increase in sperm death (38.6 ± 1% and 36.8 ± 1% respectively, $p < 0.001$). However, the sperm death rate in incubations that were treated with the same concentration of LPS from *E.coli*, *K. pneumoniae*, and *S. marcescens* did not change significantly (8.8 ± 0.6%, 8.7 ± 0.5%, 9.1 ± 0.6% respectively, $p > 0.05$) (Figure 2. 5).

In another experiment after 6h incubation with increasing concentration of these latter three LPSs, from 0.1 μg/ml to 25 μg/ml, no marked rise in sperm death was seen. Accordingly, only the result of one concentration (0.1 μg/ml) has been shown in Figure 2. 5 as an example. When the LPS concentration was increased to 50 μg/ml for *E.coli*, *K. pneumoniae*, and *S. marcescens*, spermatozoa showed a significant decrease in viability (21 ± 0.5%, 22.3 ± 1.9 %, 22.3 ± 1.1% respectively, $p < 0.001$) (Figure 2. 5).

A



B

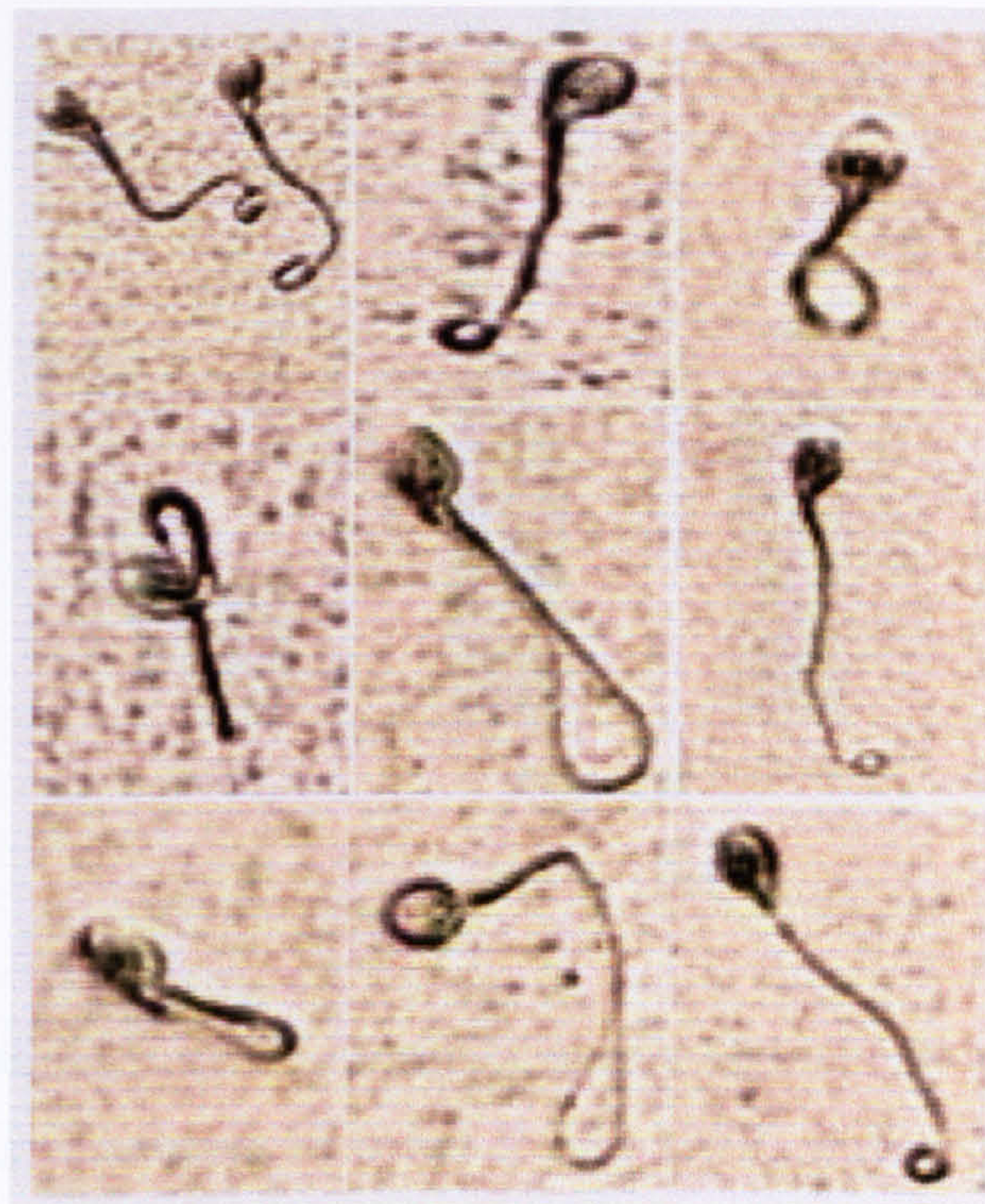
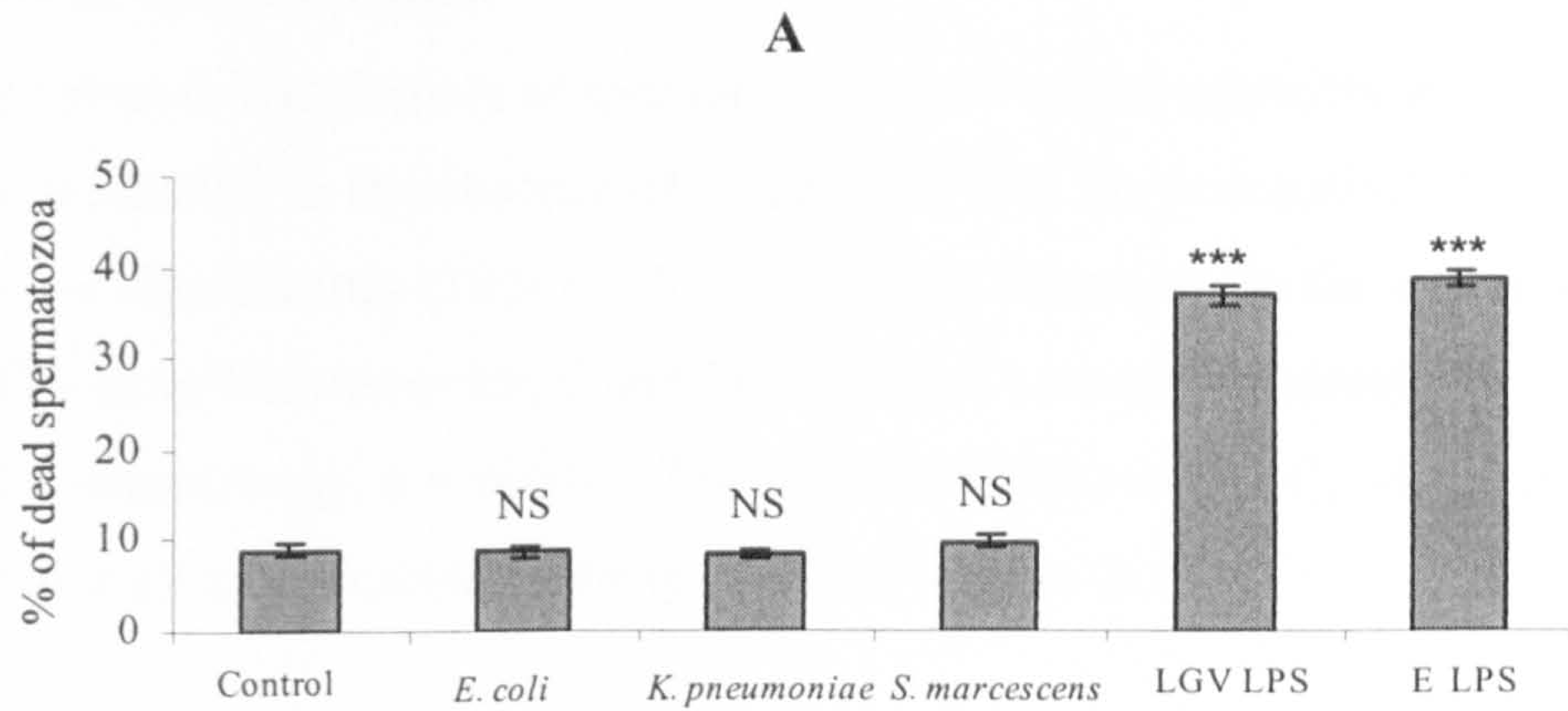


Figure 2. 4. HOS test is based on the semipermeability of the intact sperm cell. A, dead spermatozoa showing straight tails. B, live spermatozoa demonstrating various types of curled tails.

A



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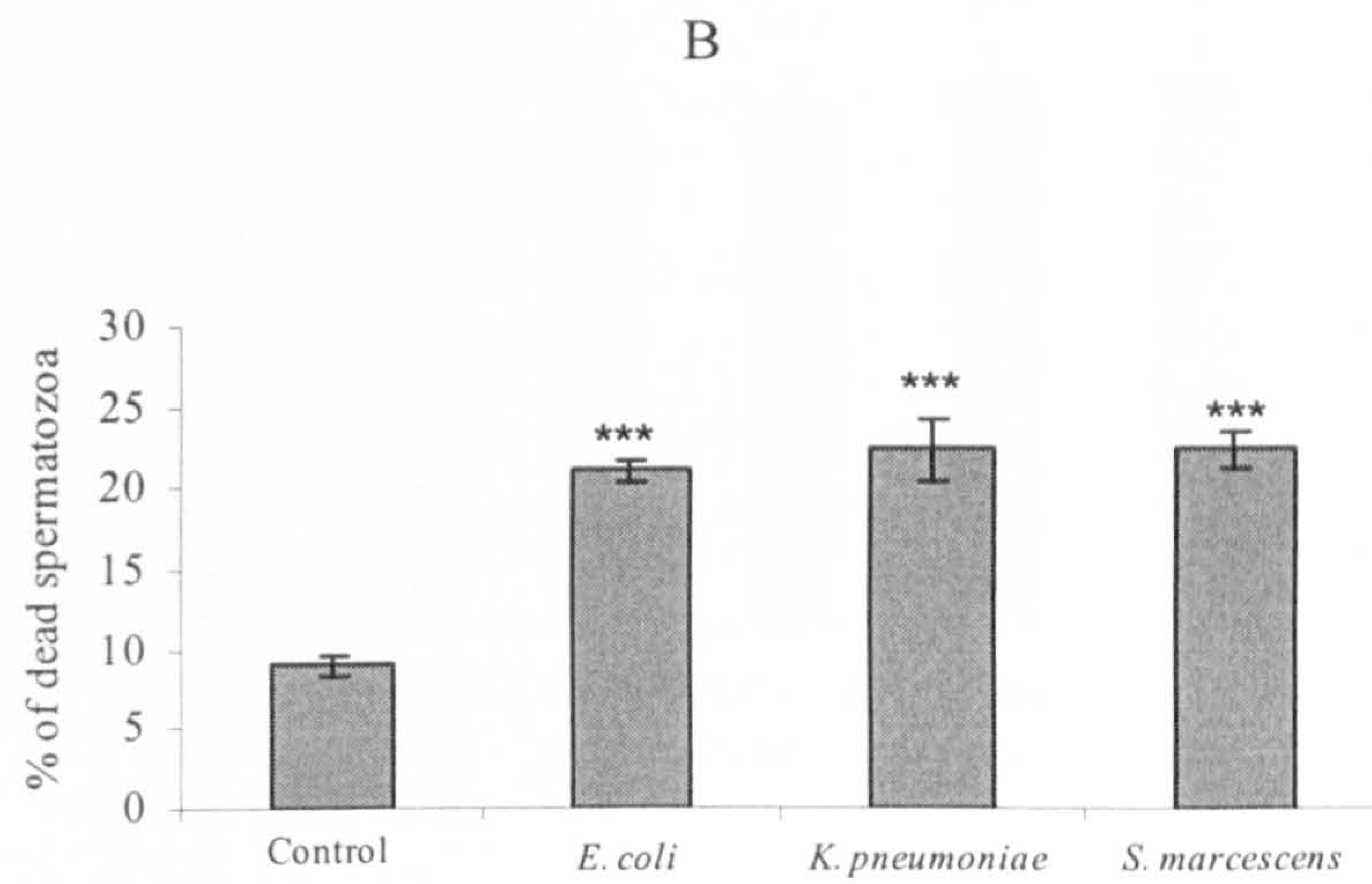


Figure 2. 5. Percentage sperm mortality over a 6h incubation period with 0.1 μg/ml of LPS from *C.trachomatis* serovars E and LGV, *E.coli*, *K. pneumoniae,* and *S. marcescens* (A) and 50 μg/ml of LPS from the last three LPSs (B). Results are mean of six experiments ± SEM. ***, significant difference between response to LPS and control, $p < 0.001$. NS, not significantly different from the control.

2.4.4 The effect of heating *C.trachomatis* EBs and their interaction with spermatozoa

At time 0, the aliquots of spermatozoa had a sperm mortality of 10.6 ± 1.9 %. After 6h incubation in the absence of chlamydial EBs the percentage of sperm death did not alter significantly (10.9 ± 1.1%, $p > 0.05$). However, in the presence of non-heated EBs from both serovars, E and LGV, values rose substantially (38.1 ± 2.1 and 34.3 ± 2.5 respectively, $p < 0.001$). The pre-heated EBs at 37° C, 56° C, and 100° C had a similar effect on spermatozoa ($p < 0.001$) (Figure 2. 6).

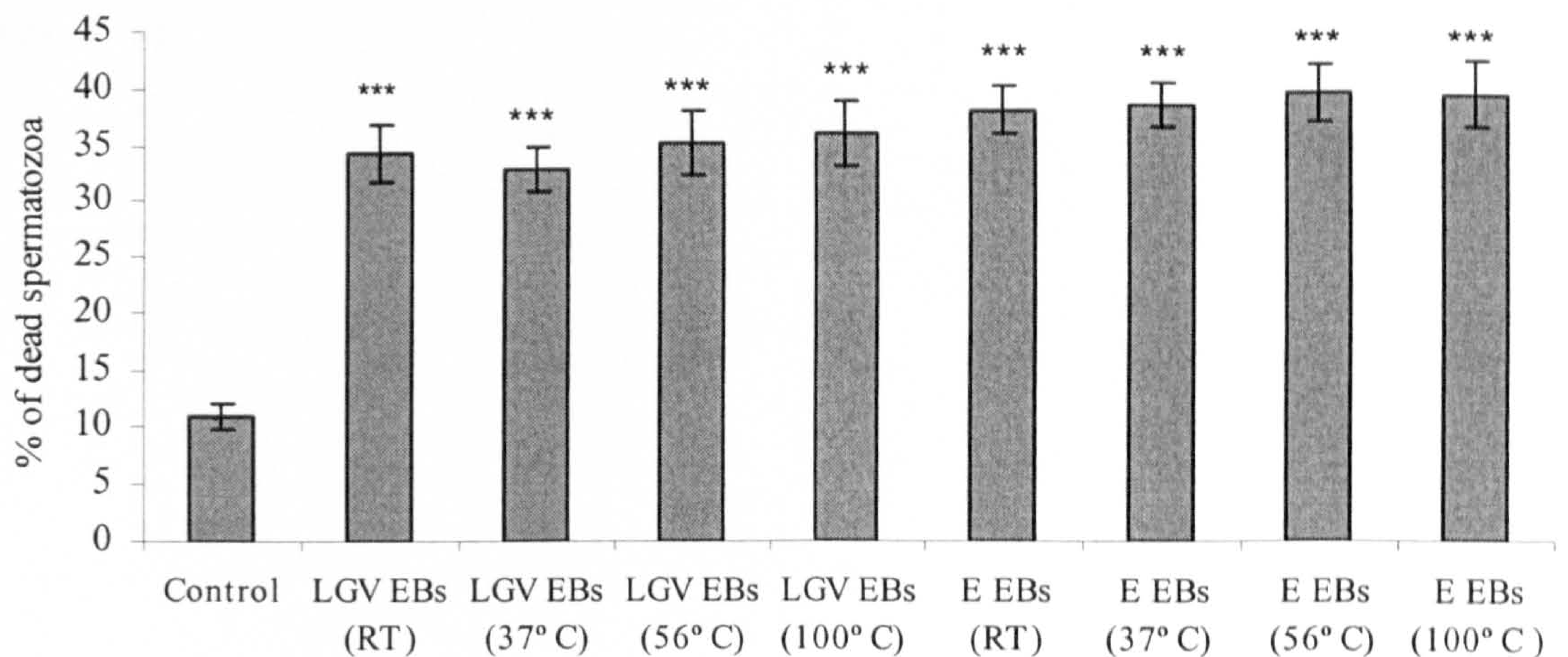
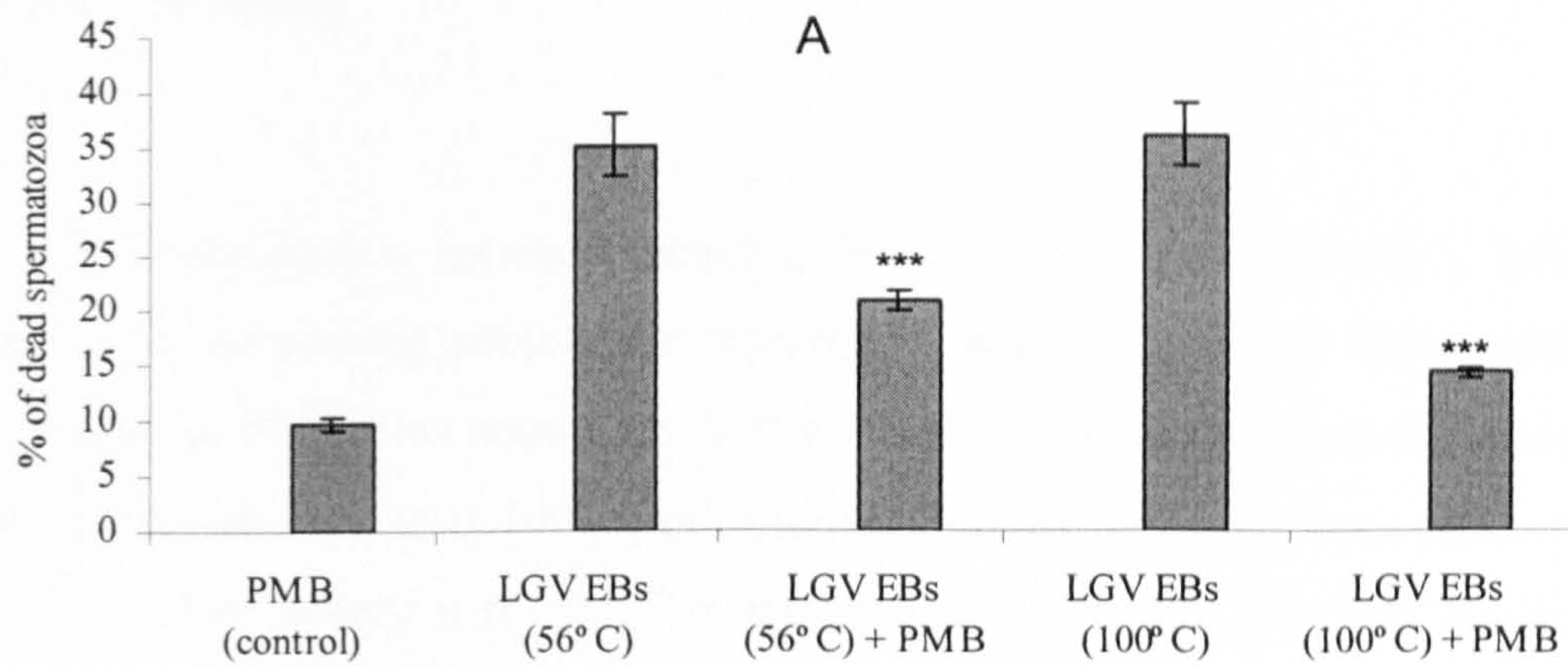


Figure 2. 6. Percentage sperm mortality over a 6h incubation period with 6×10^5 of heat-treated (37° C, 56° C, and 100° C) purified EBs from *C.trachomatis* serovars E and LGV. After 6h incubation all groups in the presence of EBs regardless of the temperature showed a marked decline in sperm viability ($p < 0.001$) in comparison with the negative control. Data represent the average of six experiments ± SEM. ***, significant difference between response to LPS and control, $p < 0.001$.

2.4.5 The effect of PMB on heated chlamydial EBs and their interaction with spermatozoa

In this experiment, the effect of 100 μg/ml of PMB on heated chlamydial EBs was investigated to confirm the spermicidal activities of heated EBs observed in Figure 2. 6 were due to LPS derived from EBs. Over the 6h incubation period, the sperm mortality rate of the negative controls (medium and PMB only) did not change markedly (12 ± 2.4%, 11.7 ± 2.9% respectively, $p > 0.05$) from that seen immediately after preparation (11.5 ± 3.1%). In the presence of non-heated E and LGV EBs this value altered significantly (36.8 ± 3.1% and 35.1 ± 1.7% $p < 0.001$). Interestingly, the mortality rate of spermatozoa in aliquots which were incubated with heated chlamydial EBs showed a dramatic reduction in the presence of PMB (Figure 2. 7).



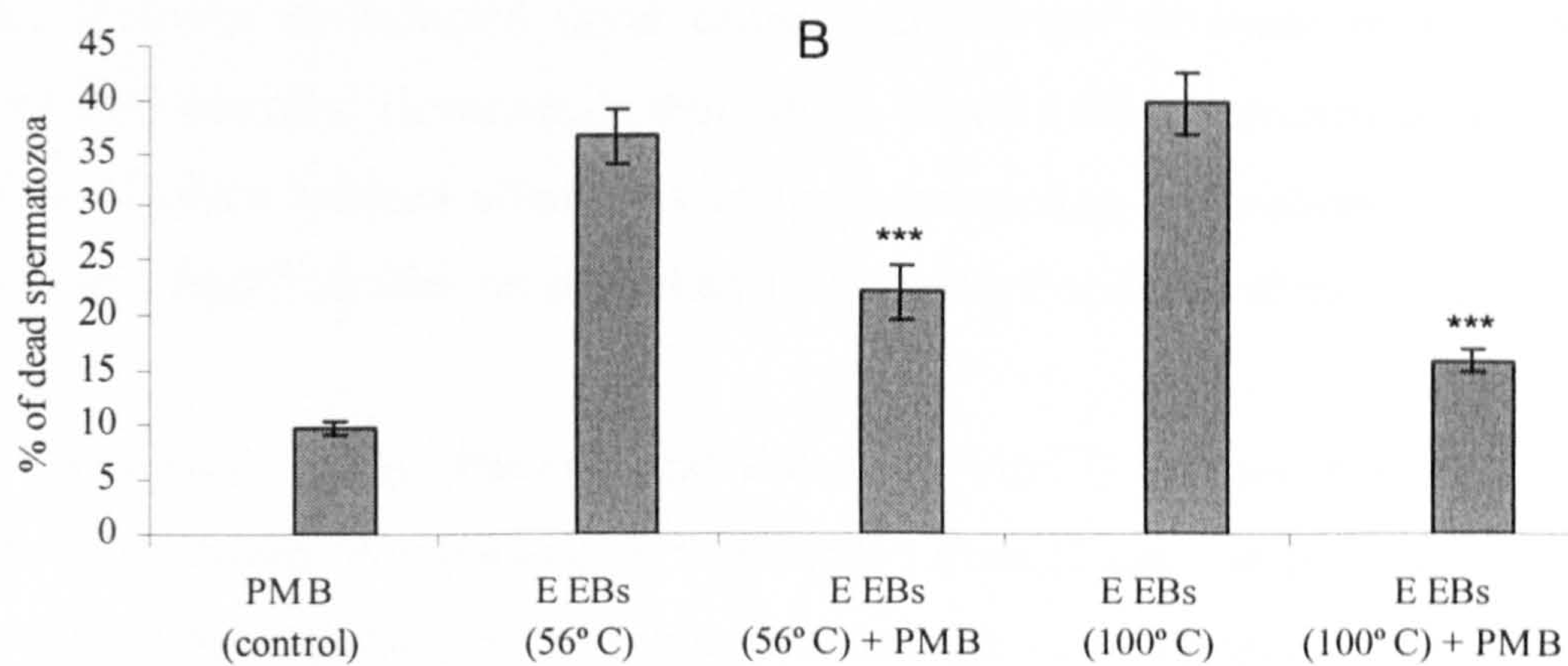


Figure 2. 7. Effect of PMB on heated chlamydial EBs serovars LGV (A) and E (B) interacting with sperm following a 6h incubation period. PMB, polymyxin B. Data shown for six experiments ± SEM. ***, $P < 0.001$).

2.5 Discussion

The relationship between bacterial infection and male infertility has been constantly an interesting subject for researchers who work in the fertility-sterility field. Dennis in 1962, was among the first workers who demonstrated that endotoxin isolated from *Vibrio fetus* at 100 μg/ml, immobilised spermatozoa from rams or bulls after 4h incubation however, the first reported study of the effect of bacteria on human spermatozoa goes back to about 10 years earlier. In their 1951 study, Matthews and Buxton showed that certain bacteria cultured from the cervix were spermicidal.

Twenty years later, Teague *et al.*, (1971) treated human spermatozoa with *E.coli* obtained from cervical cultures. They reported that *E.coli* isolated from either cervical cultures or infected urine cause a significant decrease in spermatozoa motility and viability. However, in their study, *E.coli* LPS at unstated concentration failed to produce marked alterations in the spermatozoa parameters. In 1975 Del porto *et al.*, found similar results when they exposed spermatozoa to *E.coli* at 10^6 cfu/ml.

In another study, Paulson and Polakoski (1977) isolated a low-molecular weight spermatozoal immobilisation factor (SIF) from *E. coli* cultures. However, the factor passed through the dialysis tubing and through a 500 molecular weight cut-off membrane, indicating that it was too small to be LPS. They showed this factor that was stable to heating, freezing, and lyophilization could immobilise spermatozoa.

Galdiero *et al.*, (1988) showed LPS extracted from *E. coli* killed about 80% of the spermatozoa at a concentration of 50 μg/ml after a 60 min incubation period. The influence of *E. coli* on human sperm motility was also studied by Diemer and co-workers (1996) *in vitro*. Using electron microscopy, they showed multiple adhesions of *E. coli* to spermatozoa, causing variable ultrastructural damage as probable morphological correlates of immobilisation.

The effect of another organism, *C.trachomatis*, on male infertility has been also investigated but with controversial results. While some studies with diagnosis based on mostly serologic methods have suggested that chlamydial infections

probably do not remarkably contribute to male infertility (Bjercke and Purvis, 1992; Eggert-Kruse *et al.*, 1997; Rezacova *et al.*, 1999), there are other reports that show this infection can compromise the sperm function (Galdiero *et al.*, 1994; Hosseinzadeh *et al.*, 2001). These workers showed *C. trachomatis* LPS as a major component of this organism could lead to a significant degree of sperm death during an *in vitro* incubation. By the eosin exclusion test, Galdiero and co-workers (1994) demonstrated that LPS extracted from *C. trachomatis* at 0.1 μg/ml causes an increase in spermatozoa mortality rate. In their previous experiments Hosseinzadeh *et al.*, (2000) suggested that *C. trachomatis* EBs increase tyrosine phosphorylation of sperm proteins that may be important for sperm function (Hosseinzadeh *et al.*, 2000) and the serovar E EBs also could lead to premature sperm death (Hosseinzadeh *et al.*, 2001). These workers also demonstrated that chlamydial LPS has a spermicidal effect and using polymyxin B, a polycationic antibiotic known to neutralise LPS effects (Morrison and Jacobs, 1976; Duff and Atkins, 1982), they confirmed that this effect is primarily as a result of LPS activity (Hosseinzadeh *et al.*, 2003).

Based on these considerations, in our study we incubated human spermatozoa with not only house-made *C. trachomatis* LPS and commercial *E.coli* LPS, but with commercial LPS from *K. pneumoniae* and *S. marcescens* in order to compare their potency against spermatozoa *in vitro*. The two latter LPSs along with *E.coli* LPS, belong to the *Enterobacteriaceae* family which are involved in urinary tract infections.

After 6h treatment of spermatozoa with these LPSs, the result of spermatozoa viability assessed by the HOS test showed that while *C.trachomatis* LPS at 0.1 μg/ml had a marked toxic effect on sperm viability, the other LPSs induced a significant increase in the percentage of dead spermatozoa only when their concentrations were increased to 50 μg/ml.

As was described, there are several reports regarding interaction between human spermatozoa and *C.trachomatis* LPS or *E.coli* LPS. However, little is known about the effect of the two other LPSs used in the present study. For example, to our knowledge, Dumoulin *et al.*, (1991) are the first researchers who investigated the effect of *S. marcescens* LPS on spermatozoa. In their study, they reported that these

LPS even at 500 μg/ml had no spermicidal activity and these findings contradict our results. However, there are two significant differences between their study and our investigation that might explain the controversial results of these two studies. Firstly, in their study they obtained spermatozoa pellet by centrifugation of semen using Whittingham's T6 culture medium supplemented with low-endotoxin bovine serum albumin that was totally different from the percoll gradient method that we used for sperm praparation. Secondly, to estimate the sperm survival, they counted the number of motile spermatozoa using a Makler counting chamber (see Appendix 2), however, we assessed sperm viability using the HOS test.

The difference in the concentrations of LPSs used in our investigation, possibly can be ascribed to their differences in chemical structure that are briefly described below.

There is evidence that shows *C.trachomatis* has a weak LPS with low activity (but not against spermatozoa):

a) the potency of *C.trachomatis* LPS in activating LPS-responsive cells is ~100 fold less than other bacterial LPS (Qureshi *et al.*, 1997).

b) The chlamydial LPS were at least 10 times less active than typical endotoxins using LPS antagonist or monoclonal antibodies against chlamydial LPS (Heine *et al.*, 2003).

The low endotoxic activity may be attributed to the presence of unusual long-chain fatty acids (C14–C21) in a penta-acyl arrangement and mono-phosphorylation of the diglucosamine backbone. While species expressing LOS typically attach between 10 and 20 sugars to their lipid A, chlamydial LPS makes do with only a Kdo tri-saccharide joined α-Kdo-2-8-α-Kdo-2-4-α-Kdo (Erridge *et al.*, 2002). Chlamydial LPS lacks the O-chain and this is another reason that possibly explain the low endotoxin activity of this organism.

The low potency of *C. trachomatis* LPS might help this organism to establish chronic infections like trachoma or upper genital tract infection. As LPS is an integral compound of the outer membrane of bacteria and therefore easily accessible to innate immune cells, it would be expected that the expression of highly stimulatory endotoxins results in severe and acute infections, whereas expression of low- or non-stimulatory endotoxins causes slowly progressing chronic infections.

Comparison of the known structures of core LPS from different *Enterobacteriaceae* reveals that the inner core structure contains two residues of 3-deoxy-D-*manno*-octulopyranosonic acid (Kdo*p*) and three residues of L-*glycero*-D-*manno*-heptopyranose (L,D-Hep*p*I, L,D-Hep*p*II and L,D-Hep*p*III) (Vinogradov and Perry, 2001). *E. coli* LPS has a hexa-acyl diphosphorylated lipid A with acyl chains of length C12–C14 (Erridge *et al.*, 2002). The properties of *K. pneumoniae* LPS include the unusual Kdo existing in the outer core, the existence of homo-polysaccharides in some O-chains, and bisphosphorylated lipid A substituted with six acyl chains of 12–16 carbon length (Erridge *et al.*, 2002). The chemical characteristics of *S. marcescens* LPS in terms of the size of repeating units, molecular weight and the presence of glucuronic acid, galacturonic acid, and pyruvate as acidic components, indicate that *S. marcescens* LPS closely resembles *Klebsiella* and *E.coli* LPS (Aucken *et al.*, 1997).

In addition to these differences in the chemical structure of LPSs from the above-mentioned bacteria, based on our investigations and the other relevant studies we would also suggest that the type of host cells, i.e. monocytes, spermatozoa, and etc, the conditions and environment where the host cells interact with pathogenic bacteria i.e. *in vitro* or *in vivo* possibly are important and may determine whether pathogens show a weak or a strong activity against their hosts.

Previous work from our group has demonstrated that *C.trachomatis* EBs are spermicidal. Using polymyxin B (a polycationic antibiotic known to neutralize LPS effects), they confirmed that the toxicity effect of EBs against spermatozoa was primarily a result of LPS activity which is released from EBs. It is well acknowledged that LPS is a heat-stable complex which could preserve its activities against host cells at boiling point for 30 min. Despite this fact, in one study by Teague *et al.*, (1971), it was shown that *E.coli* killed by boiling for 10 min, has no toxic effect on human spermatozoa, however, the dose of killed bacteria was not stated in their study.

Apart from LPS, a number of other bacterial cell components including porins and peptidoglycans from Gram-negative bacteria have been shown to have a toxic activity against spermatozoa (Galdiero *et al.*, 1988; Gorga *et al.*, 2001). In the

case of Gram- negative bacterial cells, the porin concentration is about 2 x 10^5 molecules of porins per cell, covering an area of approximately 1.8 μm^2 or roughly a third of the cell surface area (Galdiero *et al.*, 1990). Porins are able to induce immune and acquired immune responses and have diverse biological activities on several eukaryotic cell types. Porins are also capable of interfering with the host cell's apoptotic apparatus at different steps in the cascade of signal transduction (Galdiero *et al.*, 2003).

Contrary to LPS, most proteins are denatured above 50°C, a temperature well above the normal body temperature of 37°C. As the temperature is increased, a number of bonds in the protein molecule are weakened (www.life.sci. qut.edu.au./epping/).

In another experiment in the present chapter, we exposed human spermatozoa to heated-*C.trachomatis* EB to confirm the stability of LPS components from EBs against heating. Our findings demonstrated that the spermicidal activity of EBs were unaffected by heating at either 56°C or 100°C since the spermatozoa death rate did not alter significantly in the presence of heated-EBs compared to spermatozoa which were incubated with non-heated EBs. To inhibit the LPS toxicity and to rule out the effect of other bacterial components, which could be potentially toxic and interfere with our results, heated-EBs-induced spermatozoa were pre-incubated with 100 µg/ml of polymyxin B (PMB) in control groups. PMB interacts strongly with phospholipids and penetrate into and disrupt the structure of cell membranes. PMB also binds to the lipid A portion of endotoxin and inactivates this molecule (Brown and Wood, 1972; Warner *et al.*, 1985).

The results of control groups also showed that PMB could inhibit the spermatozoa mortality when incubated with heated-EBs. However, no activities were observed from other bacterial components suggesting that the spermicidal properties of heated-EBs could be possibly ascribed to the LPS. In conclusion, we demonstrated that the spermicidal activity of chlamydial LPS is about 500 times more active than that of enterobatcterial LPS *in vitro*. We also confirmed that chlamydial LPS, similar to other LPS is a heat-stable complex.

Chapter 3

The effect of two main LPS fractions, Kdo and lipid A, on human spermatozoa

3.1 Introduction

Endotoxin is a characteristic outer membrane entity of Gram-negative bacteria and a potent inducer of inflammatory responses. Exposure to even low amounts of LPS leads to a dramatic release of inflammatory mediators that are thought to be responsible for the deleterious effects in host cells. As described in chapter 1, LPS consists of three regions. The first and most essential part is lipid A, which is the hydrophobic anchor of LPS and has the same basic structure in practically all Gram-negative bacteria; it consists of a β-1, 6 linked D-glucosamine disaccharide bearing phosphoryl groups in positions 1 and 4', and ester and amide bound fatty acids. It forms the outer leaflet of the outer membrane and is responsible for most of the toxic activity of LPS including induction of cytokine expression by LPS (Rietschel *et al.*, 1987) and it is associated with Gram-negative septic shock (Trent, 2004). Lipid A from various Gram-negative bacteria express similar endotoxic features despite their chemical diversity (Tanamoto *et al.*, 2001).

The core, second region, including Kdo serves as a link between lipid A and the distal repeating-unit polysaccharide known as O antigen. The chemical structure of the core region, like lipid A, in LPS of many bacterial families is known to be common (Shimizu *et al.*, 1992). The core oligisaccharide constitutes a domain of limited structural diversity within the same bacterial genera, *e.g.*, only five unique core structures have been described for *E.coli* (Rietschel *et al.*, 1993).

The third moiety of the LPS molecules is the O antigen attached to the terminal sugar of the outer core composed of units of common sugars and is highly immunogenic. Only the Kdo and lipid A portions of LPS are required for cell growth and bacterial viability (Erridge *et al.*, 2002).

In order to maintain a functional outer membrane, LPS contains at least either an α-Kdo-(2→4)-α-Kdo disaccharide or a single Kdo (Brade *et al.*, 2002). The polysaccharide moiety expresses certain biological activities, such as induction of colony stimulating factors, interferon, adjuvanticity, B cell mitogenicity, and IL-1 secretion (Haeffner-Cavaillon *et al.*, 1989).

There is evidence that endotoxins devoid of Kdo probably do not exist (Caroff *et al.*, 1987). This specificity of Kdo has been as a promising target for the

development of novel antibacterials acting against Gram-negatives, as the inability to produce Kdo is a lethal event. Also since Kdo is not found in mammalian cells, agents directed against its biosynthesis should possess high selective toxicity (Hammond, 1992).

CD14 is a glycophosphatidylinositol-linked protein expressed by myeloid cells (membrane CD14) and also circulates as a plasma protein lacking the glycophosphatidylinositol anchor (soluble CD14). Membrane and soluble forms of CD14 have been reported on the surface of spermatozoa and in the seminal plasma respectively (Harris *et al.*, 2001). Both membrane and soluble CD14 function to enhance activation of cells by LPS and a number of other bacterial cell wall components from different organisms (Aderem and Ulevitch, 2000; Viriyakosol *et al.*, 2000).

The interaction between LPS and the effector cell occurs via the CD14 receptor. This receptor as the main LPS receptor, has been identified as a high affinity cell-surface receptor for complexes of LPS and serum LPS-binding protein. CD14 may play a role in immune defence and cell activation in the male reproductive tract (Harris *et al.*, 2001).

LPS is only one of many bacterial products which is recognised by CD14. peptidoglycan (Gupta *et al.*, 1996), mycobacterial cell wall component lipoarabinomannan (LAM) (Peterson *et al.*, 1995), uronic acid polymers which are common components of lipopolysaccharides (LPS) and extracellular polysaccharides from Gram-negative bacteria and are also in the cell wall (as teichuronic acid) and extracellular polysaccharides of Gram-positive bacteria (Jahr *et al.*, 1997), and a surface adhesion molecule of the yeast *Blastomyces dermatitidis* (Hogan *et al.*, 1995), have all been found to stimulate cellular responses via CD14.

3.2 Aims

The spermicidal properties of LPS are established by previous experiments. Since only Kdo and lipid A are required for bacterial bioactivity, in this chapter the effect of these fractions on sperm viability was separately investigated. The inhibitory effect of anti-CD14 antibody and polymyxin B (PMB) on Kdo and lipid A acting against sperm was also assessed.

3.3 Materials and methods

3.3.1 Preparation of lipid A from *C.trachomatis* LPS

Lipid A was isolated from in-house *C.trachomatis* LPS serovar E and LGV as previously described by Maasby-Baltzer and Kaijser (1979) and Qureshi *et al.*, (1982). Lipid A was extracted by hydrolysis of about 100 µg/ml LPS from E and LGV in 1 ml of 1% acetic acid. The suspensions were heated at 100°C for 90 min. The lipid A was precipitated from solution and was then removed by centrifugation at 10,000 x g for 10 min. The deposit was washed three times with hot distilled water and dissolved in 0.2% triethylamine. An LAL kit was used to quantify the extracted lipid A as described in chapter 2.

3.3.2 Assay for 2-keto-3-deoxyoctonate (Kdo) in the lipid A

To confirm the isolated lipid A is free of Kdo contamination, lipid A preparations were analysed for the presence of Kdo by the thiobarbituric acid method (Manthey and Vogel, 1994). 10 µl of each sample, estimated to contain about 20 µg lipid A, were added to 90 µl of 0.044 M H_2SO_4 and boiled for 20 min to release bound Kdo. 10 µl, and serial 2-fold aqueous dilutions, were then mixed with 20 µl of 0.025 M periodic acid in 0.250 M H_2SO_4 and allowed to oxidise for 40 min at RT. To each sample 30 µl of 2% sodium arsenite in 0.5 M HCl were added and briefly vortexed followed by 120 µl of 0.3% thiobarbituric acid. The samples were boiled for 20 min, neutralised by the addition of 15 µl 2M NaOH, and absorbance measured

at 548 nm. To obtain a standard curve, a stock solution of synthetic Kdo (Sigma, UK) at a concentration of 100 μg/ml was used. From the stock solution, six serial dilutions from 1 to 10 μg/ml were made and boiled for 20 min in 0.04 M H_2SO_4. Any Kdo present in a sample was determined by comparison with the standard curve.

3.3.3 Assay for endotoxin in the synthetic Kdo

To confirm the synthetic Kdo used in this study is free of LPS contamination, Kdo at 500 μg/ml was analysed for the presence of LPS by a Limulus Amebocyte Lysate (LAL) as described in chapter 2.

3.3.4 The effect of isolated *C.trachomatis* lipid A and synthetic Kdo on spermatozoa

Semen samples were obtained from 6 patients and prepared as described in chapter 2 to give 8 x 180 μl aliquots for each sample. To four aliquots, 1 and 2 μg/ml of extracted lipid A from *C.trachomatis* E and LGV and to two aliquots, 25 and 50 μg/ml of synthetic Kdo (Sigma, UK) were added. To the last two aliquots, 20 μl of EBSS and 0.1 μg/ml of LPS from *C.trachomatis* LGV were added as controls, followed by 6h incubation at 37°C in 5% CO_2.

3.3.5 The effect of PMB and anti-CD14 antibody on *C.trachomatis* lipid A and synthetic Kdo and their interaction with spermatozoa

Semen samples were obtained from a further 6 patients and prepared as described above to give 12 x 180 μl aliquots for each sample. PMB (Sigma, UK) was used at a final concentration of 100 μg/ml as previously reported (Hosseinzadeh *et al.*, 2003). Anti-CD14 antibody (BD PharMingen, Oxford, UK) was free of endotoxin and sodium azide and was used at a final concentration of 10 μg/ml (Raponi *et al.*, 1997) with a 30 min pre-incubation time prior to adding lipid A and Kdo at 2 μg/ml and 50 μg/ml respectively. EBSS and 0.1 μg/ml LGV LPS were used

as a negative and positive control respectively. All aliquots were incubated for 6h at 37° C in 5% CO_2.

In addition to the HOS test described in chapter 2, the sperm viability in all aliquots was also assessed using two additional methods, the Viability/Cytotoxicity assay and the Eosin-Nigrosin technique to confirm our HOS results.

3.3.6 Viability/Cytotoxicity assay

By using the Viability/Cytotoxicity Assay Kit (Molecular Probes, Invitrogen Technologies, Paisley, UK) containg Component A (Calcein AM) and Component B (Ethidium hómodimer-1) the spermatozoa death rate was assessed. This method is a fluorescence-based technique to assay cell vibility which allows simultaneous detection of live cells (green) and dead cells (red) in the same population.

2µl of component B was added to 1ml of PBS and then mixed followed by adding 1µl of component A. 10µl of each incubate was added to the same volume of mixed components and incubated 1h prior to making a smear. 200 sperm were counted at x 1000 under a fluorescent microscope (Leica Microsystems Ltd, Milton Keynes, UK). The ratio of viable (green) to non-viable (red) sperm was then calculated.

3.3.7 Eosin-Nigrosin technique (WHO)

This method is based on two stains, eosin that is taken up by dead cells, and nigrosin as a background stain, to increase the contrast between faintly stained cells and an otherwise bright background

10 µl of each incubate was mixed with 20 µl of 1% eosin Y. After 30 sec, 30 µl of 10% nigrosin solution was added and gently mixed. One drop of the sperm-eosin-nigrosin mixture was then placed on a microscope slide and a smear was made within 30 sec of adding the nigrosin. Care was taken not to make the smear too thick. The smears were allowed to air-dry and were examined under oil immersion (1000 x) with a light microscope.

3.3.8 Statistical analysis

The results of all experiments in this study were analysed using GraphPad InStat software version 3.0 (GraphPad Software, Inc. USA). The statistical significance of differences between two means was evaluated by Student's *t* test and/or one-way analysis of variance (ANOVA), and p values of > 0.05, < 0.05, < 0.01, and < 0.001 were considered not significant (NS), significant (*), very significant (**), and extremely significant (***) respectively.

3.4 Results

3.4.1 Lipid A quantification and Kdo assay

The concentrations of isolated lipid A from 100 µg of in-house *C. trachomatis* LPS from both serovars, E and LGV, using the LAL test were 27 µg and 35 µg respectively (Table 3. 1). No Kdo was detected in isolated lipid A. The data of the Kdo standard curve (Figure 3. 1) showed that the concentration of Kdo in lipid A was less than 1 µg (OD < 0.1).

Also, the results of the LAL test showed that the synthetic Kdo at 500 µg/ml was free of LPS contamination at the minimum detection level of 0.01 ng/ml. The data is shown in Table 3. 1.

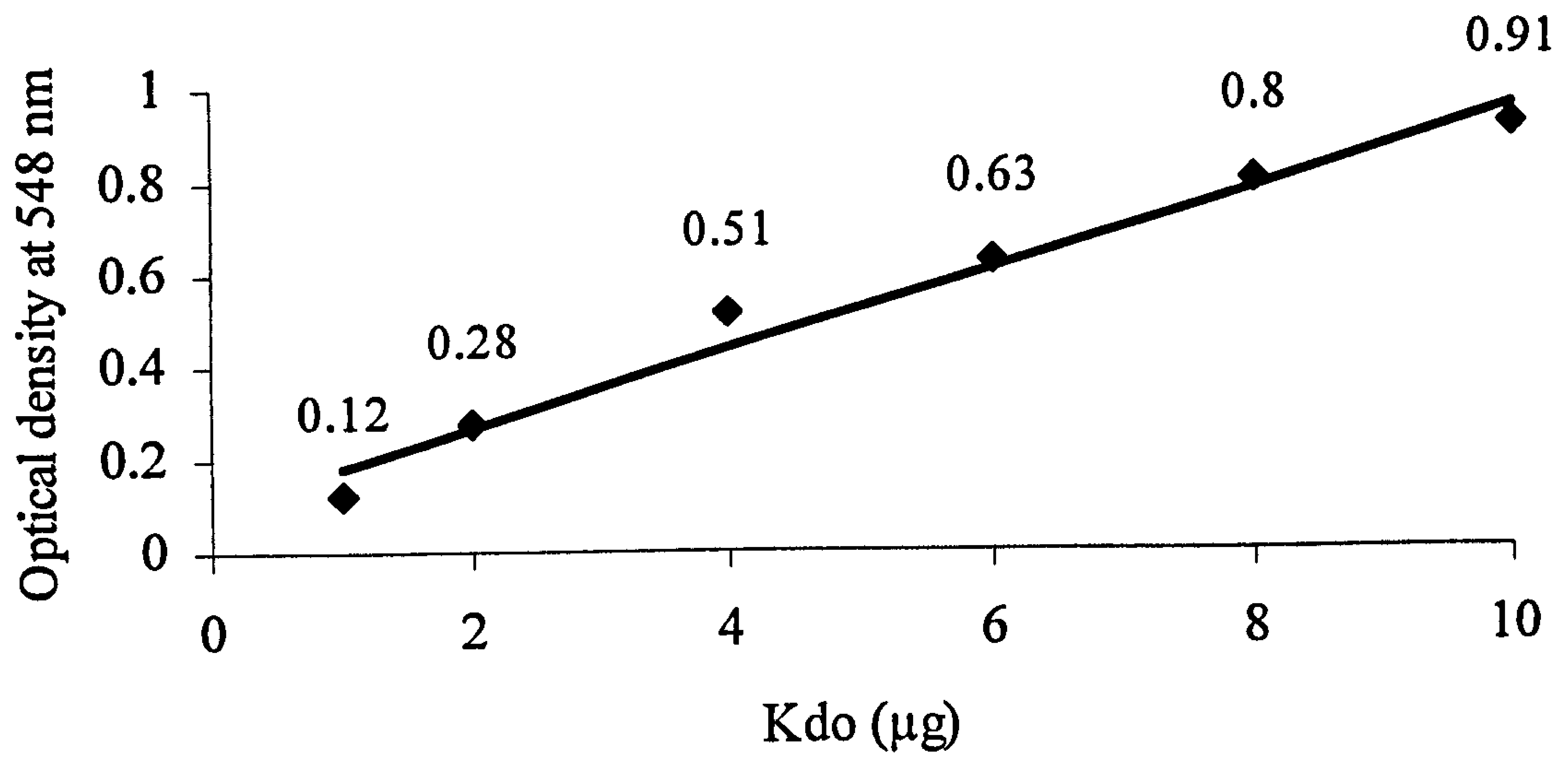


Figure 3. 1. A standard curve using 1 to 10 µg of Kdo.

	Absorbance (mean OD)
Standard* (0.1 EU**/ml)	0.119
Standard (0.25 EU/ml)	0.434
Standard (0.5 EU/ml)	0.938
Standard (1 EU/ml)	1.440
E Lipid A (df=6) (0.27 EU/ml)	0.441
LGV Lipid A (df=6) (0.35 EU/ml)	0.693
Kdo (500 μg/ml) (0.091 EU/ml)	0.108

Table 3. 1. The absorbance of each concentration of endotoxin standard and Kdo using a UV-visible spectrophotometer at 405 nm. *, Standard was prepared using *E.coli* LPS; **, Endotoxin Unit; df, dilution factor.

3.4.2 Comparison of three different methods for assessment of sperm viability

The percentage of dead spermatozoa in all incubations was investigated using three different methods. After 6h incubation, the mortality rates of spermatozoa in the control group were 9.5 ± 1.4% (HOS test), 10.8 ± 1% (Viability/Cytotoxicity assay), and 10.1 ± 1.9% (Eosin-Nigrosin technique). The spermatozoa death rate increased significantly ($p < 0.001$, Student t test) in the presence of 0.1 μg/ml of *C.trachomatis* LPS from serovar LGV; 35.5 ± 4.1% (HOS test); 33.8 ± 3.3%

(Viability/Cytotoxicity assay) and 34.4 ± 3.9% (Eosin-Nigrosin technique) (Figure 3. 2).

Since the results of these methods did not show any significant differences ($p > 0.05$, one-way ANOVA), the results of two groups, control and LGV LPS, were shown as a representative of all incubations which were assessed by these methods (Figure 3. 2) and only the results of the HOS test were chosen for experiments in this chapter. For each method, examples of viable and non-viable sperm are shown in Figure 3. 3 (Viability/Cytotoxicity assay), and 3. 4 (Eosin-Nigrosin technique). The examples of viable and non-viable sperm assessed by the HOS test are also shown in chapter 2, Figure 2. 4.

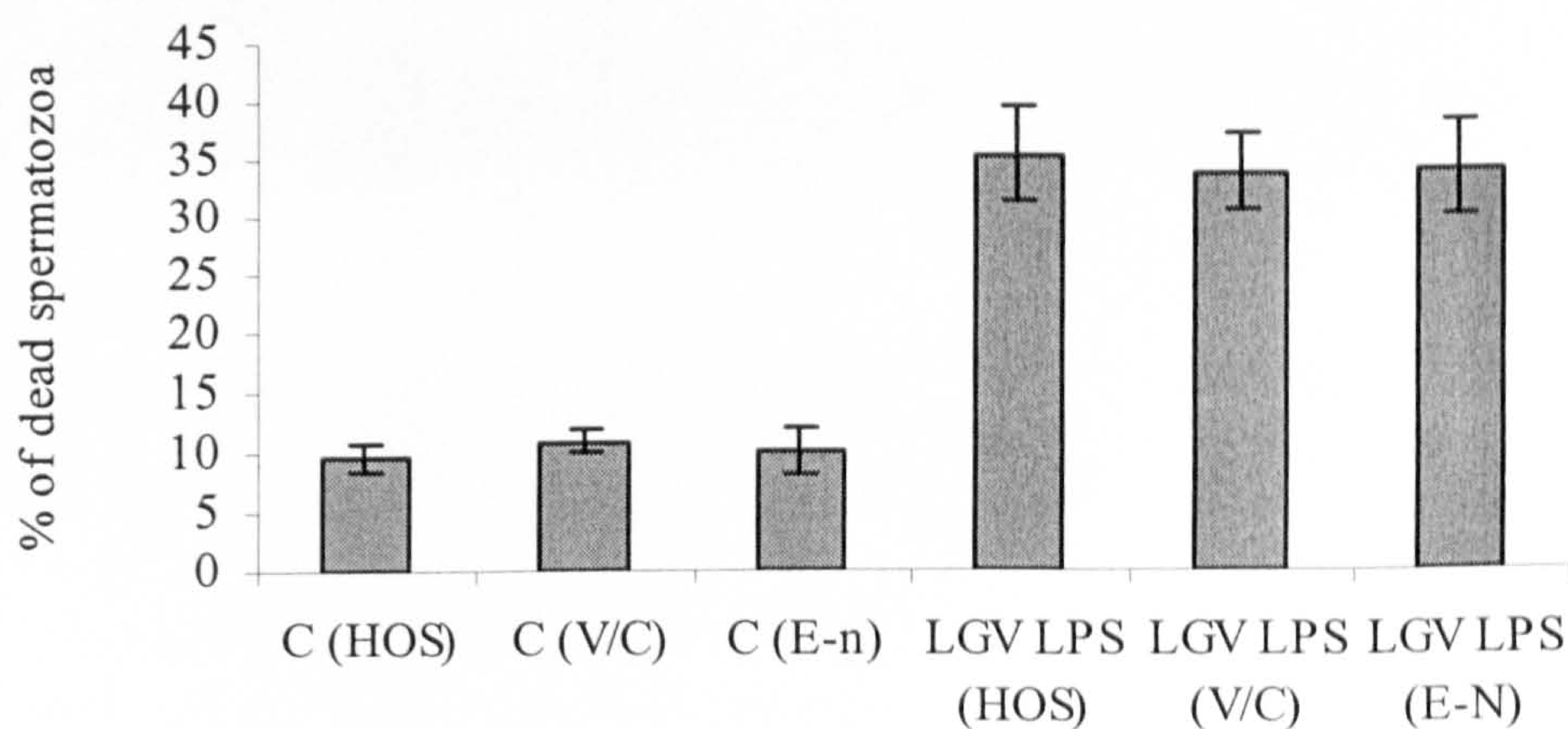


Figure 3. 2. A comparison of three methods for assessing sperm mortality after 6h incubation using; HOS, Viability/Cytotoxicity assay, and Eosin-Nigrosin technique. Results are the mean of 6 experiments ± SEM. HOS, hypo-osmotic swelling test; V/C, Viability/Cytotoxicity assay; E-N, Eosin-Nigrosin technique.

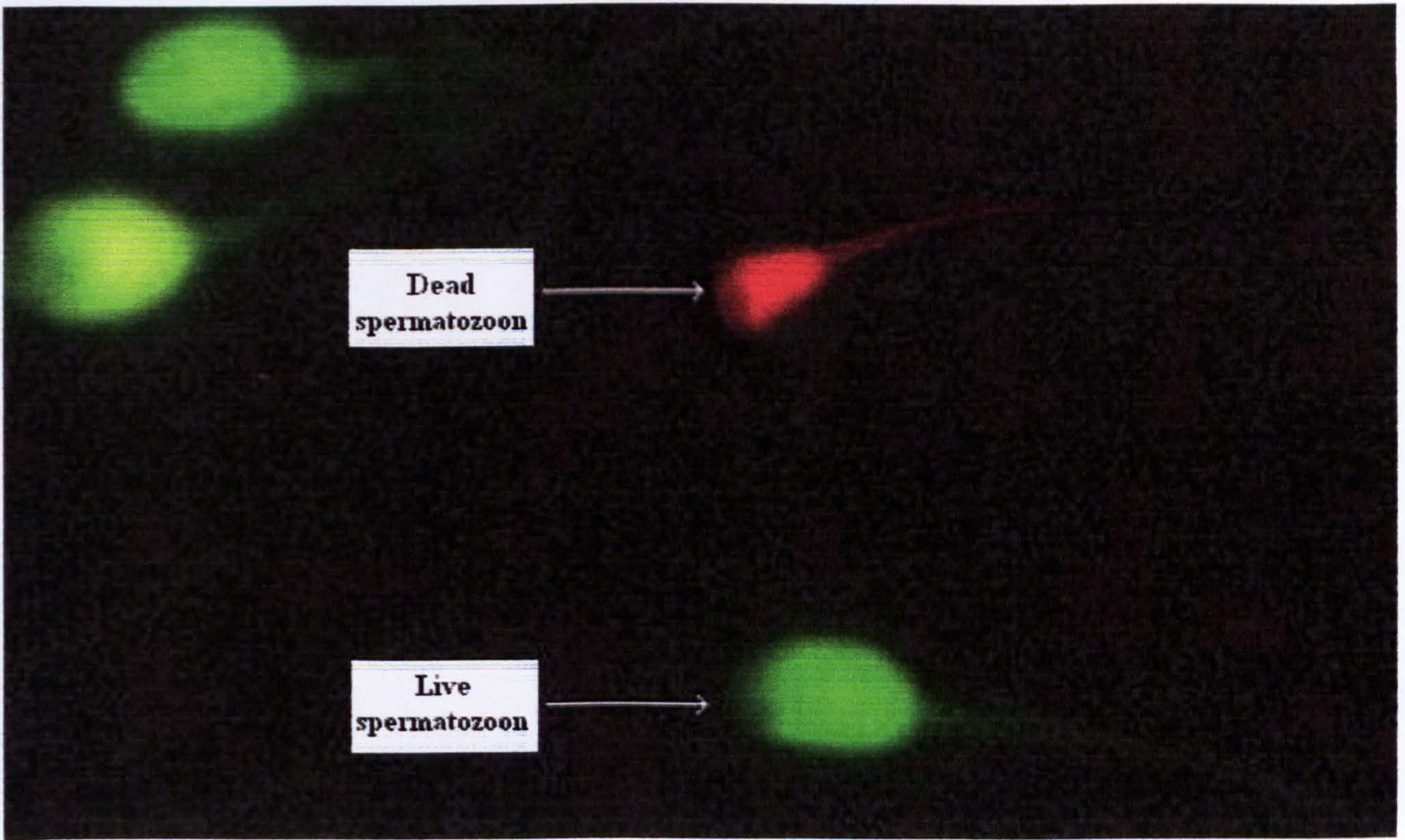


Figure 3. 3. Fluorescent microscopy pictures of viable (green) and non-viable (red) spermatozoa using the Viability/Cytotoxicity assay kit.

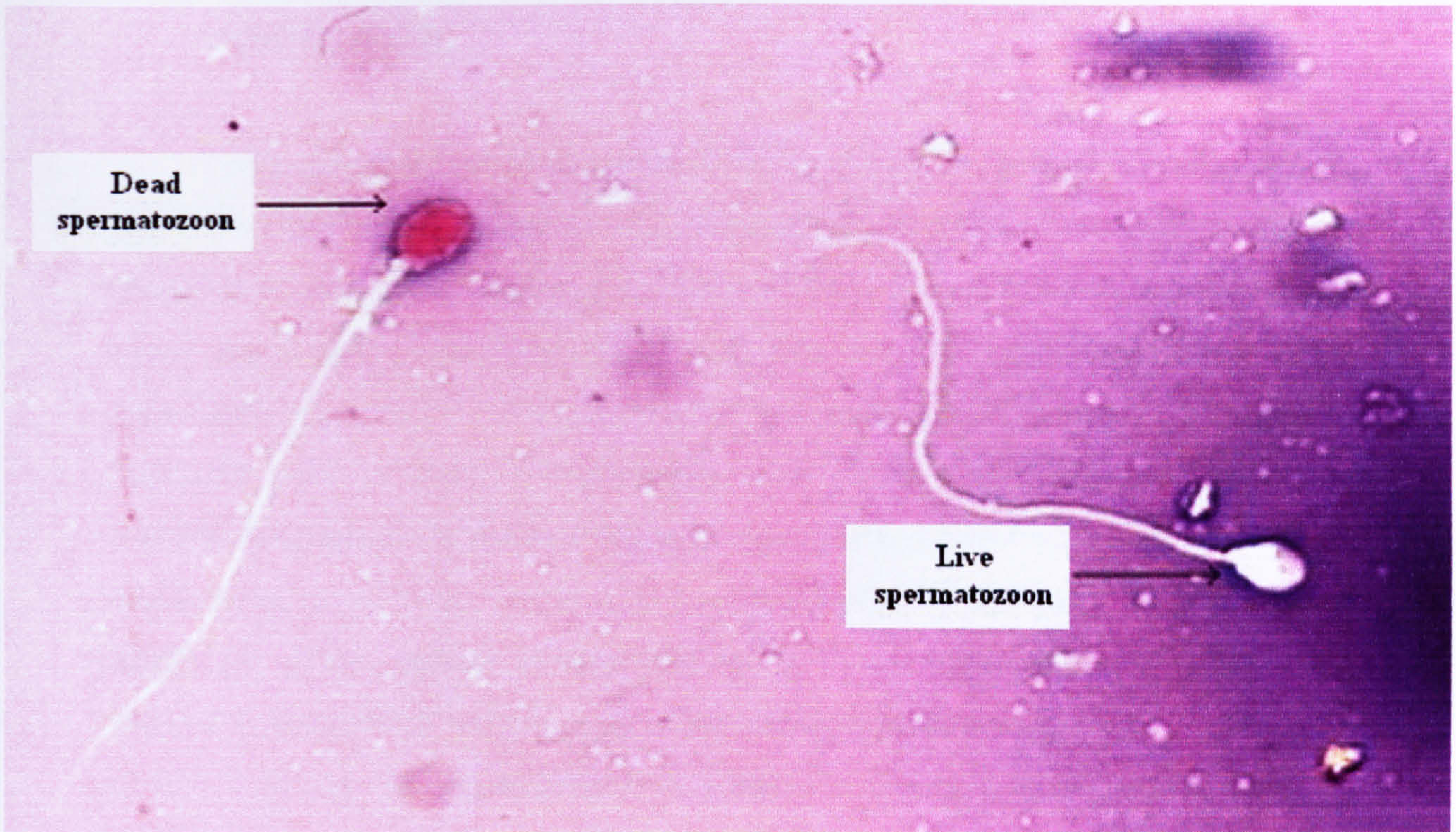


Figure 3. 4. Two spermatozoa stained by the Eosin-Nigrosin technique. The live spermatozoon is white and the dead one is red.

3.4.3 The effect of *C.trachmatis* lipid A and synthetic Kdo on human spermatozoa

The percentage of dead spermatozoa in the sperm preparation after density gradient centrifugation was 9.7 ± 0.8%. After 6h incubation this characteristic did not change markedly in the control group (9.5 ± 0.96%, $p > 0.05$) however, spermatozoa which were treated with 0.1 µg/ml of LGV LPS showed a significant change in the sperm mortality (34.3 ± 1.7%, $p < 0.001$). Spermatozoa in the presence of 2 µg/ml of extracted lipid A from *C.trachomatis* serovars E and LGV also showed a considerable increase in the percentage of sperm death (23.6 ± 1.2% and 19.7 ± 2.3% respectively, $p < 0.001$). Lipid A at a concentration of less than 2 µg/ml had no significant effect against spermatozoa ($p > 0.05$). Interestingly, synthetic Kdo showed a spermicidal activity only when the concentration increased to 50 µg/ml (20.6 ± 1.32%, $p < 0.001$) (Figure 3. 5). All data in this experiment were analysed by Student *t* test.

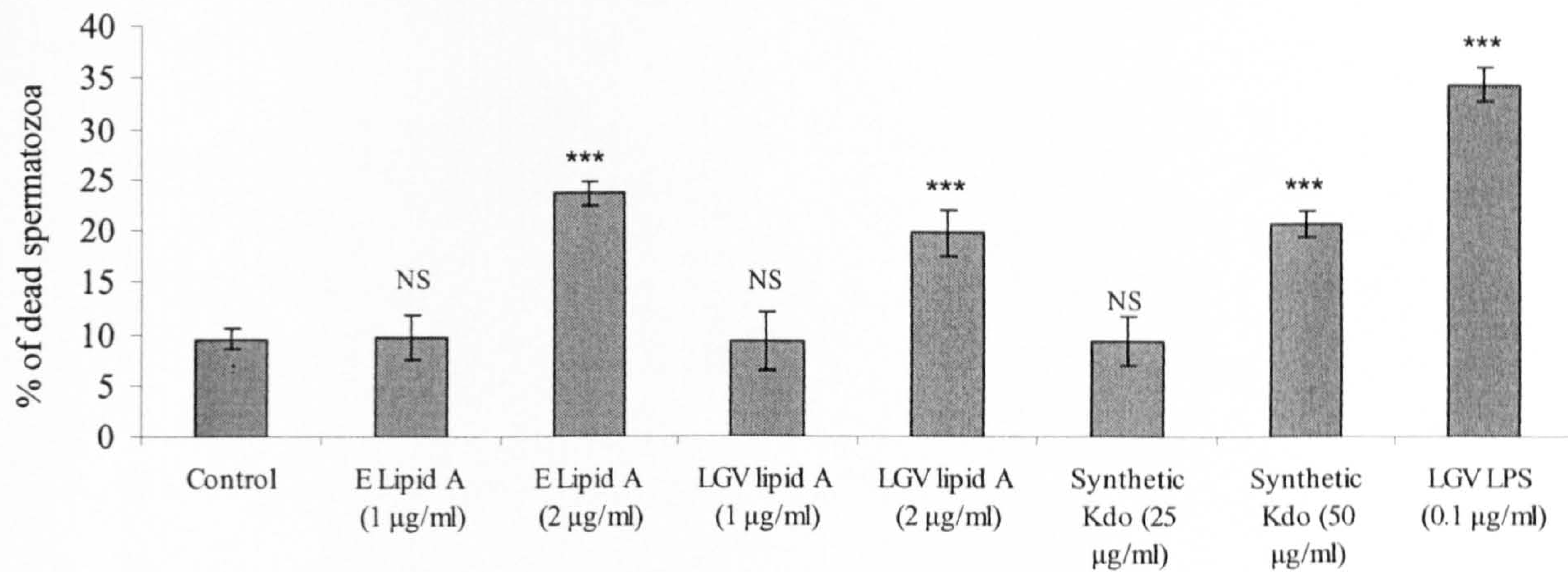


Figure 3. 5. Co-incubation of spermatozoa with extracted lipid A from *C.trachomatis* serovars E and LGV and with synthetic Kdo for 6h. The results of the negative control (EBSS) and the positive control (LGV LPS) are also shown. The data shown are the mean of six experiments ± SEM. NS. Non-significant. ***, $p < 0.001$, Student *t* test.

3.4.4 Inhibitory effects of anti-CD14 and PMB on lipid A and Kdo interactions with spermatozoa

After 6h incubation, the spermatozoa death rate in the control groups was similar to the values of previous experiment (10.2 ± 1.6% and 37.9 ± 3.1% respectively) (Figure 3. 5). The percentage of dead spermatozoa in the presence of anti-CD14 and PMB was also similar to the control group (Figure 3. 6) (9.1 ± 1.3% and 9.8 ± 1.1% respectively). Spermatozoa which were treated with 2 μg//ml of LGV lipid A and 50 μg//ml of synthetic Kdo showed similar findings to those seen previously (Figure 3. 5) (21.9 ± 2.2% and 19.5 ± 1.9% respectively, $p < 0.001$). When spermatozoa were pre-incubated with anti-CD14 antibody and PMB, the sperm death was markedly decreased in the presence of LPS (17.9 ± 2.3% and 21.3 ± 3.1% respectively, $p < 0.001$) and LGV lipid A (12.6 ± 1.9%, $p < 0.01$ and 9.6 ± 0.7%, $p < 0.001$, respectively). The sperm mortality results of E lipid A were similar to LGV lipid A (11.1 ± 2.2%, 10.4 ± 4.1 respectively). Interestingly, this effect was not seen with Kdo (18.4 ± 1.6%, and 20.1 ± 0.8 respectively, $p > 0.05$). Therefore, anti-CD14 antibody and PMB were only able to inhibit the spermicidal activity of LPS and lipid A but not Kdo (Figure 3. 6). All data in this experiment were also analysed by Student *t* test.

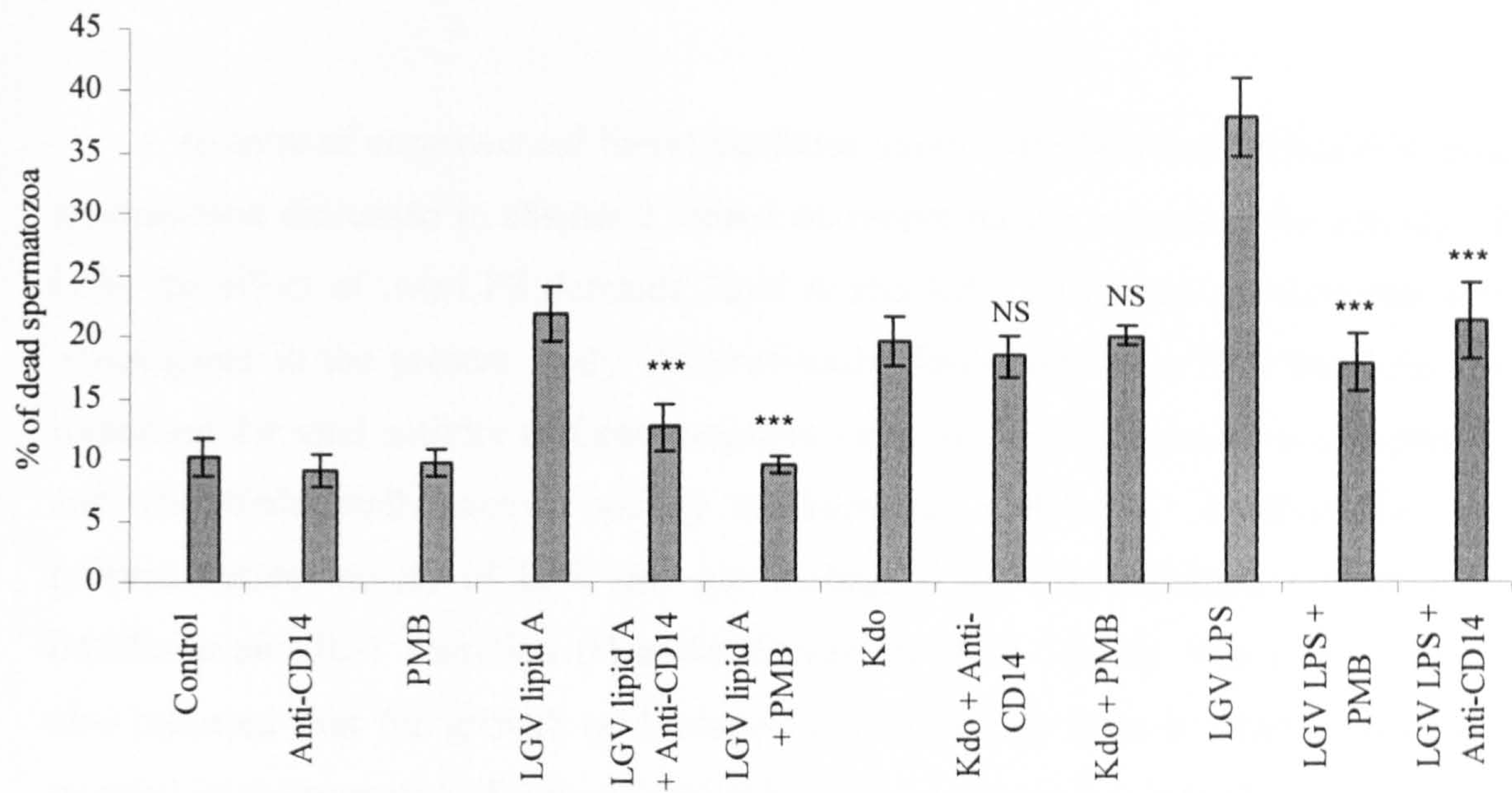


Figure 3. 6. Effect of 100 μg/ml PMB and 10 μg/ml anti-CD14 antibody on LGV LPS, LGV lipid A and synthetic Kdo on sperm viability after 6h incubation. Since the results of E lipid A was similar to LGV lipid A, only the results of the latter were demonstrated. Data shown are the mean of six experiments ± SEM. NS, Non-significant. ***, $p < 0.001$, Student *t* test.

3.5 Discussion

In spite of controversial literature about interactions between LPS and human spermatozoa discussed in chapter 2, based on recent results regarding the activity of LPS, the effect of two LPS domains, lipid A and Kdo, on human spermatozoa was investigated in the present study. As previously described, these LPS fractions are important for vital activity of Gram-negative bacteria. Lipid A is a toxic component and the biologically active moiety of bacterial endotoxin. Additionally, the polysaccharide moiety of LPS also has biological activity including induction of interferon and IL-1 secretion (Haeffner-Cavaillon *et al.*, 1989). Van *et al.*, (1996) also reported that the growth of *Haemophilus influenzae* type b (Hib) completely stopped in the presence of 5 μg of anti-Hib capsular polysaccharide (PS) MAb. This finding might confirm the role of polysaccharides in bioactivity of bacteria.

However, there is no evidence thus far to show the bioactivity of these two moieties of LPS against human spermatozoa. As lipid A is the main part of LPS and is responsible for most of the toxic activity of LPS, we could postulate that lipid A might be toxic for spermatozoa however, we demonstrated that this hypothesis cannot be generalised to Kdo due to no inhibition of its spermicidal activity by PMB. From our previous experiments we confirmed and showed that LPS from *C.trachomatis* at 0.1 μg/ml and from three genera of the *Enterobacteriaceae* family, *E.coli, K. pneumoniae,* and *S. marcescens* at 50 μg/ml has spermicidal activity. Although in our present study we demonstrated that isolated lipid A from *C.trachomatis* LPS had a marked effect against spermatozoa however, this effect was observed at 2 μg/ml which is 20 times more than LPS. Additionally, the percentage of dead spermatozoa caused by lipid A alone was about 15% less than sperm death rate induced by LPS. This discrepancy might be explained by the following evidence.

Usually, natural lipid As isolated from LPS by acid hydrolysis are very heterogeneous in their number of fatty acids and phosphates (Tanamoto , *et al.*, 2001). Erridge *et al.*, (2002) reported that the major contributing factors to endotoxicity of lipid A are the number and lengths of acyl chains present and the

phosphorylation state of the disaccharide backbone. Therefore the isolation process of lipid A could affect the potency of lipid A interacting with spermatozoa.

Furthermore, Kdo is seen in almost every LPS, being α-bound to the carbohydrate backbone of lipid A and is always absolutely required for bacterial viability (Erridge , *et al.*, 2002). For example, the LPS of the deep rough mutant of *Haemophilus influenzae* has only one Kdo residue attached to its lipid A (Helander *et al.*, 1988).

The most significant result of the present study was the identification of Kdo, which to our knowledge, has not been described so far as a spermicidal agent. Also we showed that this spermicidal activity of Kdo was not affected by two LPS inhibitors, anti-CD14 antibody and PMB. We obtained this finding with the HOS test confirmed by two other sperm viability tests, i.e. the Viability/Cytotoxicity assay, and the Eosin-Nigrosin technique. It should be noted that the Kdo used in this study was synthetic and monosaccharide however, Lebbar *et al.*, (1986) reported that synthetic Kdo alone has no biological activity. Haeffner-Cavaillon *et al.*, (1989) suggested that the polysaccharide portion of LPS is able to produce interferon and IL-1. Also, Paradis *et al.*, (1994) using extracted LPS from *Actinobacillus pleuropneumoniae* showed that the polysaccharide part of this LPS is involved in binding to porcine respiratory tract cells.

As we are in the early stages of this study, induction of death in spermatozoa using synthetic and monosaccharide Kdo, cannot be generalised to all Kdos regardless of the source and the number of sugars. There were some limitations and difficulties described below that hindered our investigation to focus on *C.trachomatis* Kdo.

Extraction of Kdo from *C.trachomatis* LPS was a big obstacle since LPS extraction from EBs as a preliminary stage for Kdo extraction was a difficult and time-consuming process. For example, for producing of about 100 μg of *C.trachomatis* LPS a large number of tissue culture flasks and a time of 2-3 months was needed; this could be increased in the case of culture contamination. Additionally, Kdo extraction at a high concentration was not achieved with only 100 μg of LPS. For these reasons, the effect of *C.trachomatis* Kdo on spermatozoa currently remains a mystery.

As described in chapter 2, polymyxin B (PMB) is a polycationic antibiotic which binds to the lipid A portion of bacterial lipopolysaccharide (LPS) and which thereby blocks many well-known biologic and toxic LPS effects. PMB is also frequently added to *in vitro* cell cultures in order to neutralize possible LPS contamination (Fibbe *et al.*, 1988). In experiments looking at the inhibitory effects of PMB, incubation of sperm with 100 µg/ml was able to inhibit the toxic effect of extracted LPS and lipid A from *C.trachomatis* LPS, however, PMB was not able to inhibit the spermicidal activity of Kdo. Interestingly, the same inhibitory effect on LPS and lipid A but not Kdo was observed when spermatozoa were pre-incubated with anti-CD14.

Although the presence of CD14 on the surface of spermatozoa and in the seminal plasma has been reported, the function of CD14 on human spermatozoa is presently uncertain (Harris , *et al.*, 2001). These workers proposed that spermatozoal CD14 may have a complementary role to that of seminal plasma CD14 (spCD14) in permitting spermatozoa to respond to LPS and other bacterial products. Since the inhibitory effect of anti-CD14 was observed in the present study, this finding may lead us to conclude that CD14 receptors existing in spermatozoa and seminal plasma have a similar role to that of other cells, plasma, and biological fluids possessing this receptor.

LPS and one of its active components, lipid A, induce various transcription factors via intracellular signalling cascades initiated by their receptor including CD14 (Reisser *et al.*, 2002). Anti-CD14 antibody blocks these signalling cascades and it is also well established that PMB binds to the lipid A portion of endotoxin and inactivates this molecules. Therefore, we would expect anti-CD14 antibody and PMB to block the activity of LPS and lipid A; Kdo would not be affected by PMB. However, the fact that spermicidal activity of synthetic Kdo was unaffected by anti-CD14 antibody was an interesting finding. It is possible that anti-CD14 antibody fails to block the activity of Kdo as it may not interact with the host cell in the same way that lipid A does. Although, the inhibitory effect of PMB on the spermicidal activity of LGV LPS used as a control in this study was statistically significant, however, this

effect was incomplete. The incomplete nature of inhibition might be ascribed to the toxic effect of Kdo components on spermatozoa which would not affected by PMB.

To expand our knowledge in terms of the mechanism of Kdo toxicity against spermatozoa, more experiments were performed including the role of Kdo in TLR induction and TNF-α secretion which are essential in recognition of pathogens and activation of host defence mechanisms. These investigations will be described in the next chapter.

Chapter 4

Mechanisms of LPS, Kdo, and lipid A acting through the TLR signalling pathway

4.1 Introduction

Bacterial LPS, a constituent of the outer membrane of the cell wall of Gram-negative bacteria, is a potent activator of cells of the immune and inflammatory systems, including macrophages, monocytes, and endothelial cells, and contributes to the systemic changes seen in septic shock including low blood pressure and low blood flow, and dysfunction of vital organs, such as the brain, heart, kidneys, and liver (Opal and Cohen, 1999). Recognition of LPS by monocytes/macrophages is a key event in host antimicrobial defence reaction which depends mainly on the presence of Toll-like receptors (TLRs) (Schletter *et al.*, 1995; Prebeck *et al.*, 2001).

Ten different TLRs are recognised to date (Philpott and Girardin, 2004), and with the exception of TLR10, they are involved in the recognition of pathogen-associated molecular patterns like endotoxin, peptidoglycan, flagellin, bacterial DNA and others (Hemmi *et al.*, 2000).

For recognition of LPS by host cells, at least four molecules are required (as described in detail in chapter 1); the serum LPS-binding protein, which delivers LPS to CD14 (Tobias *et al.*, 1995), the glycosylphosphatidylinositol-anchored cell-surface protein CD14 (Golenbock *et al.*, 1993), the Toll-like receptor 4 that is present in monocytes/macrophages, PMNs, dermal micro-vessel, umbilical vein endothelial cells, and intestinal epithelial cells (Muzio *et al.*, 2000). This receptor initiates a signalling cascade homologous to mammalian nuclear factor κB (NF-κB) (Poltorak *et al.*, 1998). The fourth molecule required for LPS recognition is the extracellular molecule MD2 that physically associates with TLR4 on the cell surface (Shimazu *et al.*, 1999).

Although, TLR2 which is predominantly distributed in monocytes, macrophages, and PMNs, is mainly involved in the recognition of Gram-positive cell wall components such as bacterial lipoproteins, and fungi (Hirschfeld *et al.*, 2000), it has also been reported to be a signal transducer of LPS. For example, LPS from *Porphyromonas gingivalis* (Hirschfeld *et al.*, 2001) and *Leptospira interrogans* (Werts *et al.*, 2001) may activate macrophages via a TLR2-dependent mechanism. Liu *et al.*, (2001) using peritoneal macrophages from normal and TLR4-deficient mice also demonstrated that TLR2 gene expression is upregulated in macrophge responses to peptidoglycan and to a high concentration (2.5 μg/ml) of *E.coli* LPS.

Interestingly, *N. meningitidis* LPS seems to engage both TLR4 and TLR2 due to structural composition of its lipid A (Netea *et al.*, 2002).

Production of pro-inflammatory cytokines by macrophages upon an infection is a preliminary stage to the activation of the effector cells of innate resistance. It is established that the early inflammatory responses are also essential to the process of generating antigen-specific effector cells of adaptive immunity. TNF-α (also known as cachectin) is one of the major mediators of inflammatory responses in mammals, and it also plays an essential role in bridging the innate and adaptive phases of immunity (Ma, 2001). TNF-α is a polypeptide cytokine with a diverse range of biological activities. Systemic exposure to recombinant TNF-α causes a syndrome of shock and tissue injury indistinguishable from septic shock syndrome. TNF-α has also been implicated in the pathogenesis of chronic processes such as autoimmunity, rheumatoid arthritis (RA), Crohn's disease and the cachexia accompanying cancer and acquired immunodeficiency syndrome (AIDS) (Tracey and Cerami, 1994).

Interferons are a class of cytokines possessing antiviral and antitumoral activity. The antiviral activity of interferons has led to the use of synthetic forms to control viral infections such as hepatitis C (Mogensen and Paludan, 2001). Interferon-γ (IFN-γ) acts as a powerful macrophage activator, increasing the molecule expression of the main class II histocompatibility complex and the potentialisation of the cell response, including the production of cytokines, nitric oxide, and the increase of cytolytic activity. In humans, individuals who presented with genetic mutations in the receptors for IFN-γ were observed to have had a high susceptibility to acquire infections caused by atypical mycobacteria (Jouanguy *et al.*, 1996), suggesting an important role of IFN-γ in the protective response against tuberculosis (TB).

Human leukocyte antigen-DR (HLA-DR), a human class II antigen of the major histocompatibility complex, is a transmembrane glycoprotein composed of an α chain (36 kD) and a β subunit (27 kD) expressed primarily on antigen presenting cells: B cells, monocytes, macrophages, and thymic epithelial cells. HLA-DR is also expressed on activated T cells.

LBP, CD14, TLR2, TLR4, and cytokines including TNF-α and IFN-γ involved in LPS signal transduction, may lead to reactive oxygen species (ROS)

induction and finally cell death. As TLRs play a critical role in early innate immunity to invading pathogens by sensing microorganisms and failing to produce TNF-α in TLR4-knockout mice, this demonstrated the importance of TLR4 in LPS signalling (Hoshino *et al.*, 1999). It is also reported that *C.trachomatis* LPS causes sperm death therefore, it would be an important question regarding the presence of TLRs on human spermatozoa. This hypothesis was strengthened when Harvey *et al.*, (2000) described the spermatozoa asialoglycoprotein receptor which binds to gonococcal lipooligosaccharide.

4.2 Aims

In the present study, we examined the contributions of TLR2 and TLR4 for the stimulation of HeLa cells by LPS from *C.trachomatis* serovars E and LGV. In chapter 3 we demonstrated that the mortality rate of spermatozoa increased in the presence of lipid A and Kdo. To understand the mechanism of sperm death by lipid A and Kdo we investigated the role of these LPS fractions in signal production by transfected HeLa cells and their roles in TNF-α production by IFN-γ-induced THP-1 cells. Another objective of this study was to assess the effect of LPS on mouse spermatozoa to explore whether we could use TLR knockout mice to show the existence of TLR in mouse spermatozoa.

4.3 Materials and methods

4.3.1 The role of *C.trachomatis* LPS, lipid A and Kdo in signal production via TLR 2 and 4

4.3.1.1 Purification of in-house *C.trachomatis* LPS, and commercial LPS and lipid A from *E.coli*

LPS from *C.trachomatis* serovars E and LGV, *E.coli* O55: B5 (Sigma, UK) and lipid A from *E.coli* F583 (Sigma, UK) were purified to remove protein and lipoprotein contamination according to the methods of Manthey and Vogel, (1994) and Hirschfeld *et al.*, (2000) as follows:

To prepare water-saturated phenol, 100 ml of endotoxin-free distilled water was added to 100 g of phenol, and then placed at 37°C overnight to dissolve. After vigorous shaking, the bi-phasic solution was mixed followed by placing at 4°C where the solution again became bi-phasic. The top layer was discarded and the lower layer, phenol layer, was used for the next stage.

At room temperature, 5 mg of LPS and lipid A were individually resuspended in 1 ml of endotoxin-free (EF) water containing 0.2% triethylamine (TEA). Each sample was split into two 500-µl aliquots, and one aliquot was stored at 4°C without further manipulation (unpurified LPS). 25 µl of 10% sodium deoxycholate (DOC) (Sigma, UK) was added to the remaining aliquot to a final concentration of 0.5%, followed by the addition of 500 µl of water-saturated phenol. The samples were vortexed intermittently for 5 min, and the phases were allowed to separate at room temperature for 5 min. Samples were placed on ice for 5 min, followed by centrifugation at 4°C for 2 min at 10,000 x *g*. The top aqueous layer was transferred to a new tube (~ 425 µl), and the phenol phase was subjected to re-extraction with 425 µl of 0.2% TEA plus 22 µl of 10 % DOC. The aqueous phases were pooled and re-extracted with 1 ml of water-saturated phenol. The pooled aqueous phases were adjusted to 75% ethanol (1100 µl of 100%) and 30 mM sodium acetate (15 µl of 3M) and were allowed to precipitate at -20° C for 1 h. The precipitates were centrifuged at 4°C for 10 min at 14,000 x *g*, washed in 1 ml of -20°C 100% ethanol and re-centrifuged at 4°C for 2 min at 14,000 x *g*. The ethanol supernatant was poured off and the pellets were then air-dried. The precipitates were resuspended in the original

volume (500 μl) of 0.2% TEA. To quantify the concentration of purified LPS and lipid A the LAL test was performed as described in chapter 2.

Because of insufficient amount of in-house *C.trachomatis* LPS, only 100 μg of E and LGV LPS were used for purification. All concentrations and volumes of reagents used for purification were also adjusted to the concentration of *C.trachomatis* LPS. However, the final LPS pellets were too small and our attempts to recover purified *C.trachomatis* LPS were insufficient. Therefore, unpurified *C.trachomatis* LPS preparations were used in this study.

4.3.1.2 HeLa cell transfection, stimulation, and luciferase activity assays

LPS, lipid A, and Kdo signalling via TLR2 or TLR4/MD-2 were assessed using an IL-8 promoter assay as previously described (Pridmore *et al.*, 2001). Briefly, 1.5 x 10^4/well of HeLa cells (named after patient Henrietta Lacks in 1951, any of the cells of the first continuously cultured human carcinoma strain, originally obtained from cancerous cervical tissue and maintained for use in studying cellular processes) were seeded into 96-well tissue culture plates 24h before transfection. Transient transfections were done according to the manufacturer's instructions using an activated dendrimer (Superfect; Qiagen, West Sussex, UK) when the cells were ~80% confluent. The reporter vectors that were used were pTK- rLUC and pIL8-pLUC containing the human IL-8 gene (Promega; Southampton, UK). Expression vectors were as follows: pSU1 (TLR2), pNM1 (MD2/TLR4), and pCDM8-CD14 (CD14).

Wells of HeLa cells received 150 μl of Dulbecco's modified eagle medium (Invitrogen; Paisley, UK) containing 10 % of heat inactivated foetal calf serum, 10^5 IU/100 ml of penicillin, and 50 mg/100ml of streptomycin, 400 ng of pIL8- pLUC, 25 ng of pCDM8-CD14, 100 ng of pTK-rLUC, 50 ng of each expression vectors, pSU1 and pNM1, and 50 ng of pcDNA3 (Invitrogen, UK). The latter were added to keep the total DNA dose constant at 625 ng/well. 2.5 μl of superfect was also added to each well to optimise the entry of DNA into the cells and also to assemble DNA into compact structures. The cells were then incubated for 2.5h at 37°C in 5% CO_2 followed by changing the medium with 100 μl of serum free Dulbecco's modified eagle medium; the cells were then re-incubated. At 24 h following transfection, cells

were treated with tenfold serial dilutions of *C.trachomatis* LPS from E and LGV (from 0.01 μg/ml to 1 μg/ml), purified lipid A and synthetic Kdo (from 0.5 μg/ml to 50 μg/ml), and 1 μg/ml of purified *E.coli* LPS and peptidoglycan from *Staphylococcus aureus* (Sigma, UK) for a further 6h. The latter were used as agonists for TLR4 and TLR2 respectively. 10 μl of PBS was also used as a negative control. Triplicate wells were transfected for each treatment and 30h after transfection, the cells were then lysed and luciferase reporter levels were measured using the Dual-Luciferase system kit (Promega, UK) as recommended by the manufacturer.

The term "Dual-Luciferase" refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system which allows more reliable interpretation of the experimental data by reducing extraneous influences. Normalized IL-8 promoter activity is the ratio of firefly to Renilla luciferase activity. Normalising the activity of the experimental reporter to the activity of the internal control minimises experimental variability caused by differences in cell viability or transfection efficiency.

4.3.2 Investigation into the role of LPS, lipid A and Kdo in TNF-α release by IFN-γ induced THP-1 cells

THP-1 cells (myelomonocytic cells line originating from a child with acute leukemia) were treated with 250 U/ml of human IFN-γ (Sigma, UK) for 48h as described by Raponi *et al.*, (1997). IFN-γ induced THP-1 cells were washed twice in RPMI 1640 medium (Invitrogen, UK) and resuspended to a concentration of 5 x 10^5 cells/ ml in a serum-free RPMI 1640 medium. The cells were then incubated with anti-HLR-DR (BD Pharmingen, UK) and anti-CD14 (BD Pharmingen, UK) at a final concentration of 20 μl/ml and 10 μg/ml respectively for 30 min at 37°C to establish the role of receptors in the release of TNF-α. 5 x 10^5 cells/ ml were stimulated with 0.1 μg/ml of *C.trachomatis* LPS from either serovar E or LGV, 50 μg/ml of *E.coli* LPS, lipid A, and Kdo for 18h in a 5 % CO_2 atmosphere at 37°C. The cells were centrifuged at 600 x g for 5min, and TNF-α content was tested in the supernatants.

4.3.2.1 TNF-α assay

The assay of TNF-α was done according to the R&D system kit (R&D Systems Europe Ltd; Abingdon, UK) as follows. 50 μl of assay diluent was added to each anti-TNF antibody coated well. 200 μl of standard or sample was then added and incubated 2h at RT. The wells were aspirated and washed three times with wash buffer followed by adding 200 μl of TNF-α conjugate to each well and incubated for 1h at RT. Aspiration and washing was then repeated prior to adding 200 μl of substrate solution for 20 min incubation time at RT in a dark place. In the final stage, 50 μl of stop solution was added and reading was done at 450 nm in an ELISA reader (Shimadzu, Japan) within 30 min.

4.3.3 Preparation of M2 medium and exposure of mouse sperm to LPS

M2 medium was prepared as described previously by Wittingham, (1971) as follows:

Five different stock solutions x 10 called A [NaCl, 5.534 g/100ml; KCl, 0.360 g/100ml; KH_2PO_4, 0.162 g/100ml; $MgSO_4H_2O$, 0.294 g/100ml; Na lactate 60% syrup, 2.608 g/100ml (3.2 ml); glucose, 1 g/100ml; penicillin, (10^5 IU), 60 mg/100ml; streptomycin, 50 mg/100ml], B ($NaHCO_3$, 2.106 g/100ml), C (Na pyruvate, 0.36 g/100ml), D ($CaCl_22H_2O$, 2.52 g/100ml), and E (HEPES adjusted to pH 7.4 with 2M NaOH before making up to 100 ml, 5.957 g/100ml) were prepared and filtered by Millipore filter (0.22 μm pore size) prior to storing at 4° C. From A-E stocks, 1 ml, 0.16 ml, 0.1ml, 0.1ml, and 0.84 ml were taken respectively and added to 7.8 ml water with 40 mg BSA to make up 10 ml of M2 media with pH 7.4.

4.3.3.1 Exposure of mouse sperm to LPS

Experiments were performed on male C3H/HeN mice aged 7-9 weeks old, obtained from the University of Sheffield Field Laboratories. The procedure was performed under UK Home Office Animal Procedures Act (1986); the project licence number was 40/2343. The vas deferentia were excised from the mice once they had been killed and were incised 5-10 times to allow sperm to exude into 1 ml

of M2 media at 37°C for 15 min in 5% CO_2. Sperm were washed twice by diluting to 1 ml and centrifuging (400g, 3 min). The final sperm concentration was 1-2 x 10^6/ml.

Sperm were incubated 6h at 37°C in 5% CO_2 with 0.1 µg/ml of LGV or E LPS or 50 µg/ml of *E.coli* LPS prior to staining with the Viability/Cytotoxicity Assay Kit (Molecular Probes, Invitrogen Technologies, UK) as described in chapter 3.

10 µl of incubate was added to the same volume of the mixed components and incubated for 1h prior to making a smear. 200 sperm were counted at x 1000 magnification under a fluorescent microscope. The ratio of viable (green) to non-viable (red) sperm was then calculated.

4.4 Results

4.4.1 Quantification of purified LPS and lipid A

Although in theory if no LPS or lipid A is lost during purification process, the percentage of recovery is 100% (Manthey and Vogel, 1994; Hirschfeld *et al.*, 2000), in our laboratory from 5 mg/ml of commercial *E.coli* LPS and lipid A used for purification, 4 mg/ml (80%) of LPS and 4.5 mg/ml (90%) of lipid A were recovered. These concentrations were calculated using the LAL test (see chapter 2).

4.4.2 HeLa cell transfections and IL-8 promoter assays

The ability of in-house *C.trachomatis* LPS from both serovars, purified lipid A and synthetic Kdo to signal via TLR2 and TLR4 was investigated. Induction of the IL-8 promoter of HeLa cells was used as an indicator of the cellular response to these agonists (Pridmore *et al.*, 2001). These workers using RT-PCR also demonstrated that the HeLa cells express endogenous TLR4 message but not MD2 or TLR2 message.

The sensitivity of the IL-8 promoter reporter is illustrated in Figures 4. 1 and 4. 2, which show the response of transfected HeLa cells to E LPS, LGV LPS, lipid A and Kdo respectively.

HeLa cells which were transfected with MD-2 responded with significantly higher IL-8 promoter activity to 1 μg/ml and 0.1 μg/ml of E LPS and LGV LPS ($p < 0.001$) than to PBS alone (Figure 4. 1A). While the cells stopped responding to LGV LPS at 0.01 μg/ml, this response was still kept in the presence of E LPS at this concentration ($p < 0.001$). Purified *E.coli* LPS at 1 μg/ml which was used as a control, was able to produce a signal through MD-2 transfected cells. This signal was comparable to the response produced by E LPS at 0.1 μg/ml, but, was higher than LGV LPS at the same concentration ($p < 0.01$) (Figure 4. 1A).

Although LGV LPS at the concentration of 0.01 μg/ml failed to produce a signal by MD-2 transfected cells, the response of TLR2-transfected cells to both serovars at all concentrations used in this study were significant ($p < 0.001$) (Figure 4. 1B). The response of these cells to the control which was peptidoglycan was similar to E LPS and higher than LGV LPS ($P < 0.01$).

When both MD-2 and TLR2 transfected cells were challenged with tenfold serial dilutions of lipid A and Kdo (from 50 μg/ml to 0.5 μg/ml), only responses to lipid A were observed in cells transfected with MD-2, and not in those transfected with TLR2; no activity from the transfected cells were detected in the presence of Kdo (Figure 4. 2). The control vector-transfected cells were unresponsive to all agonists used. Data were analysed using Student *t* test.

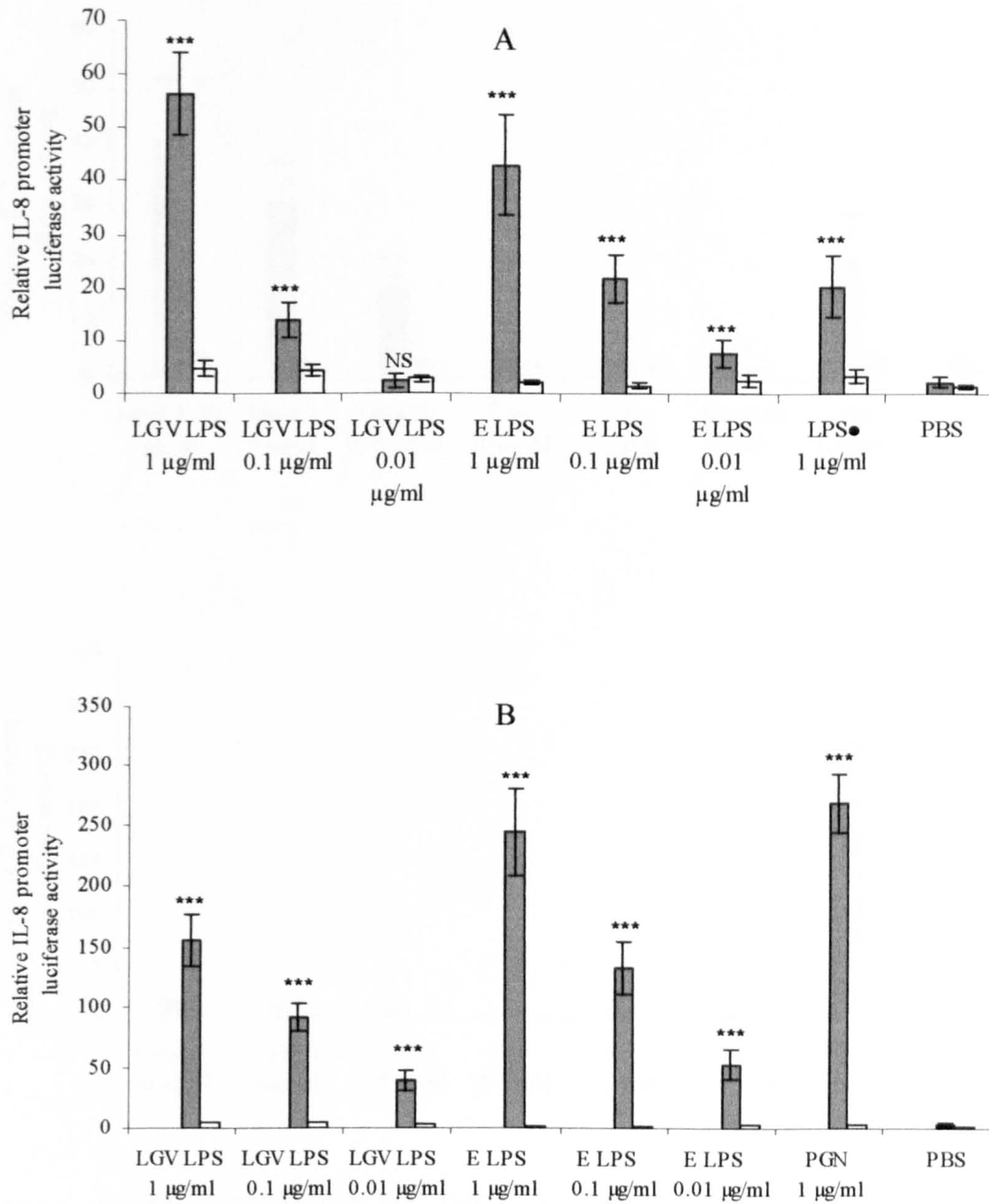


Figure 4. 1. Response of MD-2- (A) TLR2- (B) transfected HeLa cells to *C.trachomatis* LPS from E or LGV serovars. Blank bars shows the vector-transfected cells. Results are mean of six pool of transfected cells ± SEM. IL-8, interleukin-8; PGN, peptidoglycan; PBS, phosphate buffer saline. •, LPS from *E. coli* O55: B5; NS, not significant; ***, significant difference between response to LPS and PBS alone (P < 0.001, Student *t* test).

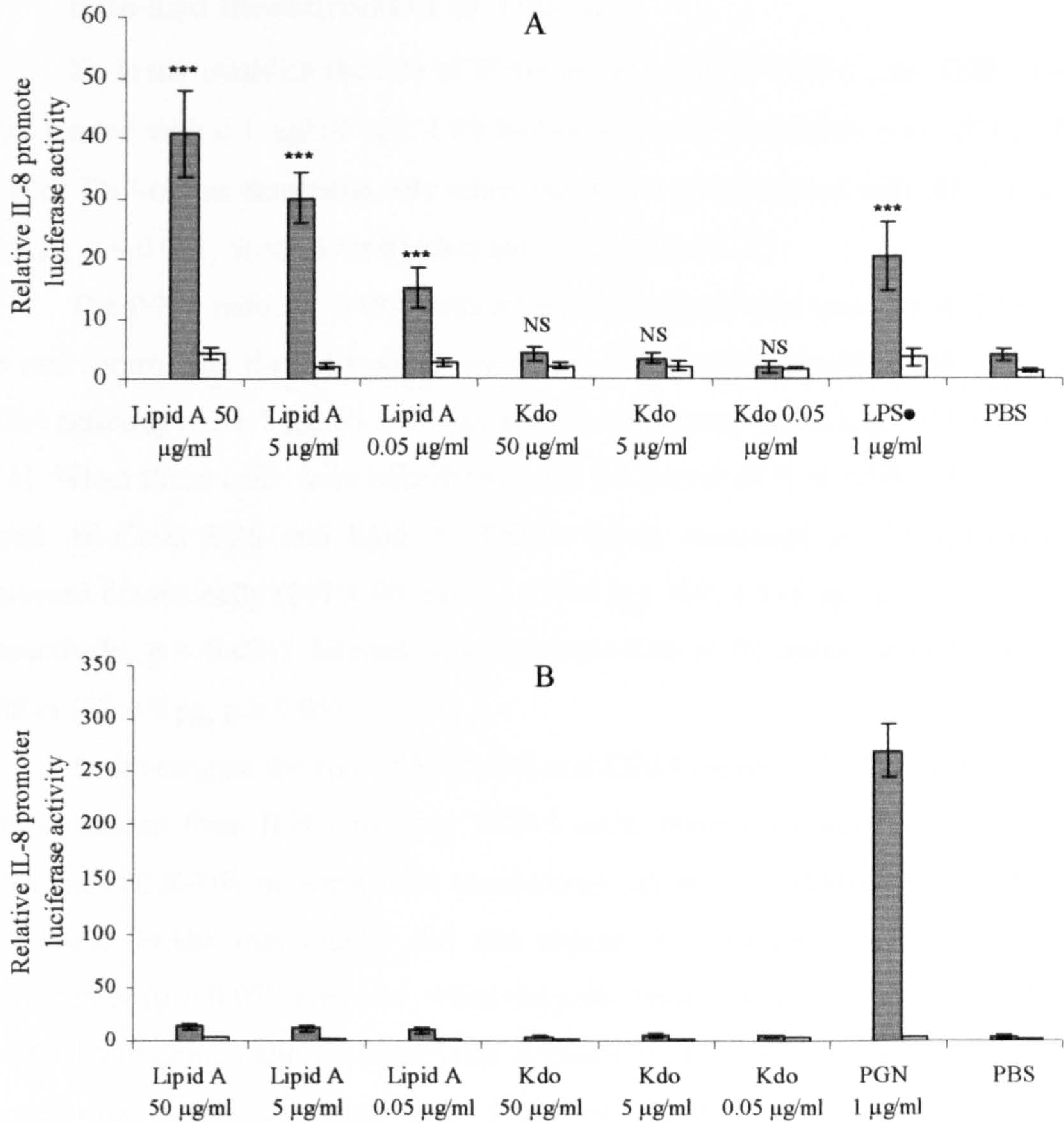


Figure 4. 2. MD-2- (A) and TLR2- (B) mediated recognition of lipid A and Kdo. Blank bars show the vector-transfected cells. Results are mean of six pool of transfected cells ± SEM. IL-8, interleukin-8; PGN, peptidoglycan; PBS, phosphate buffer saline. ●, LPS from *E. coli* 055: B5; NS, not significant; ***, significant difference between response to LPS and PBS alone ($P < 0.001$, Student *t* test).

4.4.3 Challenge of IFN-γ induced THP-1 cells with LPS, lipid A, and Kdo and measurement of TNF-α

To firstly establish the role of IFN-γ in induction of TNF-α, the THP-1 cells were treated with 0.1 μg/ml of E LPS before and after incubation with IFN-γ. The level of TNF-α was detectable only when the cells were incubated with IFN-γ (403 ± 37.5 pg, $p < 0.001$, Student *t* test) [data shown in Figure 4. 3].

The IFN-γ induced THP-1 cells released non significant amounts of TNF-α in the cell control and the cells which were only treated with anti-HLR-DR and anti-CD14 antibody (53 ± 7 pg, 53 ± 4.5 pg, and 56 ± 4 pg respectively, $p > 0.05$) [Figure 4. 4]. When these cells were stimulated with 0.1 μg/ml of E or LGV LPS, and 50 μg/ml of *E.coli* LPS and lipid A, TNF-α levels measured in the supernatants increased dramatically (601 ± 90 pg, 656 ± 108 pg, 709 ± 115 pg, and 372 ± 61 pg respectively, $p < 0.001$). Interestingly, synthetic Kdo at 50 μg/ml failed to produce TNF-α (57 ± 9 pg, $p > 0.05$).

To investigate the role of HLR-DR and CD14 receptors in the mechanism of TNF-α release from IFN-γ induced THP-1 cells, these cells were incubated with either anti-HLR-DR or anti-CD14 monoclonal antibodies (MAbs). Pre-incubation with these MAbs individually did not reduce the amount of cytokine release significantly ($p > 0.05$), however, when the cells were exposed to anti-HLR-DR and anti-CD14 in combination, a significant decrease of TNF-α levels was measured in the cell supernatants stimulated with LPSs from E, LGV, *E.coli*, and lipid A (187 ± 54 pg, 276 ± 51 pg, 268 ± 70, and 152 ± 18 pg respectively, $p < 0.001$).

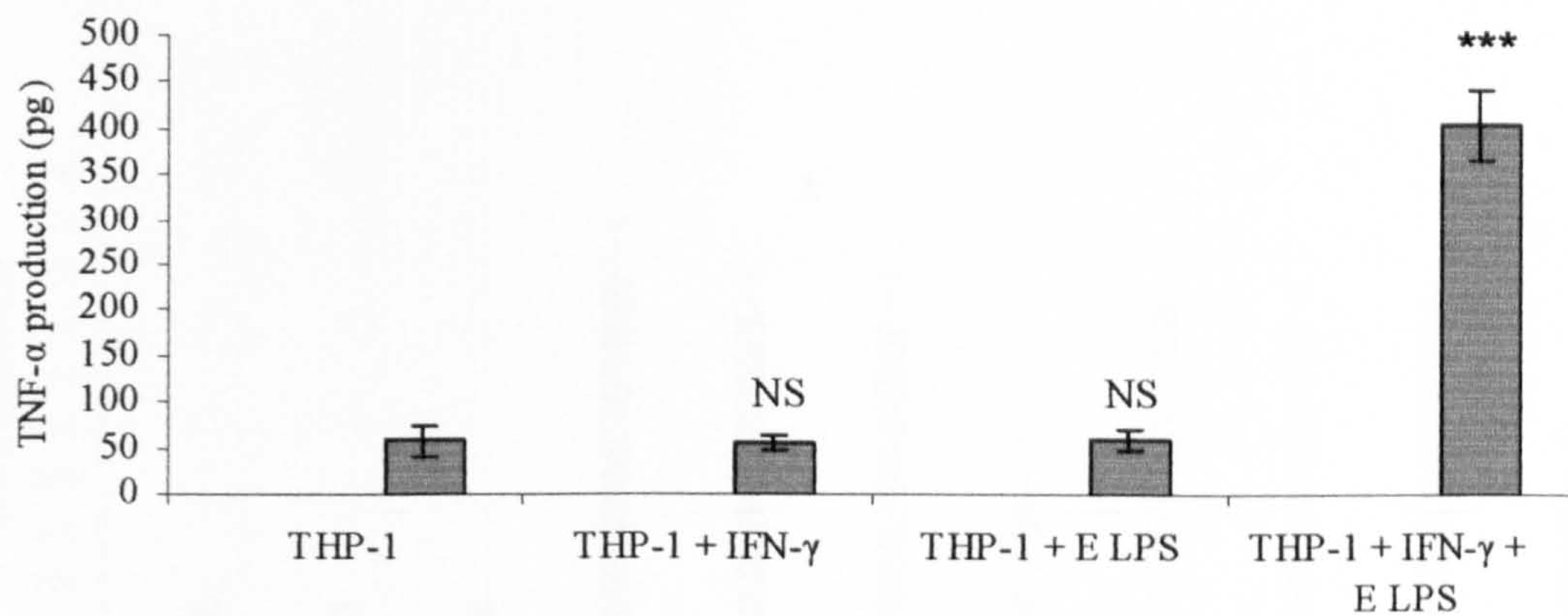


Figure 4. 3. Response of THP-1 cells to 0.1 μg/ml of *C.trachomatis* LPS serovar E in the absence/presence of IFN-γ at 250 U/ml as shown by TNF-α production. Data are the mean of three experiments ± SEM. ***, $P < 0.001$, Student *t* test.

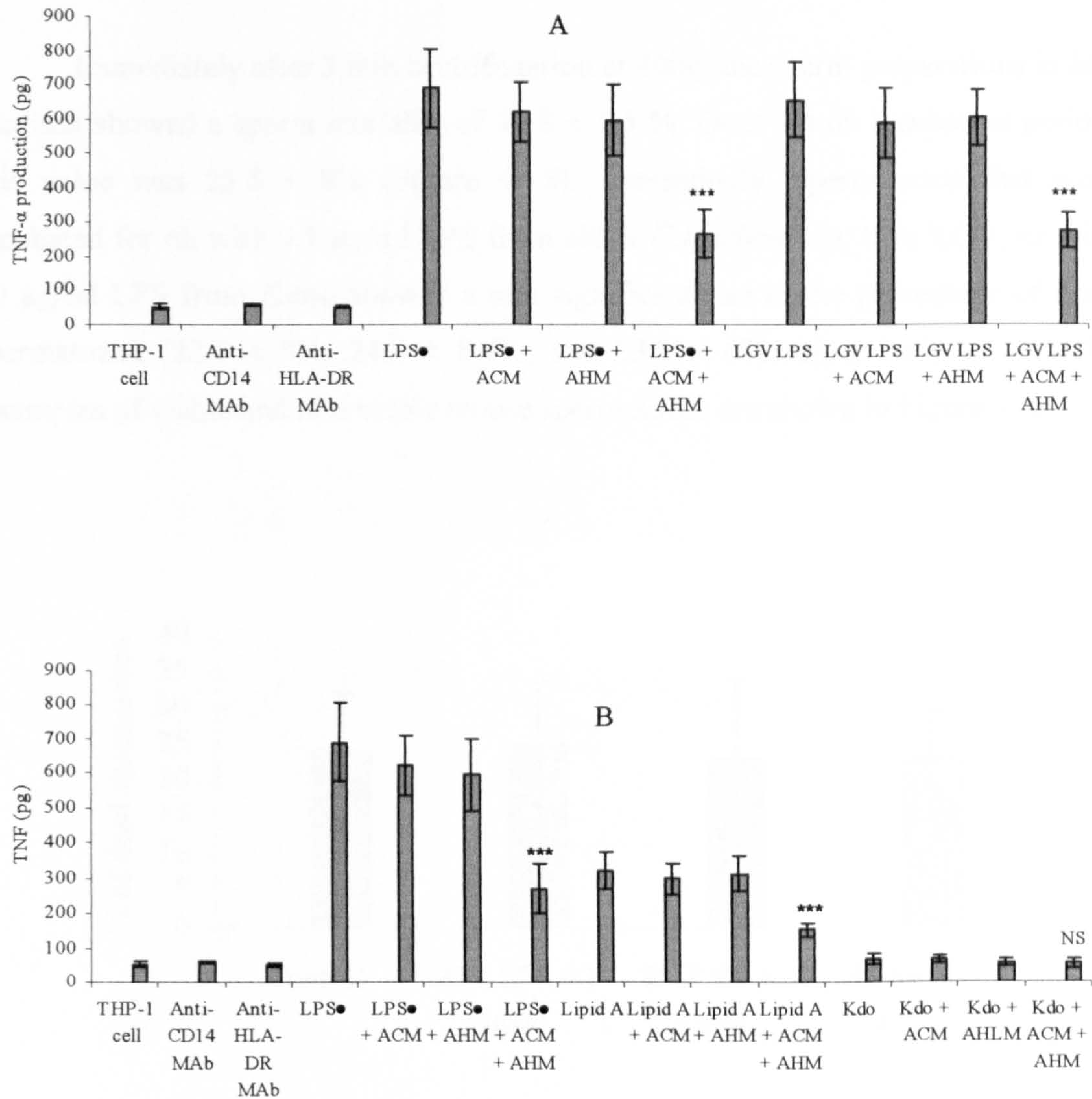


Figure 4. 4. TNF-α released by IFN-γ induced THP-1 cells stimulated with 0.1 μg/ml of *C.trachomatis* LPS serovars E or LGV (A), lipid A, or synthetic Kdo (B). 50 μg/ml of *E.coli* LPS was used as a positive control. As the result of LPS from *C.trachomatis* E and LGV was essentially the same, only the LGV LPS data are shown as an example. Results of TNF-α levels were expressed as the mean and the ± SEM of six experiments. ACM, anti-CD14 antibody; AHM, anti-HLR-DR antibody; NS, not significant. •, LPS from *E. coli* O55: B5; NS, ***, $P < 0.001$, Student *t* test.

4.4.4 Interaction between *C.trachomatis* LPS and mouse spermatozoa

Immediately after 3 min centrifugation at 400g, the sperm preparations in M2 medium showed a sperm mortality of 19.8 ± 5.5 %. Over the 6h incubation period, this value was 23.8 ± 8% (Figure 4. 5). Interestingly, spermatozoa that were incubated for 6h with 0.1 μg/ml LPS from either *C.trachomatis*, E or LGV, or with 50 μg/ml LPS from *E.coli* showed a non significant rise in the percentage of dead spermatozoa (22.7 ± 9%, 24.9 ± 8.4%, and 23.2 ± 6% respectively, $p > 0.05$). Examples of viable and non-viable mouse spermatozoa are shown in Figure 4. 6.

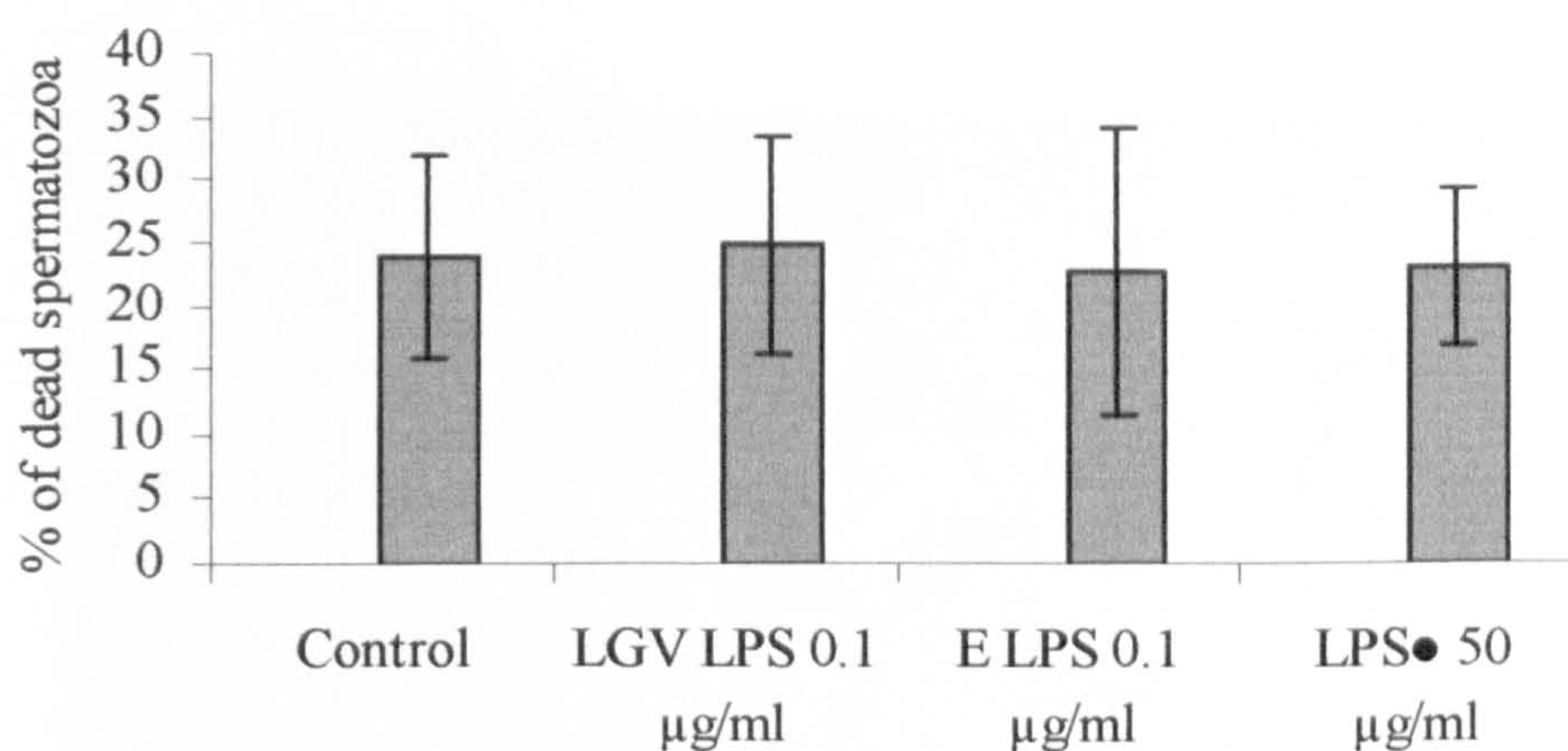


Figure 4. 5. Percentage of dead spermatozoa over 6h incubation with *C.trachomatis* LPS from either serovar, E or LGV, using fluorescence microscopy. Data shown are the mean of 6 experiments ± SEM. •, LPS from *E. coli* 055: B5.

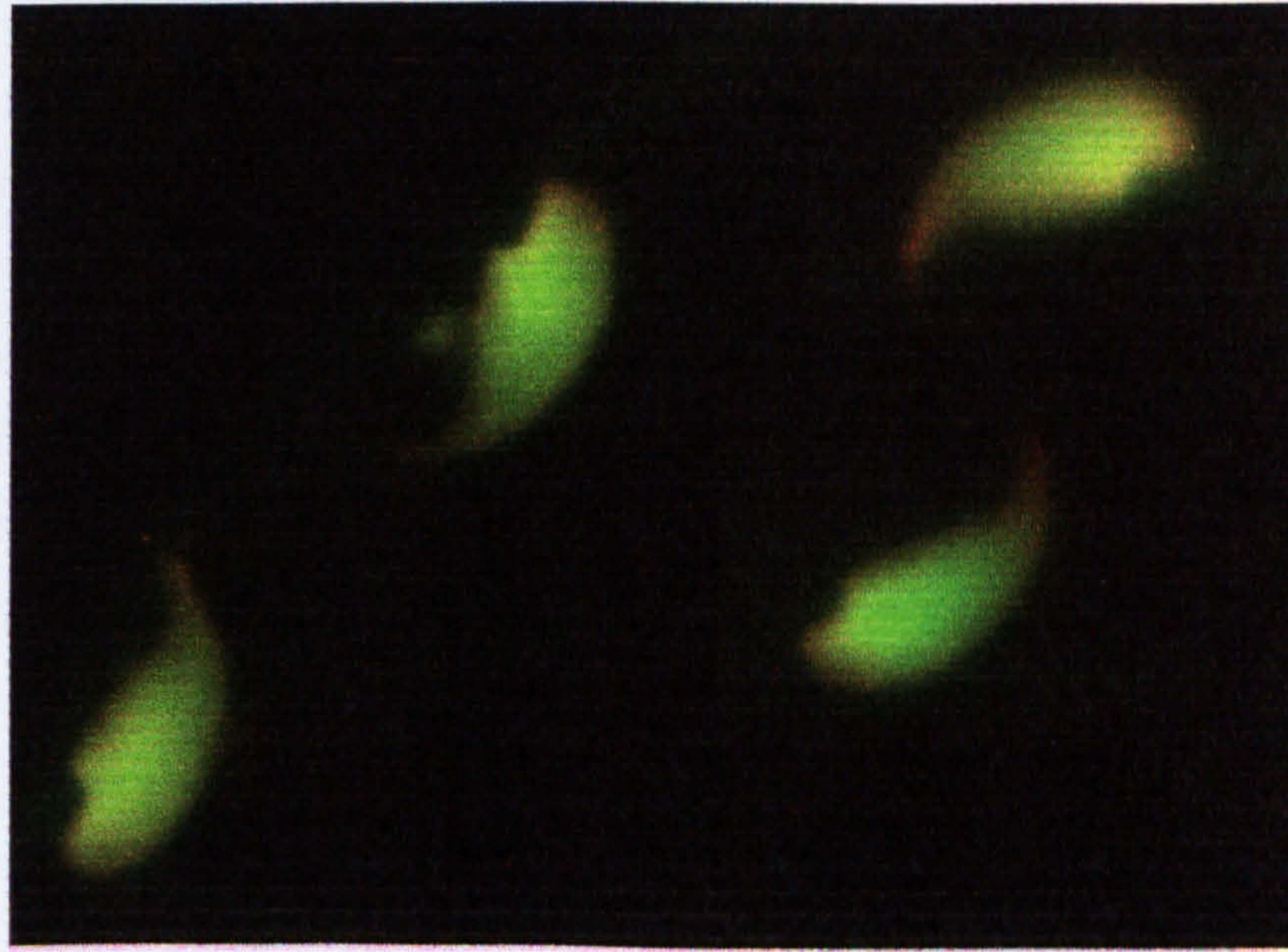


Figure 4. 6. Fluorescent micrographs using the Viability/Cytotoxicity method from four viable (green) and five non-viable (red) mouse spermatozoa incubated for 6h with LPS from *C.trachomatis* and *E.coli*. Photographs show only the head of spermatozoa.

4.5 Discussion

We demonstrated in chapter 3 that treatment of human spermatozoa with the Kdo portion of LPS at 50 μg/ml significantly compromised the sperm viability. We also showed that while anti-CD14 antibody and PMB inhibited the toxic effect of LPS or lipid A on human spermatozoa, Kdo could maintain its spermicidal properties even in the presence of anti-CD14 antibody and PMB. This observation encouraged us to investigate further whether Kdo like other PAMPs is able to initiate a signalling pathway via TLRs with involvement of TNF-α secretion. The two well-known cell lines, HeLa and THP-1, and different TLR agonists including *C.trachomatis* and *E.coli* LPS, *E.coli* lipid A, peptidoglycan and Kdo were used to attempt to answer this question.

Our results clearly demonstrated that the synthetic monosaccharide Kdo used in this study failed to produce a signal via either TLR2 or TLR4 or to release TNF-α in the IFN-γ-induced THP-1 cells stimulated by Kdo. These observations were in agreement with the previous studies described below.

Lebbar *et al.*, (1986) reported that the Kdo alone (monosccharide) is not sufficient to induce IL-1 secretion from human monocytes whereas synthetic disaccharide containing 3-deoxyoctulosonic acid can trigger IL-1 secretion like the native endotoxin. In another study by Tanamoto and Azumi, (2000) it was shown that the polysaccharide portion of the *Salmonella* LPS by itself is unable to exhibit TNF-α induction activity in human-derived cells. Although it is widely accepted that the lipid A is responsible for the biological activity of LPS, they also found that the activity of lipid A from various *Salmonella* as assessed by TNF-α induction and NF-κB activation in THP-1 and U937 human-derived cells, is very low compared with *E.coli* lipid A, although *Salmonella* LPS preparation is highly active. Later, Muroi and Tanamoto, (2002) suggested that *Salmonella* LPS requires at least a disaccharide structure (2-keto-3-deoxyoctonic acid) covalently bound to lipid A for its activity.

As we mentioned in chapter 3, because of the small amounts of *C.trachomatis* LPS extracted, only commercial lipid A and Kdo were used. Further studies using isolated lipid A and Kdo from *C.trachomatis* LPS are required to generalise our findings to the portions of *C.trachomatis* LPS. This additional

information may be beneficial for the understanding and expanding of our knowledge regarding interaction of *C.trachomatis* LPS and spermatozoa. In the next chapter we will describe the mechanism of Kdo-induced spermatozoa death in more detail.

In this work we also showed *C.trachomatis* E LPS and LGV1 LPS signalled through both TLR2 and TLR4, although MD-2 transfected HeLa cells did not respond to LGV LPS when the concentration was decreased to 0.01 μg/ml. To date, there are a few reports on the interaction between *C.trachomatis* LPS and TLR2 and TLR4. Heine *et al.*, (2003) and Prebeck *et al.*, (2003) reported that *C.trachomatis* serovars E and LGV2 respectively, only signal via TLR4. In contrast, Erridge *et al.*, (2004) suggested that *C.trachomatis* serovar LGV1 signals via TLR2 not TLR4.

These controversial findings might be explained by methodological differences of LPS extraction or of the cells transfection. For example, for extraction of LPS from *C.trachomatis* serovars E and LGV2, Prebeck *et al.*, (2001) and Heine *et al.*, (2003) used 45% phenol containing 2 % N-lauroylsarcosine sodium salt and for transfection they used HEK 293 cells, however, we used 90% aqueous phenol followed by ether and chloroform for LPS extraction and HeLa cells for transfection. Also, in their study, Erridge and co-workers used different batches of extracted *C.trachomatis* LPS.

In spite of the *C.trachomatis* LPS results, our findings showed that lipid A from *E.coli* similar to *E.coli* LPS used as a control, was able to produce a signal only via MD-2 transfected cells, indicating that lipid A was recognised by TLR4 and not by TLR2. The exact structural features of LPS, especially its main portion, lipid A, probably define whether they are recognised by TLR2 or TLR4. For example, there are differences in the number and the length of lipid A acyl chain from *C.trachomatis* and *E.coli*. The chemical structure of *E.coli* lipid A consists of six acyl chains of 12-16 carbons in length whereas, *C.trachomatis* LPS has only five chains of 14-21 carbons. Furthermore, the shape of lipid A might also determine the interaction of LPS with the TLRs (Netea *et al.*, 2002). They proposed that lipid A with a conical shape (*e.g.*, from *E.coli)* induces cytokine production via TLR4 however, cylindrical lipid A (*e.g.*, from *Porphyromonas gingivalis*) induces expression of cytokines through TLR2.

Similar to Kdo, we were not able to investigate the interaction of transfected HeLa cells with *C.trachomatic* lipid A therefore, further investigation regarding the responses of these cells to the isolated lipid A from *C.trachomatis* LPS is warranted.

As is described earlier although, TLR2 are mainly involved in recognition of variety of PAMPS, including lipoproteins and peptidoglycan rather than interacting with LPS. Apart from the separate roles of TLR2 and TLR4 in recognition of PAMPS, they both are important in the inflammatory response to Gram-negative bacterial infection because both endotoxin proteins and LPS are present in whole bacteria. These findings may be useful in the development of alternative therapies aimed at reducing excessive inflammatory responses during infections involving the TLR pathway.

A lipopolysaccharide present in the bacterial cell wall is considered to be responsible for the activation of humoral and cellular immune mechanism, resulting in increased production of cytokines including IFN-γ, TNF-α, IL-1 and IL-8. IFN-γ can cause profound changes in monocytic cells, including cellular morphology, expression of cell surface antigen and antimicrobial capacities (Murray and Cartelli, 1983; Virelizier *et al.*, 1984). Cytokines migrate to the site of infection and are also found in significant concentrations in plasma including the semen of infertile men. Depending on the rate of cytokine production, their activities range from an anti-inflammatory to a pro-inflammatory propensity (Ma, 2001).

Estrada *et al.*, (1997) demonstrated that the sperm parameters decrease as a result of a combination of TNF-α and IFN-γ *in-vitro*. They incubated washed spermatozoa from normal volunteers with TNF-α and IFN-γ for 0, 30, 60 and 180 min. The data showed a time-dependent negative effect of TNF-α and IFN-γ on sperm parameters including motility and viability.

Sikka *et al.*, (2001) also reported that when spermatozoa are exposed to LPS and IFN-γ in combination, sperm motility, viability and integrity are compromised which shows the synergistic effect of LPS and IFN-γ against spermatozoa. The results of their investigation showed that after 1h of incubation with a combination of LPS plus IFN-γ, human sperm motility, viability and membrane integrity were

significantly reduced by 20-25%. However, LPS and IFN-γ individually did not alter sperm viability or motility.

Another reason for this synergism was explained by Raponi *et al.*, (1997). In their study they observed a TNF-α release from the THP-1 cell line only when these cells were pre-incubated with IFN-γ. These findings support our results when we compared the levels of TNF-α secretion in the supernatant of the THP-1 cell line with and without pre-incubation with IFN-γ.

THP-1 is an undifferentiated cell line which reacts to chemical and immunological inducers by enhancing the expression of several receptors. In THP-1 cells, IFN-γ can enhance the expression of the HLA-DR receptor and allow the production of cytokines including TNF-α (Vey *et al.*, 1992). Furthermore, CD14 receptor acts as co-receptor and triggers cytokine secretion via a putative signal transducer-associated cell surface structure (Tobias *et al.*, 1993). In our results, a lack of significant reduction in TNF-α release in the presence of separate anti-HLA-DR and anti-CD14 antibody, and a marked decline in the production of TNF-α using combined anti-HLA-DR and anti-CD14 antibodies showed a synergy between these two receptors. This finding was consistent with the previous report by Raponi *et al.*, (1997). They suggested that HLA-DR receptor together with CD14 receptor trigger TNF-α release by IFN-γ-induced THP-1 cells.

Adherence to host cells is an important prerequisite for the pathogenesis of many bacteria. Bacterial cell components including LPS and LOS have been shown to be involved in adherence and invasion of host cells (Jacques and Paradis, 1998). Our studies showed that *C.trachomatis* LPS and synthetic Kdo have a toxic effect on human spermatozoa. Also recent studies have revealed that mammalian TLRs are important molecules for recognising pathogen-associated microbial patterns (PAMPs) including LPS and the other bacterial cell wall components (Kopp and Medzhitov, 1999).

Harvey *et al.*, (2000) demonstrated the presence of the asialoglycoprotein receptor (ASGP-R) on human sperm in the head, neck and tail regions under immunofluorescence confocal microscopy using the red nucleic acid stain ethidium bromide and fluorescein isothiocyanate (FITC)-conjugated antirecombinant ASGP-R

monoclonal antibody. They also suggested that this receptor on human sperm serves as a binding and attachment site for gonococcal lipooligosaccharide.

All these previous findings together led us to the objective of the current study, which was to determine how sperm respond initially to LPS, lipid A or Kdo and to investigate the possible presence of TLRs on human spermatozoa. To shed a light on this latter study we treated mouse spermatozoa with LPS. The rationale behind this experiment was to establish whether LPS has a deleterious effect on mouse spermatozoa similar to human spermatozoa. This experiment would then be continued with the TLR-knock-out mouse spermatozoa to assess whether they responded to LPS. Since LPS failed to kill wild-type mouse spermatozoa, continuing this study with the TLR knock-out mouse spermatozoa unfortunately would be pointless.

Weil *et al.*, (1998) also reported that treatment of mouse sperm with staurosporine (STS) and cycloheximide (CHX) or a peptide caspase inhibitor does not accelerate or delay cell death. As STS and CHX are apoptotic reagents via interaction with protein synthesis and mouse sperm did not respond to these reagents, they suggested that possibly caspases are absent in the mouse sperm and these cells have a death programme that may not depend on caspases. Although their suggestion could not exclude the possible existence of TLRs on mouse sperm, since both receptors and apoptosis enzymes, TLRs and caspases, are involved in the death pathway via apoptosis, lacking in any of these two receptors would conflict with apoptosis which will be described in detail in the next chapter.

Chapter 5

Reactive oxygen species (ROS) production and apoptosis in spermatozoa stimulated with LPS, Kdo, or lipid A

5.1 Introduction

The influence of ROS on human spermatozoa was first reported by McLeod, (1943). He observed that human spermatozoa rapidly lose their motility when incubated under high oxygen tension. Much attention has also been dedicated in recent years to the involvement of ROS in apoptosis of spermatozoa (Potts *et al.*, 2000; Agarwal and Saleh, 2002; Agarwal *et al.*, 2003; Wang *et al.*, 2003; Moustafa *et al.*, 2004).

Various ROS are generated in the body at stages where oxygen is utilised. ROS including superoxide anion, hydroxyl radical, hydrogen peroxide and oxygen ion, are unstable and fully reactive to attack molecules in the body resulting in ROS conversion to stable atoms and molecules (Ichikawa *et al.*, 1999). ROS plays an important role in the pathogenesis of many reproductive processes. ROS-induced DNA damage may accelerate the process of germ cell apoptosis, leading to the decline in sperm counts associated with male infertility (Agarwal *et al.*, 2003). Irvine *et al.*, (2000) reported that there was a strong association between the presence of nuclear DNA damage in mature spermatozoa and poor semen parameters particularly sperm concentration.

Oxygen has a paradoxical role in cells living under aerobic conditions. Oxygen is required to support life and a small amount of its metabolites such as ROS are necessary for spermatozoa to acquire fertilizing capabilities including capacitation and the acrosome reaction (Aitken *et al.*, 1989). These investigators suggested that low levels of ROS can enhance the ability of human spermatozoa to bind to the zona pellucida. de Lamirande and Gagnon (1993) found that incubating spermatozoa with low concentrations of hydrogen peroxide (H_2O_2) stimulates sperm capacitation, hyperactivation, the acrosome reaction, and sperm-oocyte fusion. Zini *et al.*, (1995) also demonstrated that nitric oxide and superoxide anion ($O_2^{\bullet-}$) promote sperm capacitation and the acrosome reaction.

However, overproduction of ROS can interfere with sperm functions and threaten the survival of spermatozoa (de Lamirande and Gagnon, 1995; Aitken, 1999). The increased formation of ROS has been correlated with a reduction of sperm motility (Armstrong *et al.*, 1999). Gomez *et al.*, (1998) reported that levels of ROS production by pure sperm populations are negatively correlated with the quality

of sperm in the original semen. Ichikawa *et al.*, (1999) showed that there is a reverse correlation between seminal ROS levels and the acrosome reaction (AR). They demonstrated that many patients with low ROS levels show increased rates of the AR. The excessive levels of ROS can also cause a reduction of sperm-oocyte fusion (Henkel *et al.*, 1997). Aitken *et al.*, (1990) suggested that high levels of ROS production in human ejaculates may originate from morphologically abnormal spermatozoa and/or seminal leukocytes.

Oxidative stress (OS) occurs as a consequence of excessive production of ROS and impaired antioxidant defense mechanisms (Sikka, 2001). It is suggested that OS precipitates the range of pathologies that currently are thought to afflict reproductive function (Sharma and Agarwal, 1996). In male-factor infertility, OS impairs the fluidity of the sperm plasma membrane and also the integrity of DNA in the sperm nucleus (Aitken, 1999). Spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFA) (Alvarez and Storey, 1995) and their cytoplasm contains low concentrations of scavenging enzymes like catalase and superoxide dismutase (de Lamirande and Gagnon, 1995). PUFA, fatty acids that contain more than two carbon-carbon double bonds and also methylene groups, are particularly susceptible to peroxidation (Halliwell, 1990). OS also attacks the other cellular components including proteins, nucleic acids, and sugars (Agarwal *et al.*, 2003).

ROS must be continuously inactivated by a defence mechanism to keep only a small amount necessary to maintain normal cell function. Seminal plasma has antioxidant defense mechanisms to protect spermatozoa against oxidants (Sikka, 1996; Armstrong *et al.*, 1998). It has been reported that levels of antioxidants in seminal plasma from infertile men are significantly lower than those in plasma from controls (Lewis *et al.*, 1995).

ROS have a tendency toward chain reaction production; that is, a compound carrying an unpaired electron will react with another compound to generate an unpaired electron therefore, the defence mechanisms are based on breaking this chain reaction by the formation of nonradical end products (Sies, 1993).

α-tocopherol (vitamin E) is a chain-breaking antioxidant that inhibits lipid peroxidation (LPO) in membranes by scavenging peroxyl ($RO^{\bullet}$) and alkoxyl ($ROO^{\bullet}$)

radicals (Wefers and Sies, 1988). Another antioxidant defense mechanism is the prevention of excessive ROS formation. An example is the binding of metal ions, iron, and copper ions in particular, which prevents them from initiating a chain reaction (Sies, 1993). In spite of these defence mechanisms, spermatozoa are still susceptible to oxidative insult because they are unable to repair the damage caused by excessive ROS due to lacking the cytoplasmic enzyme systems that are required to do this repair (Aitken *et al.*, 1989).

There are two sources of ROS within the human ejaculate, spermatozoa and infiltrating leukocytes (Kessopoulou *et al.*, 1992). Leukocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (Tomlinson *et al.*, 1992). They may be activated in response to a variety of stimuli including inflammation and infection (Pasqualotto *et al.*, 2000). Leukocytes, especially polymorphonuclears (PMNs), are the major source of ROS such as super oxygen anion and hydrogen peroxide (Wang *et al.*, 1997) in semen. Activated leukocytes can produce 100-fold higher amounts of ROS than nonactivated leukocytes (Plante *et al.*, 1994). Sperm damage from ROS that is produced by leukocytes occurs if seminal leukocytes concentrations are abnormally high, such as in leukocytospermia [$> 1 \times 10^6$ leukocytes/ml of semen] (Shekarriz *et al.*, 1995). At this concentration, LPS, IFN-γ, and TNF-α induce a significant ROS production by PMNs (Wang *et al.*, 1997).

Spermatozoa are enriched with mitochondria. The mitochondria in the sperm midpiece are the energy generators for mammalian sperm (Kao *et al.*, 2004). The integrity of mitochondria is established by the presence of cytochrome *c* in the inner membrane space (Wang *et al.*, 2003). These investigators using western blot analysis for cytochrome *c*, showed that high cytochrome *c* levels in seminal plasma associate with significant mitochondrial damage caused by high ROS in infertile men. They also suggested that release of a series of such proteins from the mitochondrial inner space is likely to accelerate the process of apoptosis, possibly leading to DNA damage.

Apoptosis, is a distinctive form of cell death characterized by a series of morphological and biochemical changes including regular DNA fragmentation

(Wyllie, 1980), mitochondrial membrane potential changes (Kroemer *et al.*, 1998) and caspase activation (Cohen, 1997) that result in efficient elimination of cells from tissues without eliciting an inflammatory response (Wyllie, 1980) (see more details in chapter 1). Recently, Print and Loveland, (2000) and Kierszenbaum, (2001) reported that germinal cell apoptosis is an underlying mechanism for normal spermatogenesis. Spermatogenesis refers to a dynamic process including the mitotic divisions of spermatogonia, meiotic divisions of spermatocytes, the morphological differentiation and maturation of spermatids, and finally the formation of sperm (Print and Loveland, 2000). Men with azoospermia or severe oligozoospermia have an increased frequency of apoptotic germ cells in their testicular tissues in comparison with those with normal spermatogenesis (Hikim *et al.*, 1998).

Caspases are cysteine proteases that promote apoptosis in mammals. Following activation of caspases, biochemical events occur that lead to DNA degradation and the characteristic morphological changes associated with apoptosis (Salvesen and Dixit, 1997).

It is acknowledged that a central component of apoptotic machinery involves members of a family of caspases. In mammals, approximately 14 caspases have been identified so far (Thornberry and Lazebnik, 1998; Said *et al.*, 2004). In healthy cells, caspases are expressed as inactive proenzymes (~30 kDa) that contain three domains: an NH_2-terminal domain, a large subunit (~20 kDa) and a small subunit (~10 kDa). These enzymes participate in a cascade in response to pro-apoptotic signals and culminate in proteolysis of proteins essential for cell homeostasis, ultimately resulting in death of the cell. Caspase-3 is the main executor within this apoptotic cascade (Weng *et al.*, 2002).

The role of caspases is not limited to apoptosis. For example, caspases-1 and -11 are predominantly involved in the process of inflammatory responses, and caspase-4, -5, -12, -13, and -14, which are homologous to caspase-1 in amino acid sequences, may function as cytokine processors (Wolf and Green, 1999).

5.2 Aims

In chapters 3 and 4, the role of LPS and its active fractions, Kdo and lipid A, in spermatozoa death were investigated. We showed that these components could compromise sperm viability. Of interest is our finding that the polysaccharide domain of LPS, Kdo, had a spermicidal activity not affected by two LPS inhibitors, anti-CD14 antibody and polymyxin B. The role of LPS, lipid A, and Kdo in signalling pathways via TLRs and in TNF-α production were also investigated. We demonstrated that although LPS and lipid A were able to stimulate TLRs in transfected HeLa cells and also to activate the IFN-γ- stimulated THP-1 cells to secrete TNF-α, Kdo was not able to do this. Since it is well established that TNF-α as a product of the TLR signalling pathway, induces ROS production and ROS in turn to induce apoptosis, the objectives of this chapter were firstly, to determine if pre-treatment with antioxidants (ROS scavengers) can reduce sperm mortality induced by LPS and its fractions, and secondly, to clarify the role of LPS and its fractions in sperm apoptosis.

5.3 Materials and methods

5.3.1 Effect of ROS scavengers on spermatozoa treated with LPS, Kdo, and lipid A

5.3.1.1 Identification and quantification of leukocytes in sperm preparation

As was described in chapter 2, the two-layer (40/80%) percoll gradient was used in all experiments for sperm preparation. There are two potential sources of ROS in semen, spermatozoa and leukocytes (macrophages and neutrophils) (Kessopoulou *et al.*, 1992; Wang *et al.*, 1997). Since the presence of macrophages and neutrophils might interfere with our results, leukocyte contamination in sperm

preparation was assessed by two independent methods, immunocytochemistry and a cytocentrifuge preparation technique.

5.3.1.1.1 Immunocytochemistry method (WHO, 2000)

5.3.1.1.1.1 Reagents

1. Dulbecco's phosphate buffered saline with the following constituents:

$CaCl_2.2H_2O$	0.132 g/l
KCl	0.2 g/l
KH_2PO_4	0.2 g/l
$MgCl_2.6H_2O$	0.1 g/l
NaCl	8.0 g/l
Na_2HPO_4	1.15 g/l

2. Tris buffered saline 10 x with the following constituents:

Trizma base	60.55 g/l
NaCl	85.20 g/l

(pH was adjusted to 8.6 with 1 M HCl)

3. Alkaline phosphatase substrate with the following constituents:

Naphthol AS-MX phosphate	2 mg
Dimethylformamide	0.2 ml
0.1 M Tris buffer. pH 8.2	9.7 ml

(1.21 g of Trizma base dissolved in water. pH was adjusted to 8.2 with 1 M HCl. Made up to 100 ml with water)

1 M Levamisole	0.1 ml
Fast red TR salt (was added just before use)	10 mg

(All above-mentioned reagents were purchased from Sigma, UK)

4. Primary antibody. A mouse monoclonal antibody was obtained against the common leukocyte antigen, encoded CD45 (Dako, Glostrup, Denmark). We used this antibody because all classes of human leukocytes express a specific antigen (CD45) that can be detected by this monoclonal antibody (WHO, 2000).

5. Secondary antibody (Antimouse immunoglobulins raised in a rabbit) (Dako, Denmark).

6. Alkaline phosphatase anti-alkaline phosphatase complex (APAAP) (Dako, Denmark).

5.3.1.1.1.2 Preparation of sperm smear and monoclonal antibody staining

0.5 ml of spermatozoa that had been through a percoll gradient was mixed with five volumes of PBS and centrifuged at 500g for 5 min at RT. This procedure was repeated twice and the cell pellet resuspended in PBS to the original volume of the sperm sample. The suspension was then diluted with three times its volume of PBS.

Two 5 µl aliquots of this cell suspension containing 5 x 10^6 spermatozoa/ml were then air-dried onto a clean glass slide. The air-dried cells were fixed in absolute acetone for 10 min followed by washing twice with Tris buffered saline (TBS), and allowed to drain. The fixed cells were covered with 10 µl of primary monoclonal antibody and incubated in a humidified chamber for 30 min at RT. The cells were then washed twice with TBS and allowed to drain.

The cells were covered with 10 µl of secondary antibody, incubated for 30 min in a humidified chamber at RT, washed twice with TBS and drained. 10 µl of APAAP was added to each specimen and incubated for 1h in a humidified chamber at RT before being washed twice in TBS and drained. In order to intensify the reaction product, staining with the secondary antibody and APAAP was repeated, with a 15 min incubation period for each reagent.

The cells were washed twice with TBS, drained, and incubated with 10 µl of alkaline phosphatase substrate for 18 min for inhibition of endogenous alkaline

phosphatase activity. After alkaline phosphatase colour development, the cells were washed with TBS and finally counterstained for a few seconds with 750 μl of haematoxylin (DAKO, Denmark) before being washed in tap water and mounted in an aqueous mounting medium. The slides were then checked for positive red-stained cells at x 400 magnification under a light microscope.

Positive and negative control slides were also prepared from human peripheral leukocytes that were isolated from whole heparinised blood as described by Jack *et al.*, (2001). Briefly, fresh whole blood (15 ml) from healthy laboratory volunteers was placed in 50 U of heparin. Dextran (6% wt/vol) in saline was mixed 1: 10 with the whole blood, and the entire mixture was left to stand at RT for 1h to allow the majority of erythrocytes to separate to the bottom of the tube (Hogg *et al.*, 1999). The upper plasma layer containing the leukocytes was gently removed by aspiration and centrifuged at 200g for 10 min. the cell pellet was washed with 20 ml of Hanks' balanced salt solution (HBSS) (GIBCO, UK) without Ca^{2+} and Mg^{2+} and centrifuged as before. The cell pellet was then resuspended in 1 ml of HBSS with Ca^{2+} and Mg^{2+} plus 0.2% (wt/vol) BSA.

For the negative control, the primary antibodies were omitted from the staining regimen.

5.3.1.1.2 Cytocentrifuge preparation technique

This procedure is commonly performed on clinical fluids to detect very small numbers of cells, which may be present in the specimens. It is also used to concentrate harvested mononuclear cells onto a small area of a slide prior to cytoplasmic antigen staining (Oertel *et al.*, 1998).

A labelled glass slide and a disposable carrier were inserted into the cytocentrifuge holder and clipped together. The holders were placed in the centrifuge in the appropriate position, ensuring that they were balanced. 0.5 ml of heparinised blood as a positive control and 0.5 ml of spermatozoa prepared through a percoll gradient were mixed with the same volume of foetal calf serum separately. Four drops of blood and sperm suspension were pipetted into the funnel of each carrier. The plastic lid over the centrifuge bowl was replaced prior to closing the centrifuge lid and centrifuging at 90 x g for 12 min. The slides were then removed from the

holders and using a diamond marker a ring of about 2 cm diam was drawn around the cell pellet. The slides were allowed to air-dry for a few min and were then stained using a modified Wright-Giemsa stain and a staining machine (ADVIA S60, Bayer HealthCare LLC Diagnostic Division, Tarrytown, USA).

When the slides are stained using Wright-Giemsa stain, the white blood nuclus and cytoplasm take on the characteristic blue or pink coloration. The combination of purified eosin and thiazine dyes in the product eliminates inconsistent staining and yields reproducible chromogenic responses.

5.3.1.2 Treatment of spermatozoa with LPS, Kdo, or lipid A with and without ROS scavengers

30 x 180 μl aliquots of sperm preparation were prepared as described in chapter 2. The aliquots were incubated with 20 μl of *C.trachomatis* LPS, Kdo, lipid A, and five different ROS scavengers [ascorbic acid, catalase, reduced glutathione, superoxide dismutase (SOD), and α-tocopherol as described previously by Urata *et al.*, 2001] with the details described in Table 5. 1. All aliquots were incubated for 6h at 37°C in 5% CO_2. The viability of spermatozoa in each aliquot was then determined using the HOS test as described in chapter 2.

5.3.2 Evaluation of the apoptotic effect of LPS, Kdo, and lipid A on spermatozoa

5 x 10^6 /ml sperm were incubated with 0.1 μg/ml of E and LGV LPS, 50 μg/ml of lipid A and 50 μg/ml of Kdo at 37°C in 5% CO_2 for 6h. 1 mM of staurosporine (Sigma, UK) and untreated percoll prepared sperm were used as apoptotic and negative controls respectively. In the other groups, prior to adding these reagents, the cells were incubated with 200 μM of pan-caspase inhibitor (Z-VAD-FMK) (Calbiochem, Darmstadt, Germany) and caspase-3 inhibitor I (Calbiochem, Germany) for 1h at 37°C in 5% CO_2. One aliquot of spermatozoa was incubated in a 56°C heated water bath for 6h as a necrosis control.

Content(s)	Number of aliquot(s)	Final concentration
Ascorbic acid (control)	1	1 mM
Catalase (control)	1	1 U/ml
Reduced glutathione (control)	1	100 nM
SOD (control)	1	100 U/ml
α-tocopherol (control)	1	10 mM
EBSS (negative control)	1	-
E or LGV LPS	2	0.1 μg/ml
Lipid A or Kdo	2	50 μg/ml
E LPS + each ROS scavenger	5	0.1 μg/ml + *
LGV LPS + each ROS scavenger	5	0.1 μg/ml + *
Lipid A + each ROS scavenger	5	50 μg/ml + *
Kdo + each ROS scavenger	5	50 μg/ml + *

Table 5. 1. Experiment design for the exposure of human spermatozoa to *C.trachomatis* LPS from serovars E and LGV, lipid A, and Kdo with and without ROS scavengers. *, ROS scavenger concentration of above.

5.3.2.1 Measurement of apoptosis by the Annexin V/PI binding assay

To prepare the sperm samples for flow cytometry, the cells were then washed twice with PBS and then resuspended in 1 x annexin binding buffer (Pharmingen, UK) at a final concentration of 1 x 10^6 sperm/ml as described by Oosterhuis *et al.*, (2000). 100 μl of the solution (1 x 10^5 cells) was then transferred to a 12 x 75 mm polystyrene test tube (Elkay, Basingstoke, UK) followed by adding 5μl of annexin V-FITC (Pharmingen, UK) and 50 μg/ml propidium iodide (PI) (Sigma, UK). The cells were then mixed by vortexing gently and incubated for 15 min at RT in the dark. 400 μl of 1 x annexin binding buffer was added to each tube and the cells were then analysed by flow cytometry within 1h. A minimum of 10,000 spermatozoa were examined for each test. The sperm population was gated by using forward-angle light scatter. The FITC-labelled annexin V positive sperm cells were measured

in the FL1 channel and the PI labelled cells were measured in the FL2 channel of the flow cytometer.

5.3.2.2 Quantitation of caspase-3 activity in spermatozoa treated with LPS, Kdo, or lipid A

Sperm lysates were prepared as described by Weng *et al.*, (2002). Briefly, 1 x 10^7 sperm (after 6h incubation with 0.1 μg/ml of E or LGV LPS or 50 μg/ml of lipid A or Kdo) were centrifuged to form a pellet. The pellet was then placed on dry ice (5-10 min) prior to lysing in 400 μl of 10 mM Tris buffer with 0.5% Triton X-100 (Sigma, UK), pH 7.5, containing general protease inhibitors; 2 mM phenylmethylsulfonyl fluoride (Sigma, UK), 10μg/ml leupeptin (Sigma, UK), 10μg/ml pepstatin (Sigma, UK), 10 mM dithiothreitol (Sigma, UK) for 15 min at 4°C. The lysates were vortexed and sonicated twice for 15 sec at amplitude of 12 microns interspersed by 1 min intervals before centrifugation at 12000 x g for 20 min at 4°C to remove particular matter. The supernatant was then removed and protein quantification was performed using the Bradford method (Bradford, 1976) as follows:

5.3.2.2.1 Bradford method

5.3.2.2.1.1 Reagents

Coomassie Brilliant Blue G-250 (Sigma, UK) was used in the preparation of the Bradford reagent as follows:

Coomassie blue	100 mg
95% (v/v) ethanol	50 ml
85% (v/v) orthophosphoric acid (Sigma,UK)	100 ml

The final volume was adjusted to 1 L GDW, and the solution stored at 4°C.

For the Bradford method a series of standard dilutions ranging from 3 μg/ml to 800 μg/ml were prepared from a freshly prepared stock solution of bovine serum albumin (BSA) in PBS. Subsequently 100 μl of each standard, test samples and PBS as a blank were placed into separate plastic cuvettes to which 1.2 ml of Bradford

reagent was added. After allowing between 5 min to 60 min for colour development, the OD of each sample was read at 595 nm and the protein concentration of the test samples determined directly from a standard curve.

5.3.2.2.2 Measurement of fluorescent emission

Aliquots of sperm lysate (100 μg, according to the Bradford method) were then diluted with 1 ml of buffer containing; 50 mM HEPES, 10% sucrose, 0.1% Chaps, {(3-[(3-chloamidopropyl) dimethylammoniol]-1-propanesulfonate}, pH 7.5, and 50 μM of caspase-3 substrate, Ac-DEVD-AFC [(Asp-Glu-Val-Asp (DEVD) conjugated with 7-amino-4-trifluoromethylcoumarin (AFC)], (Bachem, Merseyside, UK) and incubated for 45 min at 37° C. The fluorescent emission (excitation 400 nm and emission 505 nm) was measured using a fluorimeter (Perkin Elmer, Beaconsfield, UK). Prior to performing the main experiments, the fluorimeter machine was tested at least three times using the AFC standard. AFC is a fluorescent molecule which is released from the substrate and can be used for standardisation and measurement of caspase-3 activity. Caspase-3 activity in the samples is proportional to the amount of free AFC produced. When AFC is attached to the substrate, it produces a blue fluorescence upon exposure to UV light (400 nm). Caspase-3 cleaves the AFC-substrate and releases free AFC, which produces a yellow-green fluorescence at 505 nm when exposed to UV light. Dimethyl sulfoxide (DMSO) that is a solvent for Ac-DEVD-AFC and AFC, was also tested by the fluorimeter as a zero control.

Background fluorescence was determined using blanks without sperm. Standards containing 1-5 μM AFC were utilised to determine the amount of fluorochrome released, and apoptotic human neutrophils treated with 1 mmol/l cycloheximide were used as a positive control. Neutrophils were isolated from whole blood as follows:

5.3.2.2.3 Isolation of neutrophils by density centrifugation using dextran

Heparinised (~1 unit/ml) venous blood was obtained from healthy adult volunteers. Neutrophils were purified by sedimentation through a Ficoll-Paque

gradient, dextran sedimentation, and hypotonic lysis as described previously (Frenck *et al.*, 1989) with a few modifications. Firstly, 25 ml of heparinised whole blood were layered over 12.5 ml of Ficoll-Paque (Amersham Biosciences, Bucks, UK) followed by centrifuging at 400 x g for 35 min. The top layer containing plasma, monocytes, and Ficoll-Paque was discarded. To separate erythrocytes from granulocytes, PBS 1 x at the same volume of the bottom layer and 6% dextran at ¼ of total volume (for example, 25 ml of bottom layer + 25 ml of PBS 1 x + 12.5 ml of 6% dextran) were then added and gently mixed once by pipetting.

The suspension was then incubated at RT for about 1h to allow the granulocytes to settle above the precipitation of erythrocytes. The supernatant was transferred into a separate tube followed by centrifuging at 115 x g for 8 min. The top layer was discarded and to the pellet 9 ml of GDW gently added and mixed prior to adding 1 ml of PBS 10 x. The suspension was then centrifuged as previously. The supernatant was discarded and the pellet was washed with normal PBS and re-centrifuged as before. The final pellet was re-suspended in 1 ml of HBSS without Ca^{2+} and Mg^{2}. To confirm the successful separation of neutrophils, 2 slides from the suspension were prepared and stained as described earlier.

5.4 Results

5.4.1 Challenge of LPS-, Kdo-, lipid A-induced spermatozoa with ROS scavengers and measurement of sperm viability

The microscopic assessment of prepared slides from sperm preparations, using immunocytochemistry and a cytocentrifuge preparation technique showed that the spermatozoa used for the recent study were free of leukocyte contamination at the minimum detection level. This equates to $\leqslant$143 leukocytes per 1 x 10^7 spermatozoa in the 80% percoll fraction for the immunocytochemistry technique or $\leqslant$1000 leukocytes/ml for the cytocentrifuge preparation technique. However, in the control group, leukocytes were detectable as expected (Figure 5. 1).

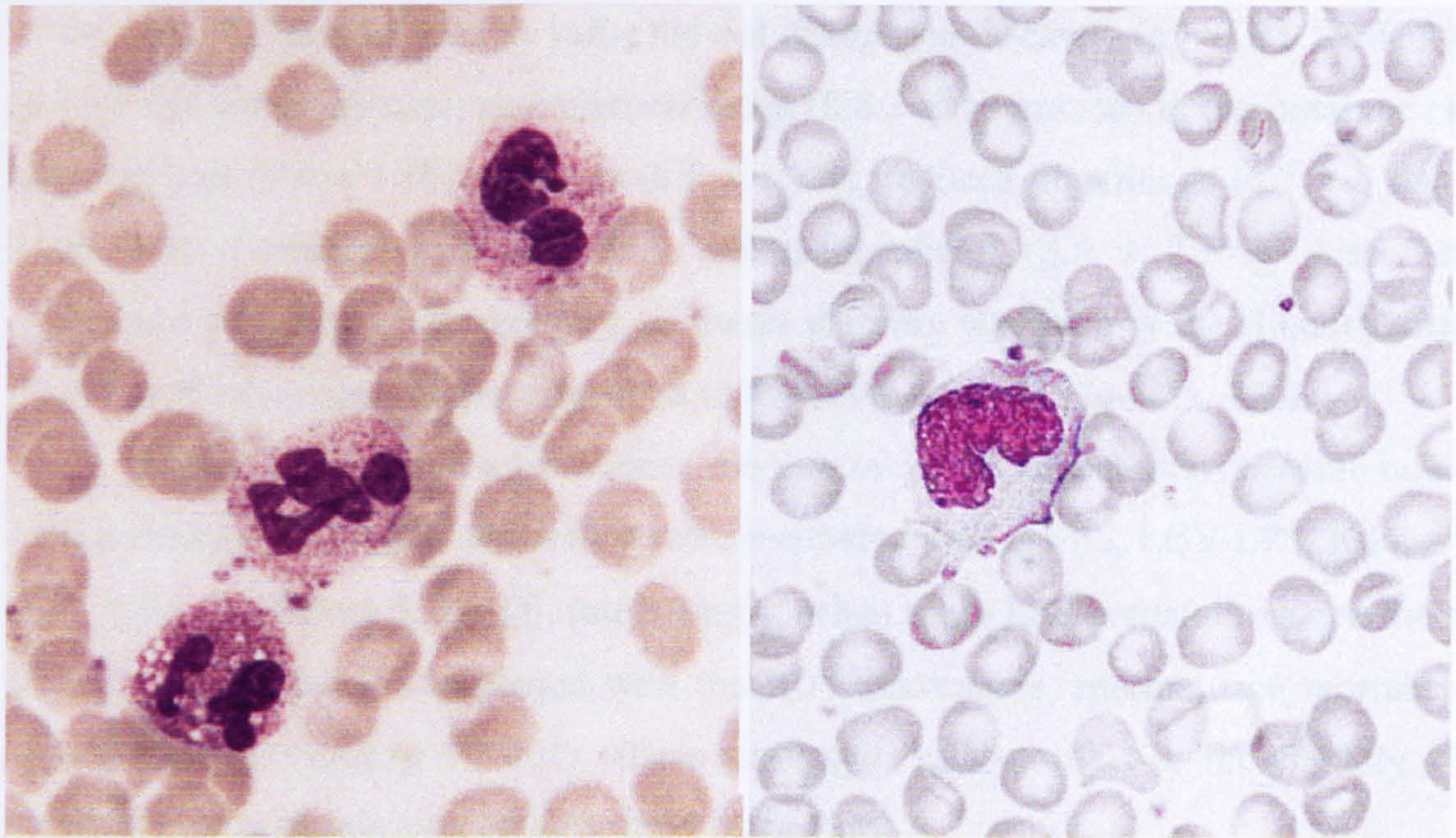
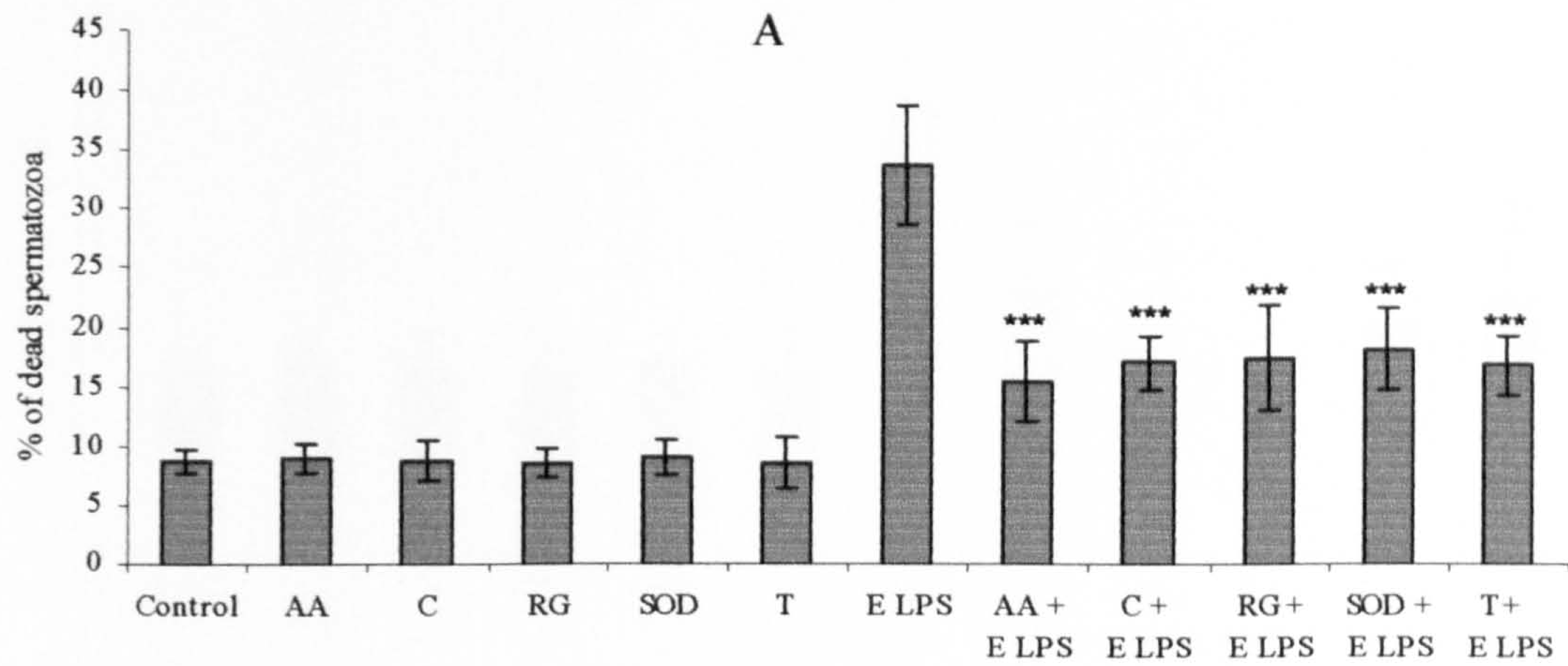
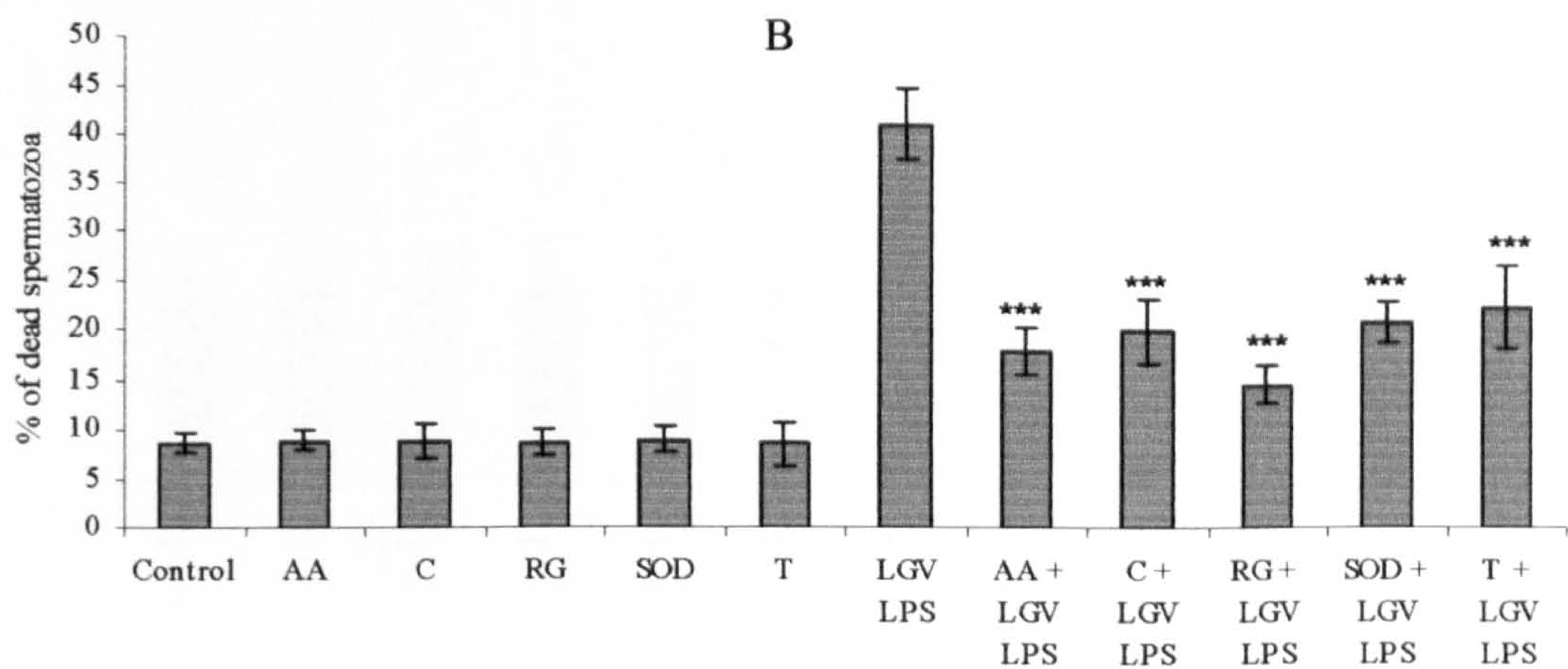


Figure 5. 1. Two slides from whole blood prepared using the cytocentrifuge preparation technique. Three neutrophils (left) and one monocyte (right) are shown. Neutrophils have segmented nuclei, typically with 2 to 5 lobes connected together by thin strands of chromatin. Monocytes are the largest cell type seen in blood smears. Their nuclei are not multilobular like granulocytes, but may be deeply indented or U-shaped, with reticular-appearing chromatin.

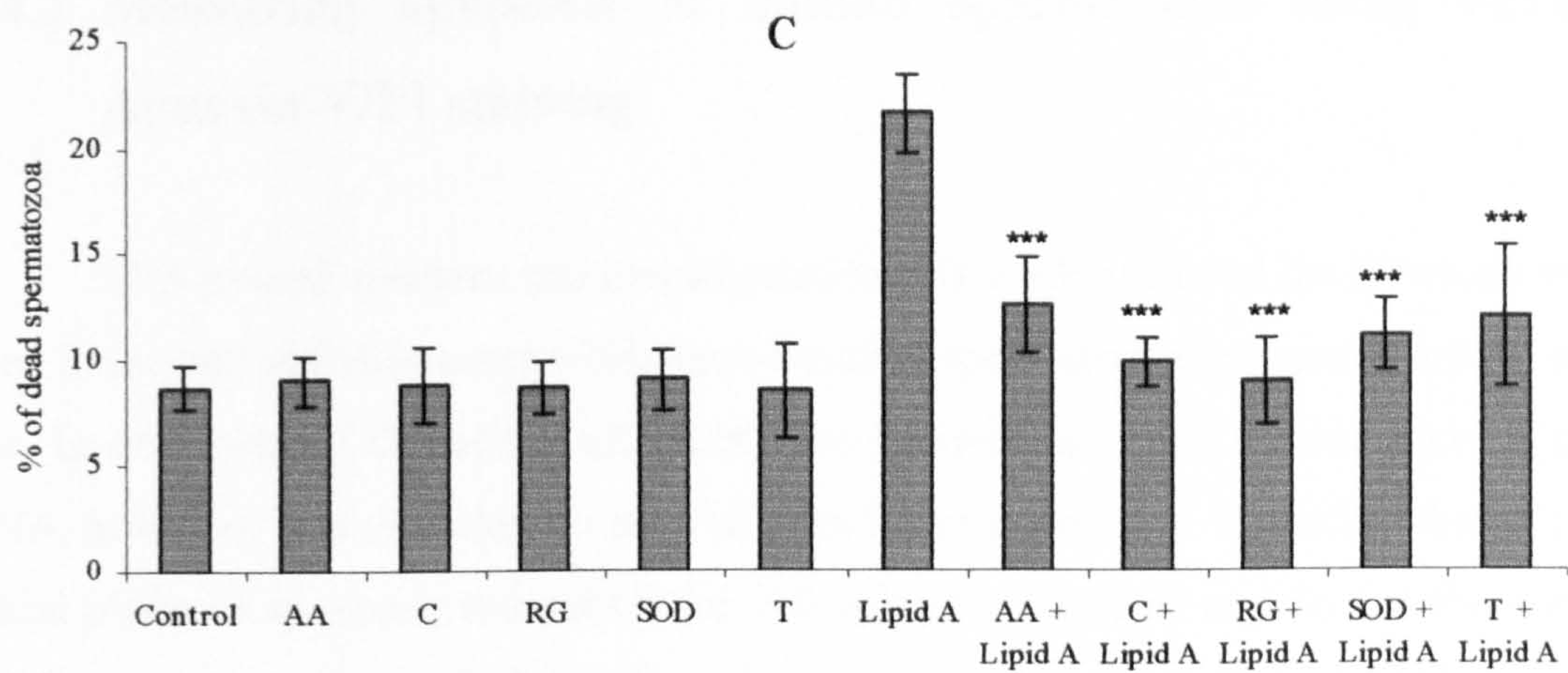
Immediately after sperm preparation, spermatozoa had a sperm death of 8.9 ± 1.5%. After 6h incubation, this value did not change significantly ($p > 0.05$, Student *t* test) in the control groups; spermatozoa alone (8.8 ± 1%) and also in the presence of ascorbic acid (9.0 ± 1.1%), catalase (8.8 ± 1.7%), reduced glutathione (8.7 ± 1.2%), superoxide dismutase (9.0 ± 1.4%), and α-tocopherol (8.5 ± 2.2%). Also, no significant variation in the recovery of sperm viability was seen in the aliquots with added ROS scavengers alone, compared to the control group (Figure 5. 2A-D).

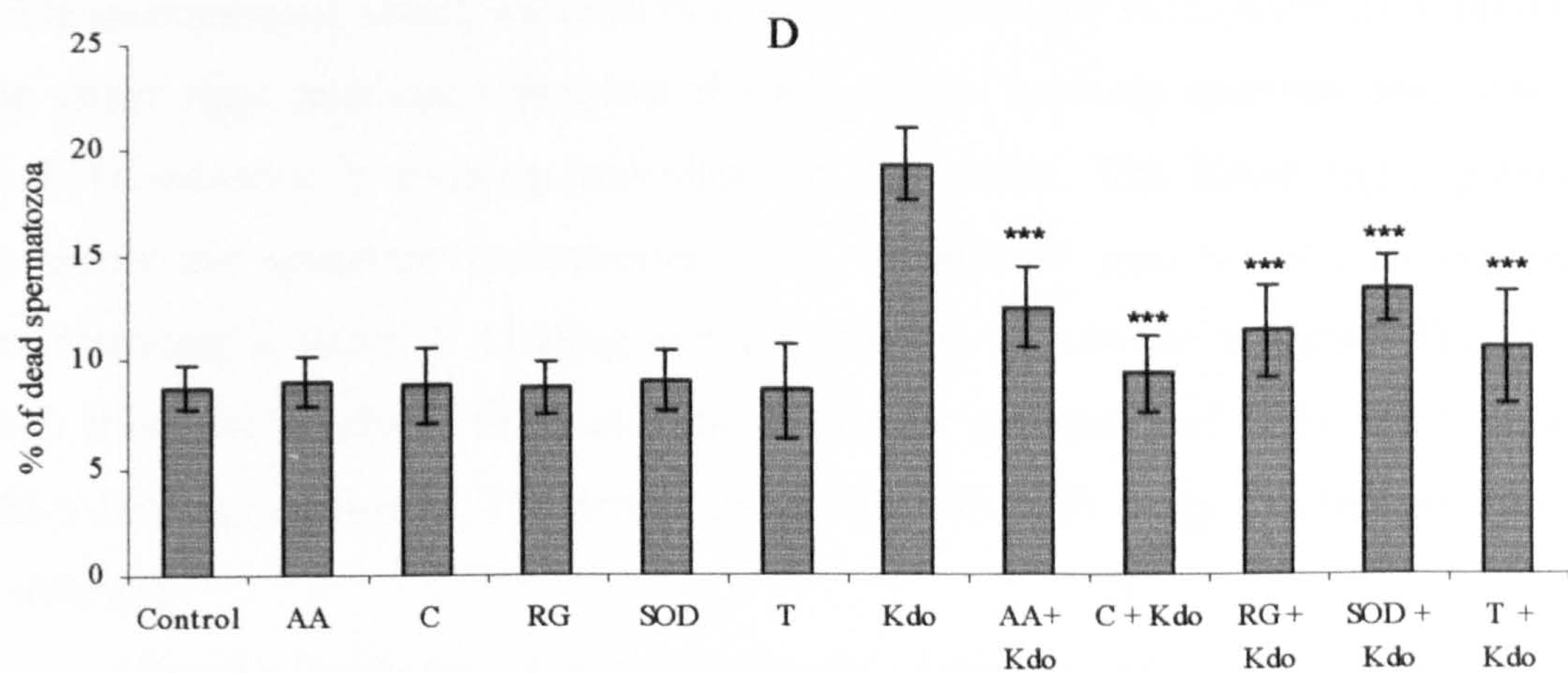
Similar to previous experiments, the rate of which spermatozoa became non-viable increased markedly when they were incubated with E LPS, LGV LPS, Kdo, or lipid A for 6h (Figure 5. 2A-D). Interestingly, when these LPS preparations and their fractions were also pre-incubated with the ROS scavengers, spermatozoa mortality significantly declined ($p < 0.001$). These scavengers had no effect on the viability of spermatozoa in the absence of LPS, lipid A, or Kdo.





Figures 5. 2A and B. Effects of ROS scavengers on spermatozoa when co-incubated with *C.trachomatis* E LPS (A) or LGV LPV (B). LPSs added to spermatozoa already co-incubated with five ROS scavengers; ascorbic acid (AA), catalase (C), reduced glutathione (RG), superoxide dismutase (SOD), and α-tocopherol (T) for 6h. The mortality of spermatozoa was investigated using the HOS test. Data are mean of six experiments ± SEM. ***, $p < 0.001$, Student *t* test.





Figures 5. 2C and D. Effects of ROS scavengers on spermatozoa when co-incubated with commercial lipid A from *E.coli* (C) or synthetic Kdo (D). These two LPS fractions were added to spermatozoa already co-incubated with five ROS scavengers; ascorbic acid (AA), catalase (C), reduced glutathione (RG), superoxide dismutase (SOD), and α-tocopherol (T) for 6h. The mortality of spermatozoa was investigated using the HOS test. Data are mean of six experiments ± SEM. ***, $p < 0.001$, Student *t* test.

5.4.2 Measuring apoptosis in human spermatozoa using FITC-Annexin V/PI staining

LPS-treated spermatozoa investigated in this work excluded the PI which was used in the cell viability assay while heat-shocked spermatozoa (necrosis control) did not. In cells with a damaged cell membrane PI induces a red fluorescence of the DNA, however, it is excluded by cells with an intact membrane. Therefore during the initial phase of apoptosis the cells were still able to exclude PI and do not show any red fluorescence signal, similar to that of living cells. Figures 5. 3a and 5. 3b show a number of dot-plots of annexin-V and PI staining in which three distinctive cell populations are demonstrated. The lower left quadrant of the cytograms shows the viable spermatozoa, which exclude PI and are negative for FITC-annexin V binding. The upper right quadrant represents the non-viable, necrotic spermatozoa, positive for FITC-annexin V binding and showing PI uptake. The lower right quadrant represents the apoptotic spermatozoa, FITC-annexin V positive and PI negative, demonstrating annexin V binding and cytoplasmic membrane integrity. Figures 5. 4A-D show the results of FITC-annexin V/PI flow cytometry of LPS-, lipid A-, and Kdo-treated spermatozoa. The details of the annexin V/PI assay can be summarised as follows:

After 6h incubation, the percentage of apoptotic cells in the control group; spermatozoa alone, spermatozoa + pan-caspase inhibitor, spermatozoa + caspase-3 inhibitor were 9.8 ± 0.53%, 8.42 ± 0.55%, and 8.7 ± 0.8 respectively (Figures 5. 4A-D). The percentage of apoptotic cells in the necrosis control group; heat-shocked spermatozoa alone, heat-shocked spermatozoa + pan-caspase inhibitor, and heat-shocked spermatozoa + caspase-3 inhibitor was 6.4 ± 0.6%, 6.2 ± 0.9%, and 6.5 ± 2.2% respectively (Figures 5. 4A-D). Spermatozoa incubated with staurosporine (apoptosis control), *C.trachomatis* LPS, lipid A or Kdo showed a significant apoptosis rate of 22.5 ± 1.48% - 37.2 ± 3.4% compared with the sperm only control ($p < 0.001$, Student *t* test). Pre-incubation of spermatozoa with pan-caspase inhibitor and caspase-3 inhibitor markedly reversed the percentage of apoptosis in aliquots which were treated by the above-mentioned apoptosis inducers (Figures 5. 4A-D).

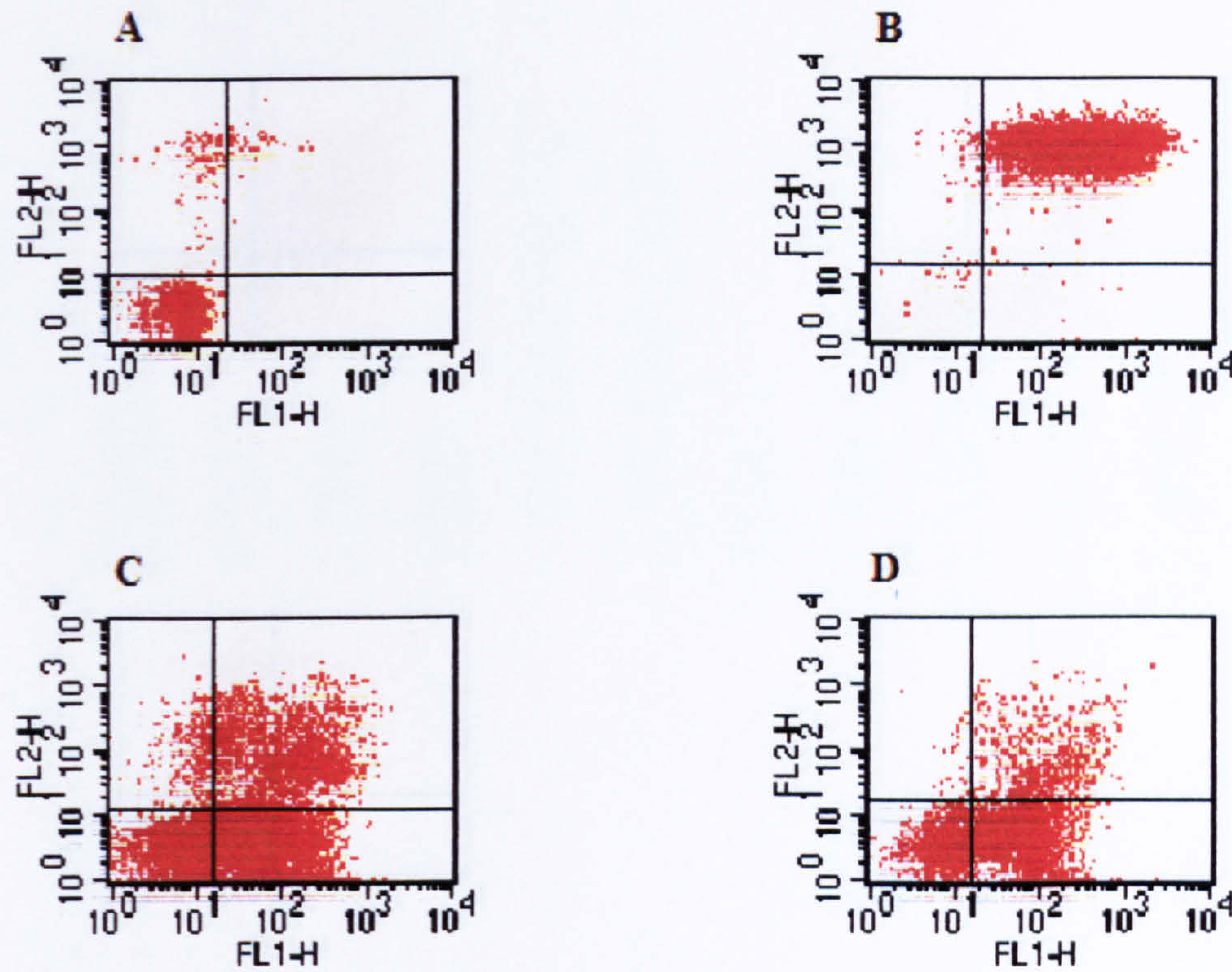


Figure 5. 3a. Examples of a counter diagram of FITC-annexin V/PI flow cytometry of spermatozoa after 6h incubation with *C.trachomatis* LPS, Kdo, and lipid A with and without the pan-caspase and caspase-3 inhibitors. Heat-shocked spermatozoa and staurosporine-induced spermatozoa were used as necrosis and apoptosis controls respectively. Three cell populations are distinctive in each diagram; the lower left quadrant contains viable, non-apoptotic cells (both annexin-V- and PI-negative), the lower right quadrant shows apoptotic cells [(annexin-V (+)/PI (-)], and the upper right quadrant represents non-viable, necrotic cells [(annexin-V (+)/PI (+)]. A, spermatozoa control; B, heat-shocked spermatozoa; C, staurosporine-induced spermatozoa; D, LGV LPS-induced spermatozoa.

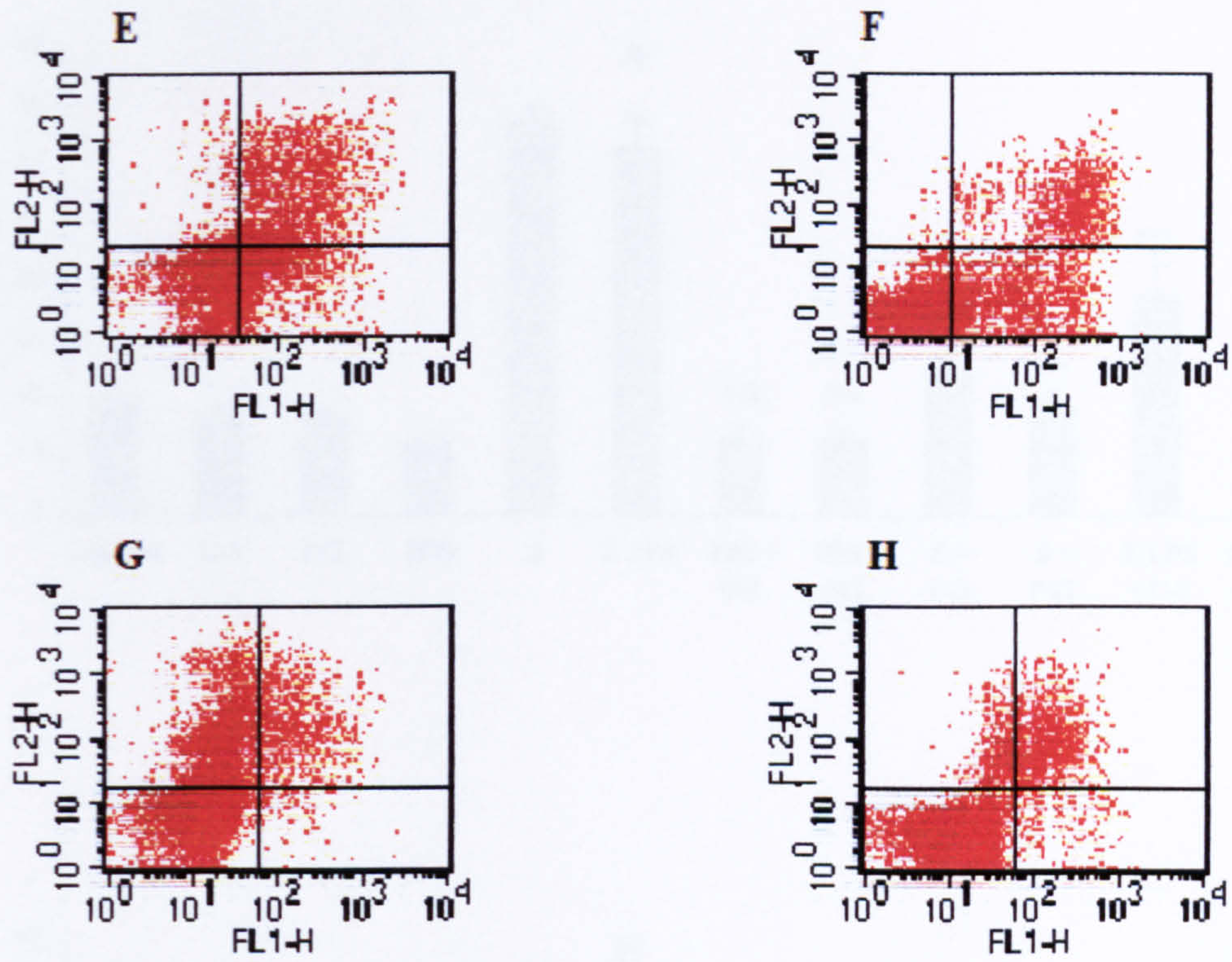
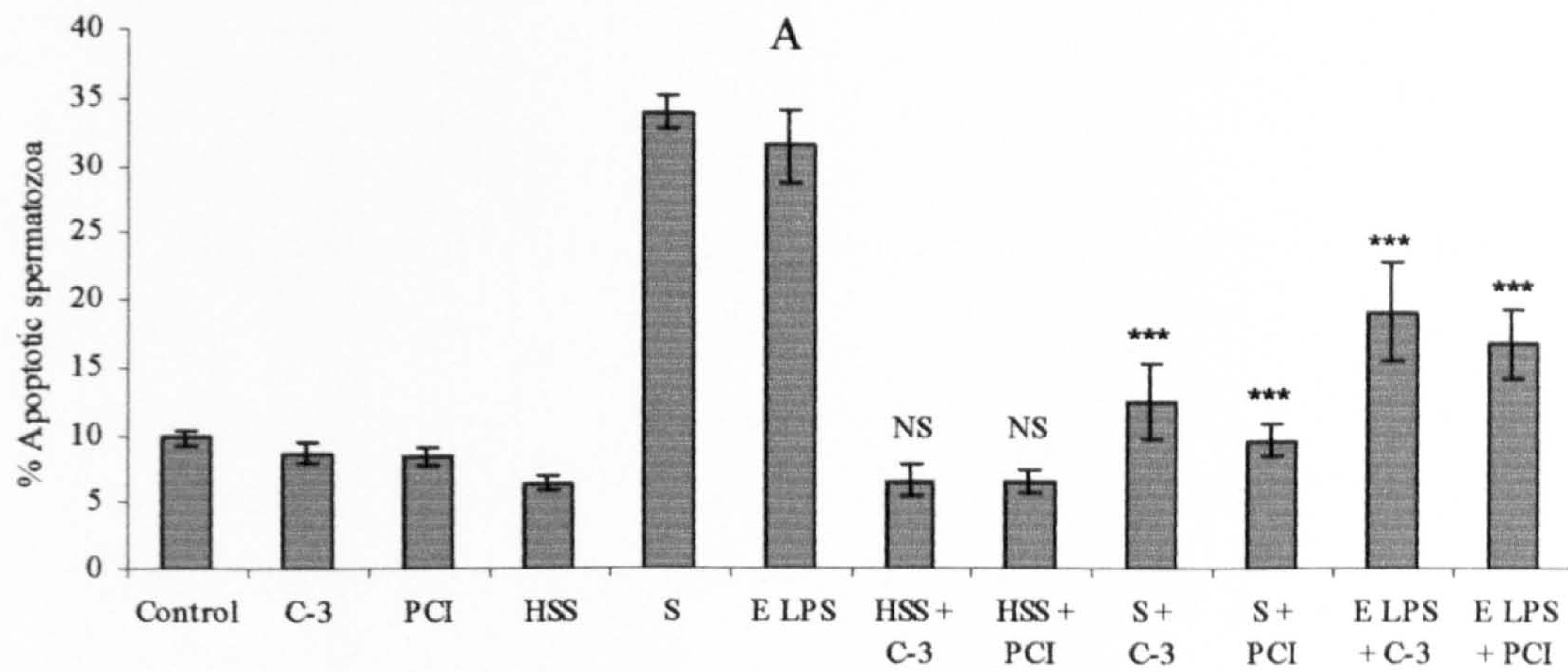
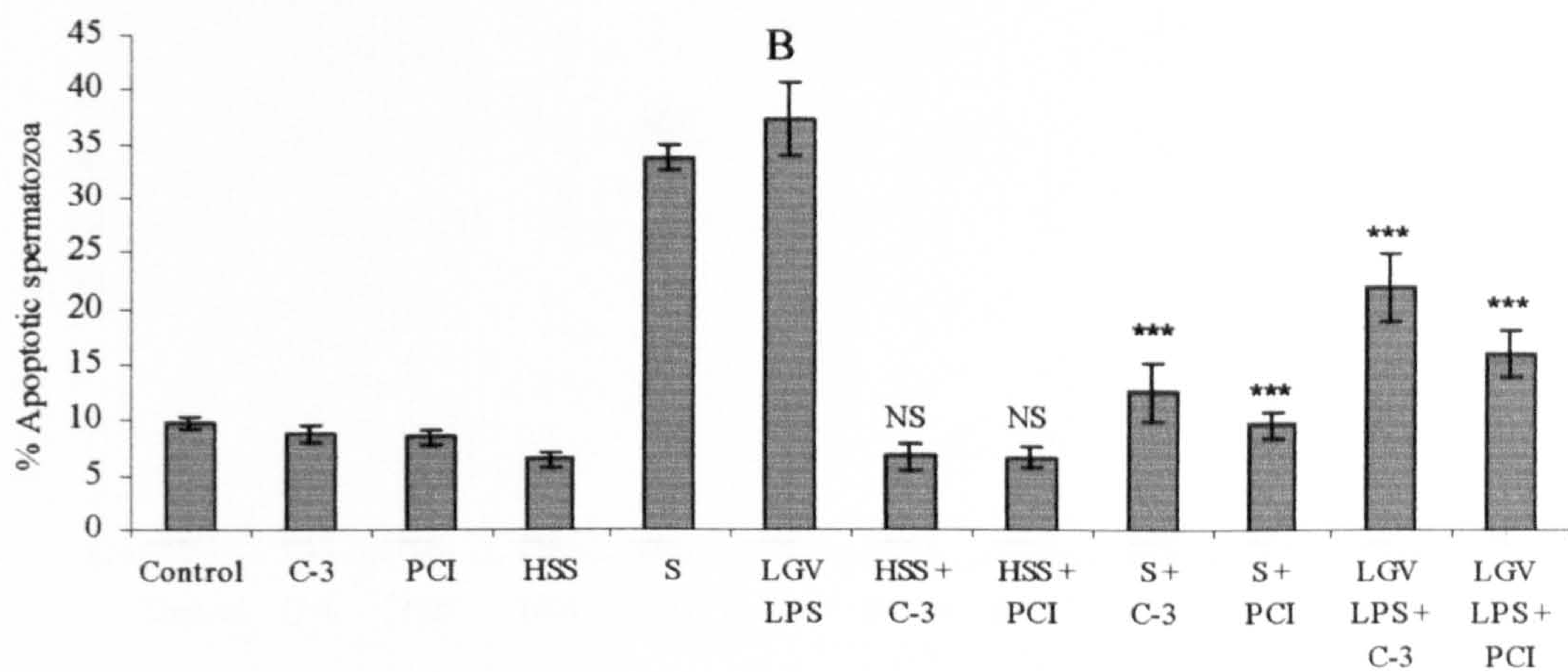
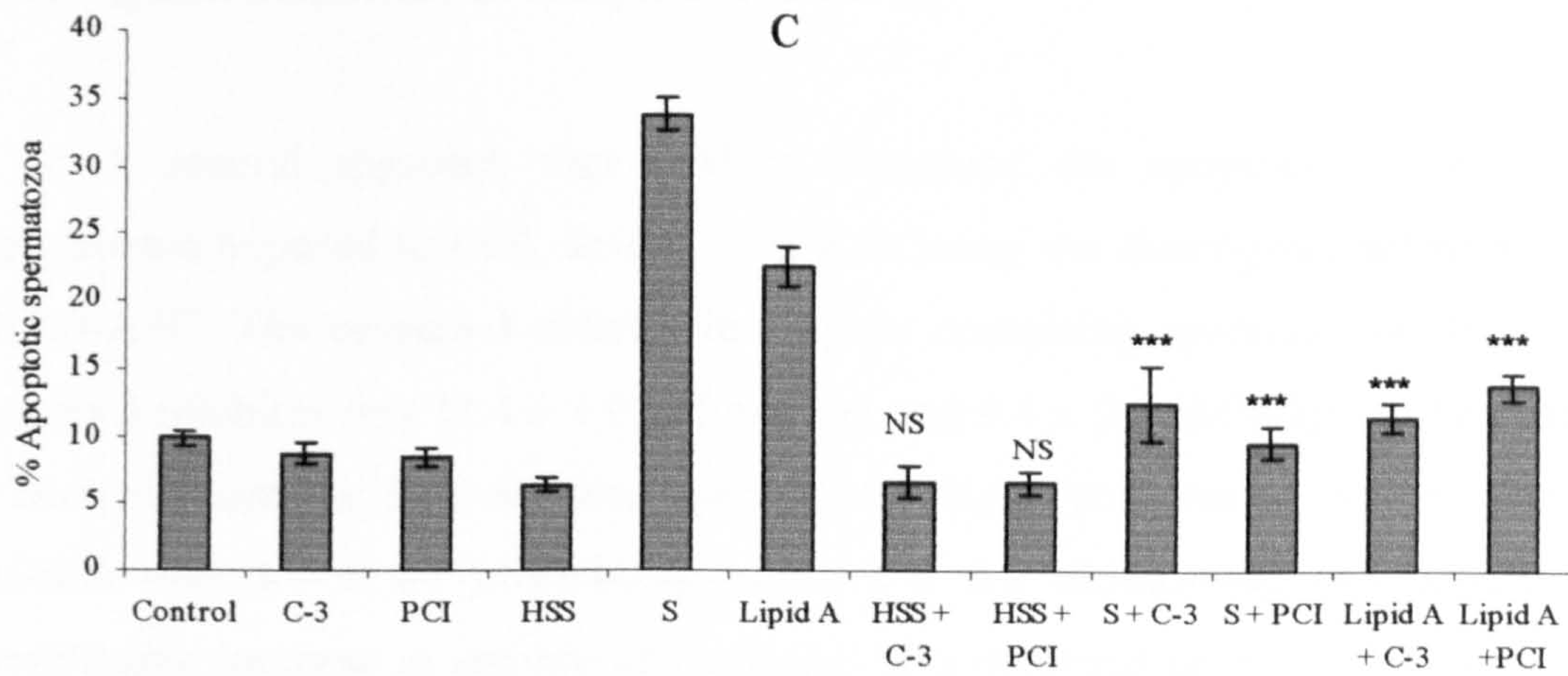


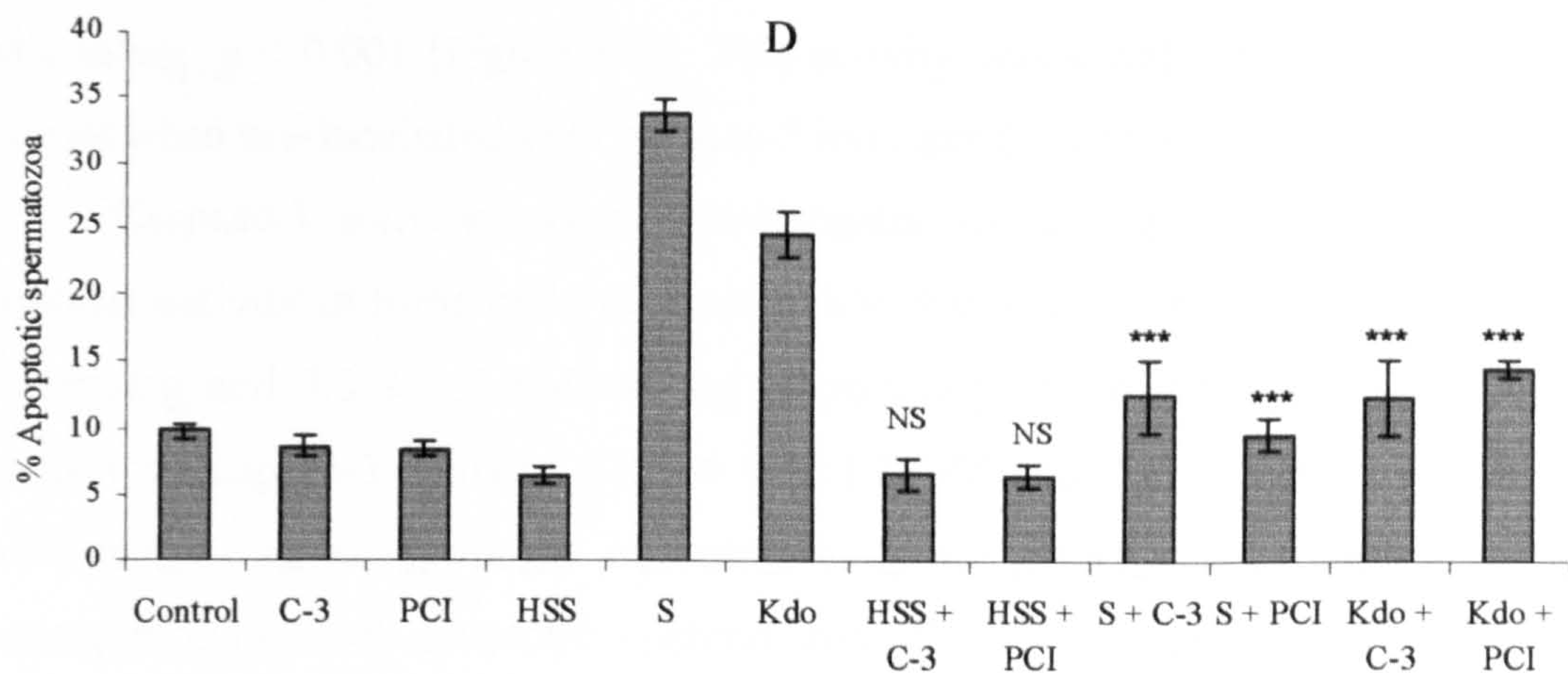
Figure 5. 3b. Examples of a counter diagram of FITC-annexin V/PI flow cytometry of spermatozoa. For details see legend to Figure 5. 3a. E, lipid A-induced spermatozoa; F, Kdo-induced spermatozoa; G, staurosporine + pan-caspase inhibitor; H, staurosporine + caspase-3 inhibitor.





Figures 5. 4A and B. Induction of apoptosis in spermatozoa incubated with *C.trachomatis* LPS from serovars E (A) or LGV (B) for 6h, with and without pan-caspase inhibitor and caspase-3 inhibitor. Heat-shocked spermatozoa and staurosporine-induced spermatozoa were used as necrosis and apoptosis controls respectively. Data are mean of 6 experiments ± SEM. C-3, caspase-3 inhibitor. HSS, heat-shocked spermatozoa. PCI, pan-caspase inhibitor. S, staurosporine. NS, not significant. *******, $p < 0.001$, Student *t* test.





Figures. 5. 4C and D. Induction of apoptosis in spermatozoa incubated with commercial lipid A from *E.coli* (C) and synthetic Kdo (D) for 6h, with and without pan-caspase inhibitor and caspase-3 inhibitor. Heat-shocked spermatozoa and staurosporine-induced spermatozoa were used as necrosis and apoptosis controls respectively. Data are mean of 6 experiments ± SEM. C-3, caspase-3 inhibitor. HSS, heat-shocked spermatozoa. PCI, pan-caspase inhibitor. S, staurosporine. NS, not significant. *******, $p < 0.001$, Student *t* test.

5.4.3 Quantification of caspase-3 activity

A second approach was used to determine the apoptotic properties of spermatozoa exposed to LPS, lipid A, and Kdo using the fluorogenic substrate Ac-DEVD-AFC. The caspase-3 activity in aliquots containing spermatozoa alone and caspase-3 inhibitor was 11.4 ± 1.9 nM/min/mg and 9.4 ± 2.1 nM/min/mg. The value in necrosis controls; heat-shocked spermatozoa alone and treated with caspase-3 inhibitor was 3.1 ± 1.1 nM/min/mg and 1.3 ± 0.9 nM/min/mg respectively. A considerable increase in activity of caspase-3 was observed in spermatozoa treated with either E LPS, LGV LPS, lipid A, or Kdo (55.8 ± 13.7 nM/min/mg, 47.8 ± 5.8 nM/min/mg, 43.9 ± 5.2 nM/min/mg, and 51.3 ± 6.6 nM/min/mg respectively, $p < 0.001$, Student *t* test) (Figure 5. 5). Spermatozoa in the presence of staurosporine as an apoptosis control also showed a significant rise in caspase-3 activity, 41 ± 5.5 nM/min/mg, $p < 0.001$ (Figure 5. 5). This activity was significantly inhibited in all aliquots when pre-incubated with caspase-3 inhibitor ($p < 0.001$).

Caspase-3 activity was also investigated in neutrophils as a control. The apoptosis activity in these cells with and without caspase-3 inhibitor was 8.1 ± 1.7 nM/min/mg and 7.3 ± 1.3 nM/min/mg respectively. Neutrophils showed a highly significant caspase-3 activity (129.4 ± 11.8 nM/min/mg) in the presence of cycloheximide as an apoptosis inducer which was about three times more than staurosporine-induced apoptotic spermatozoa. Similar to spermatozoa, apoptosis activity in neutrophils was considerably blocked by caspase-3 inhibitor (36.8 ± 8.9 nM/min/mg). The standard curve of AFC, raw data of this work and the method of calculation are shown in Figure 5. 6 and Table 5. 2 respectively.

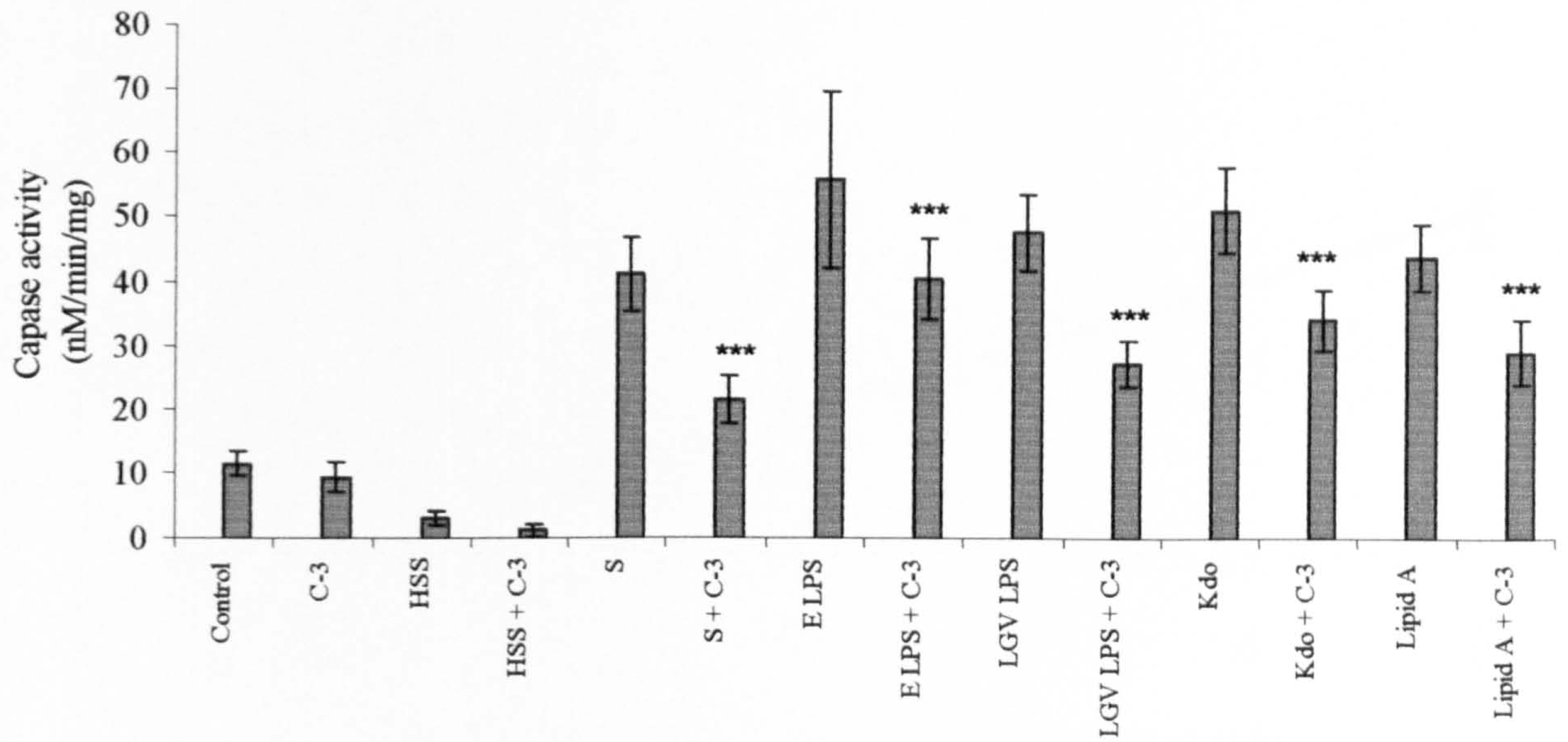


Figure 5. 5. Caspase-3 activity in spermatozoa exposed to *C.trachomatis* LPS from either serovar E or LGV, lipid A, or Kdo. Caspase-3 activity was assessed in treated spermatozoa with and without pre-incubated caspase-3 inhibitor. Heat-shocked spermatozoa and staurosporine-induced spermatozoa were used as necrosis and apoptosis controls respectively. Data are shown as the mean ± SEM from six independent experiments. C-3, caspase-3 inhibitor. HSS, heat-shocked spermatozoa. S, staurosporine. ***. $p < 0.001$, Student *t* test.

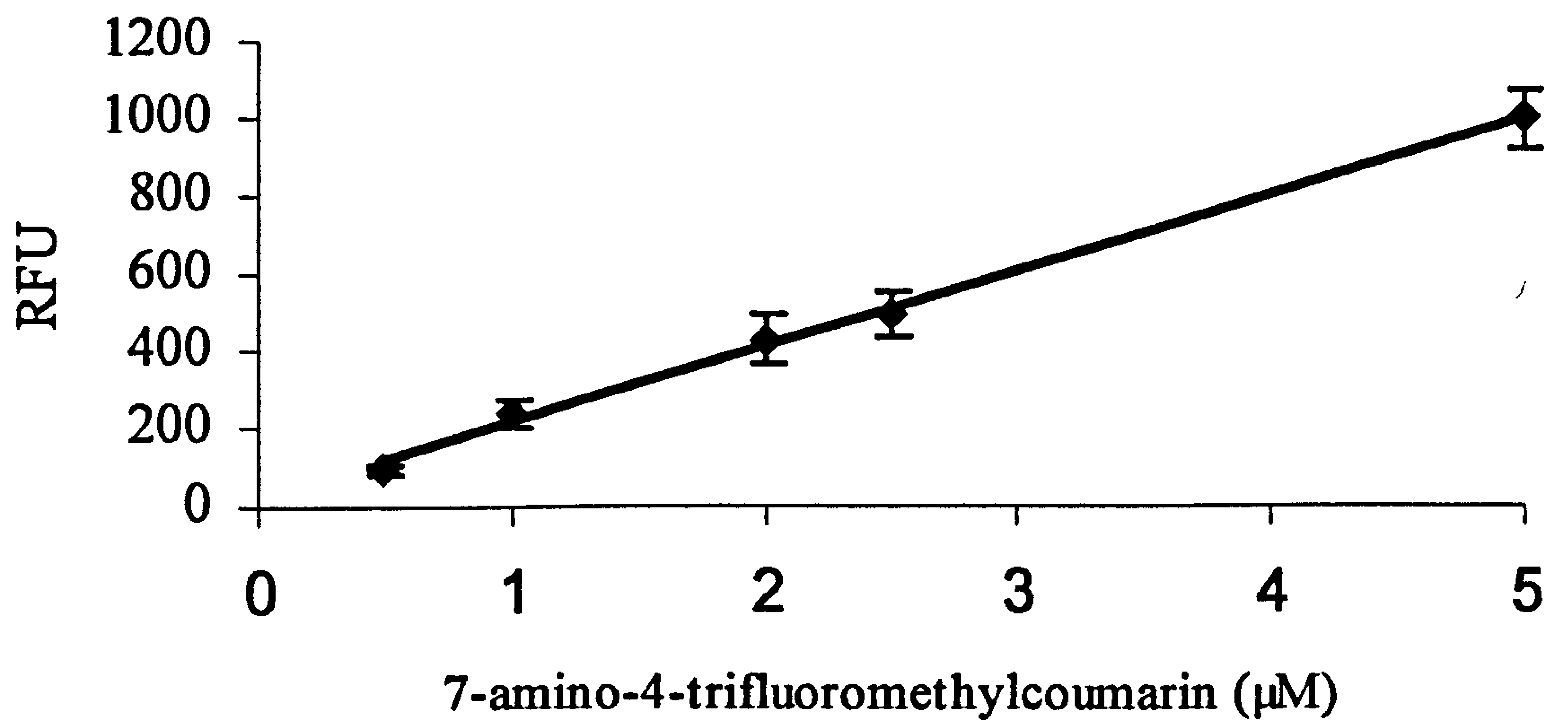


Figure 5. 6. Standard curve to determine the caspase-3 activity of samples. The corresponding free AFC concentration released by the samples was determined. Data are the average of three tests ± SEM. RFU, Relative Fluorescence Unit.

	RFU	nM (AFC concn)	AFC concn/180*	AFC concn/180/0.1**	SEM
DMSO	0	0	0	0	0
Control	41.2	206	1.14	11.4	1.95
C-3	33.9	169.5	0.94	9.4	2.15
HSS	11.16	55.8	0.31	3.1	1.15
HSS + C-3	4.7	23.5	0.13	1.3	0.9
S	147.8	739	4.105	41.05	5.55
S + C-3	77.8	389	2.16	21.6	3.75
E LPS	201.1	1005.5	5.58	55.8	13.75
E LPS + C-3	145.6	728	4.044	40.44	6.15
LGV LPS	172.1	860.5	4.78	47.8	5.85
LGV LPS + C-3	98.8	494	2.744	27.44	3.5
Kdo	184.7	923.5	5.13	51.3	6.65
Kdo + C-3	123.1	615.5	3.419	34.19	4.5
Lipid A	158.2	791	4.394	43.94	5.2
Lipid A + C-3	105.6	528	2.933	29.33	5

Table 5. 2. The raw data of quantification of caspase-3 activity using a fluorimeter and the method of calculation are shown. AFC, 7-amino-4-trifluoromethylcoumarin. C-3, caspase-3 inhibitor. DMSO, Dimethyl sulfoxide, HSS, heat-shocked spermatozoa. RFU, Relative Fluorescence Unit. S, staurosporine. *, incubation time (min). **, sperm lysate concentration (0.1 mg determined by Bradford method).

5.5 Discussion

Oxidative stress caused by ROS in semen has been suggested to be an important factor in the aetiology of poor sperm function. There is a potential association between ROS generation and male urogenital tract infection (Wang *et al.*, 1997). In our previous work and the present study, we demonstrated that *C.trachomatis* LPS, commercial lipid A and Kdo decreased sperm viability, which was modified by treatment of the spermatozoa with ROS scavengers such as ascorbic acid, catalase, reduced glutathione, SOD, and α-tocopherol. The reversibility of the sperm viability by these ROS scavengers suggests that LPS-, lipid A-, and Kdo-induced production of ROS may be related to the decreased viability caused by these treatments. Urata *et al.*, (2001) using these ROS scavengers demonstrated that there is a relationship between endotoxin-induced ROS production and a decrease in sperm motility. In their chemiluminescence studies, they also showed ROS-production is significantly higher in the LPS-induced spermatozoa than in control groups. In another study, Wang *et al.*, (1997) reported that treatment of spermatozoa with cytokines including TNF-α and IFN-γ or LPS at optimal concentrations (1 ng/ml, 2000 unit/ml, and 100 ng/ml respectively) stimulate ROS production. In chapter 4, we demonstrated that LPS and lipid A, but not Kdo stimulated TNF-α production in IFN-γ-induced THP-1 cells. Since TNF-α production in turn, stimulates ROS production (Schulze-Osthoff *et al.*, 1993), and ROS induction might cause cellular death (de Lamirande and Gagnon, 1995; Aitken, 1999) accordingly, this suggests a close relationship between our results and these mentioned studies.

Fraga *et al.*, (1991) indicated that the level of oxidative damage to human sperm DNA that could affect sperm quality and increase the risk of genetic defects is inversely related to seminal plasma ascorbic acid levels. They also suggested this ROS scavenger, complements the sperm antioxidants including the sperm antioxidant defence enzymes SOD, glutathione peroxidase, and catalase that are required for optimal sperm motility.

Kobayashi *et al.*, (1991) measured the level of SOD, a highly specific scavenging enzyme for superoxide anion radicals ($O_2^{\cdot-}$), and lipid peroxide produced by oxygen free radicals in human seminal plasma and spermatozoa. They showed that there is a close relationship between the SOD activity rate in human spermatozoa

and the number of motile spermatozoa, while the activity in seminal plasma does not relate to the sperm concentration or motility. These workers and Aitken (1995), suggested that an endotoxin-induced increase in ROS may cause the lipid peroxidation of spermatozoa and disturb its membrane conformation leading to loss of motility and could be a mechanism underlying male infertility. Because the sperm membrane has a high level of PUFA containing two or more double bonds, this could be readily attacked by oxygen radicals. Moreover, spermatozoa are unique cells because, during the terminal stages of differentiation, a considerable portion of their cytoplasm is removed and the remnants are confined to the mid-piece. Accordingly, the limited antioxidant systems within these cells are localized to this part of spermatozoa and as a result, they are not able to protect the plasma membrane bordering most of the the surface of the spermatozoa (Aitken, 1994).

Endotoxin from Gram-negative bacteria including *E.coli*, *C.trachomatis*, and *Neisseria gonorrhoeae* which could be present in male genital tract infections, has the potential to activate leukocytes in the immune system. LPS-stimulated leukocytes produce a markedly higher level of ROS compared with sperm (Wang *et al.*, 1997). Since inflammation may induce a defect in the ROS scavenging system (Rajasekaran *et al.*, 1995) and this system is able to reverse the pathogen-induced ROS production either by leukocytes or spermatozoa, antioxidant therapy is another possible useful option for treatment of bacterial genital tract infections or improving fertilization.

Since the possible presence of leukocytes in sperm preparations could interfere with our results, leukocyte contamination was investigated using immunocytochemistry and a cytocentrifuge preparation technique. The microscopic assessment of sperm preparations using a percoll gradient method, demonstrated that spermatozoa which were used in our experiment were free of leukocytes at the before-mentioned detection level.

This finding is consistent with the previously published report that leukocyte concentrations in semen cells collected from the bottom of 80% percoll gradient were extremely low (Aitken and West, 1990). Kessopoulou *et al.*, (1992) also showed that the median leukocyte number per 1 x 10^7 spermatozoa in the 80% percoll fraction was 143 (range 0-55000) however, this value in the 40-80% fraction was 4221 (range 0-326333).

Although we tried to investigate the direct effects of *C.trachomatis* LPS, lipid A, and Kdo on ROS production by spermatozoa, for three reasons our attempts failed. Firstly, as described before, isolation of lipid A and Kdo from *C.trachomatis* LPS required a large quantity of LPS, and LPS extraction from *C.trachomatis* in turn was a time-consuming procedure. Secondly, in many cases because of a lack of the sperm donor's consent, the availability of good quality human spermatozoa was limited. Finally, because of a technical problem, the chemiluminescence machine failed to read a limited number of samples correctly. For example, for testing the machine at the first stage, we used spermatozoa with and without luminal or phorbol. The EBSS was also used as a control. However, no activity was recorded by the machine. At the second stage, the LPS-treated spermatozoa were compared with spermatozoa alone, the results were the same for all samples. The experiment was then repeated and different programmes were used, no activity was again recorded. Therefore, ROS production by spermatozoa induced by LPS or its fractions remains to be investigated further. An example of the chemiluminescence data are shown in Appendix 3.

The second aim of this work was to analyse the type of cell death which occurred in spermatozoa incubated with LPS, Kdo, or lipid A. We investigated two independent assays for measurement and quantification of apoptosis in treated spermatozoa that might be adequate tools to prove the apoptotic properties of treatments used in this study. One of these investigations was performed by flow cytometry, which allows measurement of apoptosis at the single-cell level as well as automatic analysis of thousands of cells in a few seconds. The other method was by fluorimetry, which allows a quantification of caspase activity.

High levels of ROS disrupt the inner and outer mitochondrial membranes. This results in the release of cytochrome *c* protein from the mitochondria that activates caspases and induces apoptosis. Apoptosis may also be initiated by ROS-independent pathways involving the cell surface protein Fas (Lee *et al.*, 1997). Fas is a type I membrane protein that belongs to the TNF-α receptor family and mediates apoptosis (Krammer *et al.*, 1994).

The release of pro-apoptotic factors such as cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria will then initiate the downstream caspases such as caspase 9 or caspase 3 to execute the apoptotic events (Desagher and Martinou, 2000).

Caspase-3 activation is considered as a key element in the apoptosis execution programme. Caspase-3 activity, responsible for activation of specific DNases, DNAse I and DNAse II, which induce apoptosis, has been strongly implicated in human pathologies (Reed, 2001). The presence of caspase-3 in human sperm has been recently detected which suggests the existence of a caspase-dependent apoptosis in human semen (Kim *et al.*, 2001; Weng *et al.*, 2002).

Apoptosis is a cell death process, characterized by morphological and biochemical features occurring at different stages. A critical stage of apoptosis involves the acquisition of surface changes by dying cells that eventually results in the recognition and the uptake of these cells by phagocytes. Different changes on the surface of apoptotic cells such as the expression of thrombospondin binding sites (Pytela *et al.*, 1985), loss of sialic acid residues (Savill *et al.*, 1993) and exposure of a phospholipid-like phosphatidylserine (PS) (Fadok *et al.*, 1992) have been previously described.

Phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane with phosphatidylcholine and sphingomyelin exposed on the external leaflet of the lipid bilayer, and phosphatidylserine predominantly observed on the inner surface facing the cytosol (Op den Kamp, 1979). Exposure of PS on the external surface of the cell membrane has been reported for activated platelets and aged erythrocytes (Schroit and Zwaal, 1991). It has been also reported that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose PS which is translocated to the outer layer of the membrane. This occurs in the early phase of apoptotic cell death during which the cell membrane remains intact (Fadok *et al.*, 1992). This PS exposure possibly represents a hallmark in detecting dying cells (Barroso *et al.*, 2000; Oosterhuis *et al.*, 2000).

Annexin V, belonging to a recently discovered family of proteins, the annexins, with anticoagulant properties (Reutelingsperger *et al.*, 1985) has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively

charged phospholipids like PS. This is normally absent in the outer leaflet of the plasma membrane (Andree *et al.*, 1990) in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyeline, which are constitutively present in the outer leaflet of plasma membranes (Raynal and Pollard, 1994; Vermes *et al.*, 1995). Therefore, this protein can be used as a sensitive probe for PS exposure upon the cell membrane, however, translocation of PS to the external cell surface is not unique to apoptosis, but occurs also during cell necrosis. The difference between these two forms of cell death is that during the early stages of apoptosis the cell membrane remains intact, however, when the cell membrane loses its integrity and becomes leaky, necrosis occurs (Vermes *et al.*, 1995). Therefore, the measurement of annexin V binding to the cell surface as indicative for apoptosis has to be performed in conjunction with a dye exclusion test to establish integrity of the cell membrane. By conjugating fluorescein isothiocyanate (FITC) to annexin V it is possible to identify and quantify apoptotic cells on a single-cell basis by flow cytometry. Staining cells simultaneously with FITC-annexin V (green fluorescence) and the non-vital dye propidium iodide (PI, red fluorescence) allows the discrimination of intact cells (FITC-annexin V negative/PI negative), early apoptotic (FITC-annexin V positive/PI negative), and late apoptotic or necrotic cells (FITC-annexin V positive/PI positive) (Vermes *et al.*, 1995; Oosterhuis *et al.*, 2000; Shen *et al.*, 2002).

Our results using flow cytometry and fluorimetry showed that LPS and its constituents, lipid A and Kdo were able to induce spermatozoa death via apoptosis. These results were consistent with the findings of ROS scavenger experiments performed in the present study. In these experiments we showed indirectly that LPS or its fractions exert their toxicity via ROS production which could be a potential factor for apoptosis induction.

Although it is established that the PS exposure on the external surface of the cell membrane possibly represents a hallmark in detecting apoptotic cells (Barroso *et al.*, 2000; Oosterhuis *et al.*, 2000), Goth and Stephens, (2001) demonstrated that chlamydiae can induce transient exposure of PS, making annexin V-based measurements unreliable for identifying apoptosis in chlamydia-infected cultures. There is also another recent report that shows anti-apoptotic activities during chlamydial infection (Greene *et al.*, 2004). These workers using 17 different

chlamydial serovars, HeLa cells, and MS74 cells (endometrial epithelial cells) showed that chlamydia-infected cultures can continue to undergo DNA synthesis and mitosis rather than involving apoptosis. Although these two reports might put our present results in question, the latter researchers used HeLa and MS74 cells not spermatozoa for their experiments. Moreover, we confirmed our data using quantitation of caspase-3 activity.

In our experiment, the results of flow cytometry in the necrosis control group indicated that after 6h incubation at 56° C there were still a small percentage of apoptototic spermatozoa present (6.4 ± 0.6%). The pan-caspase inhibitor and caspase-3 inhibitor used in the present study failed to inhibit apoptosis activity in this small number of heat-shocked spermatozoa which were FITC-annexin V positive/PI negative. However, results of fluorimetry using the caspase-3 inhibitor showed that it was able to block even this non-significant percentage of necrotic spermatozoa therefore showing some apoptotic activity.

In the neutrophil control group, the result of fluorimetry showed that caspase activity in this group was much higher than in spermatozoa. The reason(s) might be that the apoptosis phenomenon and caspases in blood cells including neutrophils are more common than in spermatozoa. Additionally, the cytoplasmic volume of spermatozoa is small compared with neutrophils.

In conclusion, we observed that the results from flow cytometry were closely correlated to the findings of fluorimetry. Also, all LPS preparation used in the present work showed more or less a similar behaviour regarding ROS production and induction of apoptosis. However, the results of Kdo experiments indicated that this unsual sugar failed to produce a signal through the TLR pathway in transfected HeLa cells or to stimulate TNF-α secretion in the THP-1 cell line. However, it had a similar effect comparable to LPS or lipid A regarding ROS induction and induction of apoptosis in spermatozoa. Taken together, we could suggest that Kdo might stimulate ROS production or apoptosis induction via a different route to LPS and lipid A. Therefore, more experiments are required to clarify how Kdo is able to exert its toxicity against spermatozoa.

Understanding the mechanism of sperm death via ROS production and caspase-dependent apoptosis stimulation might be useful in treatment of infectious

male-factor infertility by blocking the pathways that start with the attachment of LPS to the LBP and terminates in apoptosis. Detection of early apoptosis in spermatozoa with apparently normal standard sperm parameters can also be helpful to test sperm before in-vitro fertilization (IVF) or artificial insemination.

Chapter 6

General discussion

6.1 Interaction between LPS and human spermatozoa

Chlamydia trachomatis infection is one of the most common sexually transmitted diseases especially in western countries (Bollmann *et al.*, 2001). While there is agreement on the manifestations and, in particular, the negative effects of *Chlamydia* infection on female fertility, the role of this organism in male fertility is still controversial (Vigil *et al.*, 2002a). *C.trachomatis* causes 20-50% of nongonococcal salpingitis, inflammatory pelvic diseases, tubal occlusion and extra-uterine pregnancy in women. This organism affects female fertility through salpingitis (Eggert-Kruse *et al.*, 1996) and probably through an alteration of the immune response. It has been suggested that antigen-antibody reactions to chlamydial heat-shock proteins might be an important factor for the morphological tissue damage and scarring leading to impaired fertility (Westrom, 1996).

In men *C.trachomatis* is also the main cause of nongonococcal urethritis and epididymitis (Vigil , *et al.*, 2002a). Depending on the diagnostic method employed and the population studied, there is a remarkably variable incidence of *C.trachomatis* infection in male partners of infertile couples from < 10% (Ruijs *et al.*, 1990) to > 50% (Hellstrom *et al.*, 1987). *C.trachomatis*, like other bacteria, can adhere to spermatozoa (Erbengi, 1993). The study by Vigil *et al.*, (2002) also confirmed the presence of elementary bodies and reticulate forms of *C.trachomatis* associated with spermatozoa but without alterations in sperm quality or function. These findings might provide a mechanism whereby *C.trachomatis*-infected spermatozoa could become an active transmission agent, i.e. act as 'vehicles' for the pathogens, and spread the disease to the female genital tract causing inflammatory processes and promoting the generation of antisperm antibodies.

In spite of the report by Vigil *et al.*, (2002) and the other studies (Bjercke and Purvis, 1992; Eggert-Kruse *et al.*, 1997; Rezacova *et al.*, 1999), there have been several reports especially in recent years, that confirm chlamydial infection is able to affect the physiology and the normal parameters of spermatozoa including motility, viability, and acrosome reaction (Galdiero *et al.*, 1994; Radouani *et al.*, 1996; Hosseinzadeh *et al.*, 2000; Hosseinzadeh *et al.*, 2001; Hosseinzadeh *et al.*, 2003; Veznik *et al.*, 2004). For example, in the latter study it was shown that normal sperm

morphology, semen volume, sperm concentration, motility and velocity in *Chlamydiae*-contaminated ejaculates were lower than *Chlamydiae*-negative samples. Previous work from our group (Hosseinzadeh, *et al.*, 2000-2003) have also shown that some serovars of *C.trachomatis* can effect tyrosine phosphorylation of human sperm proteins which may be important to sperm function including capacitation (Sakkas *et al.*, 2003). They also demonstrated that serovar E EBs and chlamydial LPS have a spermicidal effect.

Members of the family *Chlamydiaceae* are Gram-negative bacteria containing, in their EBs, an LPS which consists of two components; lipid A, and Kdo. This LPS structure is similar to the rough forms of enterobacterial LPS. It is well known that LPS of Gram-negative bacteria in general is one of the most potent stimulators of innate immunity and that the lipid A moiety of LPS is responsible for this activity (Rietschel *et al.*, 1994), however, Kdo is also always required for bacterial growth and viability, and along with lipid A is the minimal structure compatible with bacterial viability (Erridge *et al.*, 2002).

C.trachomatis possess a lipopolysaccharide of low endotoxic activity , which is attributed to the higher hydrophobicity of its lipid A moiety with fatty acids of longer chain length and the presence of nonhydroxylated fatty acids ester-linked to the sugar backbone (Nurminen *et al.*, 1985; Brade *et al.*, 1986). The fact that chlamydial LPS is endotoxically less active than typical LPS, *e.g.*, enterobacterial LPS, does not exclude its participation in the pathogenesis of chronic inflammation in chlamydial infections. The low potency of *C.trachomatis* LPS might also provide a biological explanation for the asymptomatic infections caused by this organism. Although according to these reports based on clinical findings, the potency of chlamydial LPS is less than enterobacterial LPS, by several *in vitro* experiments we demonstrated that the toxicity of *C.trachomatis* LPS against human spermatozoa is about 500 times more than enterobacterial LPS including *E.coli*, *K.pneumoniae*, and *S. marcescens.*

Based on these considerations and the previous results of our group, the objectives of this thesis were:

Firstly, to compare the potency of *C.trachomatis* LPS and enterobacterial LPS derived from bacteria, which are involved in urinary tract infections in humans,

acting against human spermatozoa and also to confirm that *C.trachomatis* LPS similar to other LPSs is heat-stable. Secondly, it is well established that lipid A is the active component of LPS and it is also presumed that this fraction of LPS is primarily responsible for activity against human spermatozoa. We aimed to investigate this characteristic of lipid A and thirdly, to examine the effect of the other main component of LPS, i.e. Kdo on human spermatozoa.

Fourthly, it is suggested that the TLRs especially TLR2 and TLR4 along with the co-receptor CD14, are essential for recognition of a variety of pathogen-assisted molecular patterns (PAMPs) including LPS. Stimulation of TLR pathway by PAMPs can initiate a signalling cascade that involves a number of proteins including the secretion of pro-inflammatory cytokines such as TNF-α. Previous experiments by our group showed that either elementary bodies or LPS from *C.trachomatis* have deleterious effects on spermatozoa parameters including viability. To expand our knowledge, regarding how and by which route(s), this organism could exert its spermicidal activity; we investigated the behaviours of the aforementioned LPS fractions in terms of stimulation of TLR pathways and induction of TNF-α secretion in experimental models on HeLa and THP-1 cell lines.

Finally, it is also known that stimulation of TNF-α induces an increase in ROS production via mitochondria, and that ROS in turn may be detrimental and even lethal to the host via the activation of apoptotic pathways. From our hypothesis of the effect of *C.trachomatis* LPS leading to increased sperm death it was important to understand whether it was via apoptosis or necrosis or both.

C.trachomatis serovar E is the most frequently occurring serotype in genital *C. trachomatis* infections. *C.trachomatis* serovar LGV which is endemic in parts of Africa, Asia, and south America, also leads to an infection with a more significant infection of the lymphatic system and may cause a disseminated infection (Moulder, 1991; Morre *et al.*, 2000). For these reasons, we decided to focus on these two strains in our study regarding the effects of either EBs or LPSs on human spermatozoa. Although we succeeded to produce a reasonable amount of EBs and LPS from these two serovars which could be enough for our designed experiments, however, we also

aimed to isolate two main LPS fractions namely Kdo and lipid A from *C.trachomatis* LPS.

Unfortunately, since *C.trachomatis* EBs are hazardous and difficult to grow in large quantities which is time-consuming, this hampered the preparation of large amounts of chlamydial LPS required for isolation of its fractions. Alternatively, we used commercial lipid A and Kdo to further understand their effect against spermatozoa.

Endotoxin (LPS) is a heat-stable complex which can maintain its activities against host cells at boiling point for 30 min. Since to the best of our knowledge no conclusive previous work has been reported on the effect of heated-*C.trachomatis* EBs on human spermatozoa, we wanted to confirm the stability of LPS components of EBs against heating. Our results showed that the toxicity of EBs against spermatozoa was unaffected by heating at either 56 °C or 100 °C since the mortality rate of spermatozoa did not alter markedly in the presence of heated EBs, compared to spermatozoa which were treated with non-heated EBs. To neutralise EB toxicity and to exclude the effect of cell wall proteins which could be toxic (Galdiero *et al.*, 1988) and interfere with our results, in control groups, heated-EBs were pre-incubated with polymyxin B (PMB) which interacts strongly with phospholipids and binds to the lipid A portion of endotoxin and inactivates these molecules. The results from the control group spermatozoa also showed that PMB could inhibit the death of spermatozoa incubated with heated-EBs and that we speculated that no activity would be present from the other components of EBs i.e. proteins. Based on these results, we suggested that the LPS component of EBs was primarily responsible for the toxicity of heated EBs against spermatozoa. Although, in their 1971 study, Teague *et al.*, demonstrated that *E.coli* killed by boiling for 10 min, had no effect on human spermatozoa, the dose of killed bacteria incubated with spermatozoa was not stated in their study.

Another facet of *C.trachomatis* LPS investigated in this study was the potency of this endotoxin compared to enterobacterial LPS against human spermatozoa. Although there are a number of reports claiming that *C.trachomatis* LPS is 10 (Heine *et al.*, 2003) to 100 (Qureshi *et al.*, 1997) times less active than typical LPS such as enterobacterial LPS, this activity is not related to spermatozoa.

Our findings, previous work of our group, and also Galdiero and co-workers's results (1994) have shown that LPS from *C.trachomatis* is able to interfere with normal parameters of spermatozoa including viability and motility at a concentration of 500 times less than *E.coli* LPS that was used by Galdiero *et al.*, (1988) (0.1 µg/ml vs 50 µg/ml respectively).

In this study we compared not only the spermicidal activities of *E.coli* LPS against *C.trachomatis* LPS but two other enterobacterial LPSs, *K. pneumoniae* and *S.marcescens*. Our results indicated that while LPS from *C.trachomatis* killed spermatozoa at 0.1 µg/ml, three other LPSs from the *Enterobacteriaceae* family had a deleterious effect on spermatozoa viability when the concentration was increased to 50 µg/ml.

Dumoulin *et al.*, were to the best of our knowledge the first researchers who investigated the effect of *S. marcescens* LPS on spermatozoa. In their 1991 study, they reported that this LPS even at 500 µg/ml had no spermicidal activity which contradicts our results. However, there are two significant differences between their study and our investigation that might explain the controversial results of these two studies. Firstly, in their study they obtained a spermatozoa pellet by centrifugation of semen using Whittingham's T6 culture medium supplemented with low-endotoxin bovine serum albumin that was totally different from the percoll gradient method that we used for sperm praparation. Secondly, to estimate sperm survival, they counted the number of motile spermatozoa using a Makler counting chamber, however, we assessed sperm viability using the HOS test.

This range in potency of LPSs from two different families, *Chlamydiaceae* and *Enterobacteriaceae*, used in this research might be related to a number of factors including the chemical conformation of these LPSs, especially their lipid As which are responsible for toxicity. Detailed studies on the structure-function relationships of lipid A have indicated that the number, type and distribution of fatty acids in lipid A (Heine , *et al.*, 2003) and also the shape of the lipid A component in terms of being conical or cylindrical (Netea *et al.*, 2002) determine whether LPS exhibits weak or strong bioactivity against host cells.

The structure of LPS from serovar E is very similar to that of serovar L2 with two GlcN, three Kdo residues, and two phosphates. The pentasaccharide fatty acid

composition for serovar E is also very similar to that reported for serovar L2 (Heine, *et al.*, 2003). Although we were not able to find any studies describing the chemical structure of serovar L1 used in this study, it seems that the LPS of different serovars of *C. trachomatis* show only minor variations, especially if these serovars belong to a similar biovar such as serovar L1 and L2, which have been classified in the LGV biovar along with two other serovars, $L2_a$ and L3.

A comparison of structures of lipid A from different *Enterobacteriaceae* and *C.trachomatis* reveals that this component in the latter organism contains five fatty acids with up to 22 carbon atoms however, in *Enterobacteriaceae*, lipid A possesses six fatty acids with up to 16 carbon atoms. Organisms within the two families also have two different inner core structures. While enterobacterial LPS contains two residues of Kdo and three residues of heptose, chlamydial LPS has only a Kdo tri-saccharide.

Apart from the differences in the chemical structure of these LPSs, based on our investigations and other relevant studies we would also suggest that the type of host cells, the conditions and environment where the organism challenges the host cells i.e. *in vitro* or *in vivo*, might be important for determining whether pathogens show a weak or a strong activity against their host.

Although it is well established that lipid A is the principal virulence fraction of LPS and consequently is responsible for the pathogenicity of bacterial LPS, nevertheless, little is known about the toxicity of Kdo which is bound to lipid A and is also indispensable for bacterial bioactivity. Our attempts to separate Kdo from *C.trachomatis* LPS failed for the aforementioned reasons, however, we were able to isolate a small amount of lipid A from chlamydial LPS which unfortunately was insufficient for all experiments. Accordingly, Kdo and further amounts of lipid A were obtained commercially with the aim of studying their direct effects on spermatozoa, and further understanding their roles in stimulation of TLR signalling pathways and TNF-α production using HeLa and THP-1 cell lines respectively.

Based on the immunobiological and endotoxic properties of lipid A reported in the recent literature (Schromm *et al.*, 2000; Tanamoto *et al.*, 2001; Hashimoto *et al.*, 2003; Ogawa *et al.*, 2003), we hypothesised that lipid A could also exert its toxicity against spermatozoa. This hypothesis was established when the mortality

rate of spermatozoa markedly increased in the presence of lipid A. Further supporting evidence was that anti-CD14 antibody and PMB inhibited the spermicidal activity of lipid A.

In the TLR signalling pathway, LPS binds to LPS binding protein (LBP) and CD14 acts as a co-receptor which presents the complex of LPS-LBP to TLRs. This pathway could finally terminate in cell death. Blocking this potentially lethal pathway by different routes including anti-CD14 antibody could prevent cell death. PMB treatment of spermatozoa incubated with LPS or its fractions is also another alternative method that was utilised in our studies to investigate the pathogenicity of these components.

In the case of Kdo and its interaction with spermatozoa, Kdo exhibited two different behaviours in our earlier experiments. Similar to lipid A, Kdo was able to significantly induce death of spermatozoa. However, unlike lipid A, pre-incubation of spermatozoa with anti-CD14 antibody or PMB did not alter the spermicidal activities of Kdo. Although the role of the polysaccharide moiety of LPS which includes Kdo in induction of colony stimulating factors, interferon or IL-1 secretion has been described (Haeffner-Cavaillon *et al.*, 1989), to the best of our knowledge the spermicidal property of Kdo, has not been reported.

These interesting findings led us to clarify the mechanism of sperm death induced by Kdo and also by LPS or lipid A using experimental models. In these experiments, two cell lines, HeLa and THP-1, were exploited to enable us to investigate the role of these LPS components in stimulation of the TLR pathway and in TNF-α production which potentially culminates in cell death.

6.2 LPS and TLR signalling

Recognition of microbial infection and initiation of host defense responses is controlled by multiple mechanisms including Toll-like receptors. TLRs recognize molecular products derived from all the major classes of microbes including bacteria, viruses, yeasts and fungi. Mammalian cells express at least 10 TLRs, and among them, TLR4 is the major LPS receptor; TLR2 recognizes, peptidoglycan, lipoteichoic acid, and also LPS. These receptors have recently emerged as a key component of

the innate immune system that detects microbial infection and triggers antimicrobial host defense responses. TLRs activate multiple steps in the inflammatory reaction that help to eliminate the invading pathogens and coordinate systemic defenses. Induction of these receptors results in the expression of pro-inflammatory cytokines, such as TNF-α. However, overproduction of cytokines is toxic and could threaten host cell life.

Our data showed that LPS from both serovars of *C.trachomatis*, E and LGV, signalled through TLR2 and TLR4, however, lipid A from *E.coli* was able only to produce a signal through TLR4 in transfected HeLa cells and interestingly, Kdo failed to signal via either TLR2 or TLR4. In a similar study, Erridge *et al.*, (2004) reported that LPS from *C.trachomatis* LGV1, signals only via TLR2. However, our results showed that this serovar could also signal via TLR4. It would appear that this apparent difference in TLR signalling may be due to different batches of extracted LPS.

Based on the importance of TLRs in recognition of pathogens and also our results which demonstrated that chlamydial LPS, lipid A, and Kdo are toxic for spermatozoa, we tried to find the mechanisms of pathogen-induced cell signalling in spermatozoa that could be via TLRs. Previous work of our group using anti-TLR antibodies with the aim of blocking these receptors in human spermatozoa, did not produce significant results. In another attempt, we treated wild-type mouse spermatozoa with these LPS fractions under the same conditions of human spermatozoa. This study would be continued with TLR knock-out mouse spermatozoa if the results of the former experiment were conclusive. However, the mortality rate of spermatozoa from the wild-type mouse incubated with chlamydial LPS, lipid A or Kdo did not alter appreciably from the control. These results are consistent with the earlier research performed by Weil *et al.*, (1998). They demonstrated that mouse spermatozoa do not respond to staurosporine or cycloheximide. As these reagents are apoptotic, they suggested that caspases involved in apoptosis are absent in mouse spermatozoa.

Although our attempts at finding a role of TLRs in recognition of LPS fractions by spermatozoa were inconclusive, the question of the mechanism of cell

signalling in spermatozoa remains to be solved and further work should determine whether TLRs are involved in interaction of spermatozoa with LPS fractions.

Further investigations into the IFN-γ-induced THP-1 cell line treated with chlamydial LPS, lipid A or Kdo revealed that all these spermicidal components, except Kdo, stimulated TNF-α secretion from this cell line. Spermicidal activity was the only property of Kdo which was observed in these experiments.

6.3 ROS production, apoptosis and human spermatozoa

The next stage of our studies focused on the mechanism of sperm death induced by chlamydial LPS, commercial lipid A and Kdo. Following the suggested TLR pathway and its products, namely cytokines including TNF-α, it was suggested that using TNF-receptor 1 (TNF-R1), TNF-α stimulates ROS production via mitochondria (Schulze-Osthoff *et al.*, 1993). Male genital tract infection may lead to several consequences, including the release of pro-inflammatory cytokines and the production of ROS (Jeremias and Witkin, 1996). ROS in turn plays an important role in the pathogenesis of many reproductive processes which might lead to male infertility. Since ROS can interfere with sperm functions such as motility, the acrosome reaction and threaten the survival of spermatozoa (de Lamirande and Gagnon, 1995; Aitken, 1999), exposure of spermatozoa to ROS has been associated with cellular injury, that includes DNA damage and lipid peroxidation (Potts *et al.*, 2000).

In the study by Segnini *et al.*, (2003), sperm membranes of infertile males with positive IgA antibodies against *C. trachomatis* showed a higher level of lipid peroxidation than that of infertile males with negative IgA antibody, and it is possible that this could be a route by which *C.trachomatis* affects fertility.

In order to counteract the toxic effects of ROS, seminal plasma contains a number of antioxidant mechanisms. Semen contains high levels of ascorbate, urate and thiol groups and also lower amounts of glutathione and α-tocopherol (Ochsendorf *et al.*, 1998). Moreover, the antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase have been detected in seminal plasma (Yeung *et al.*, 1998).

In indirect experiments using five different ROS scavangers; ascorbic acid, catalase, reduced glutathione, superoxide dismutase, and α-tocopherol, since the viability of spermatozoa incubated with LPS and lipid A was preserved, and these two products were also active in TLR signalling and TNF-α production in our experimental models, we concluded that these products possibly exert their toxicity via ROS production induced by TLR pathway stimulation. Although, ROS scavengers counteracted the spermicidal activity of Kdo, as we described earlier this fraction was not active in TLR signalling or TNF-α secretion; this might suggest a different route for its activity.

Granulocytes and macrophages are known to produce ROS, which could increase the level of lipid peroxidation or DNA damage. To avoid significant interference by these cells, in ROS scavenger experiments, we utilized spermatozoa preparations using a percoll gradient method and the results of immunocytochemistry and the cytocentrifuge preparation technique confirmed that spermatozoa preparations were free of cellular contamination at the minimum detection level ($\leqslant$143 leukocytes/ 1 x 10^7 spermatozoa for the immunocytochemistry technique or $\leqslant$1000 leukocytes for the cytocentrifuge preparation technique). However, it is acknowledged that contaminating leukocytes could be producing ROS and therefore complicating our findings.

Our findings from ROS scavenger experiments, encouraged us to expand our knowledge concerning the type of LPS-induced spermatozoa death and to differentiate whether this was due to, necrosis or apoptosis. That is why we followed our investigations employing flow cytometry for measurement of apoptosis and fluorimetry for quantification of caspase activity.

Apoptosis is a process of cell death manifested by fragmented nuclei with condensed chromatin and shrunken cytoplasm. One of the crucial intracellular signalling events in apoptosis is the sequential activation of caspases, a family of cysteine proteases (Villa *et al.*, 1997). Among the fourteen caspases described to date, caspase-2, -8, -9, and -10 facilitate the caspase activation cascade and are called initiators. Activation of these initiator caspases can lead either to cytochrome *c* release from mitochondria, which in turn activates downstream effector caspases, or to direct activation of downstream caspases (Wolf and Green, 1999). The

downstream effector caspases, such as caspase-3, -6, and -7, execute apoptotic responses by cleaving various cellular protein substrates vital for cell functions (so-called death substrates). Caspase-3 is considered to be the main effector caspase of apoptotic cell death.

Caspases 1, 3, 8, and 9 are also present in human spermatozoa and can become activated, particularly after freezing and thawing, and are associated with changes in the outer cell membrane. Caspases 1, 3, and 8 and 9 are localised in the postacrosomal region and in the the midpiece respectively (Paasch *et al.*, 2004).

The fluorimetry results of our investigations showed a considerable increase in activity of caspase-3 in spermatozoa treated with either *C.trachomatis* LPS, lipid A, or Kdo. Measurement of apoptosis using flow cytometry confirmed that these fractions were able to induce spermatozoa death via apoptosis. These results were also consistent with the findings of HOS tests used for testing the viability of spermatozoa.

Interestingly, apart from the results of TLR signalling and TNF-α production, in all other experiments performed in this study including the HOS test, use of ROS scavengers, FITC-annexin V/PI staining, and quantification of caspase-3 activity, chlamydial LPS, lipid A and Kdo all behaved in a similar manner. In Figure 6. 1, we illustrate a hypothetical model of how chlamydial LPS exerts its spermicidal activity.

Although we tried to isolate lipid A and Kdo from *C.trachomatis* LPS and investigate their effect on spermatozoa, as described earlier, our attempts were inconclusive as we produced only a small amount of lipid A, and instead the commercial alternative of these reagents were used. To better understand the pathophysiology of chlamydial lipid A and Kdo, further work should therefore focus on these two LPS fractions derived from *C.trachomatis* LPS.

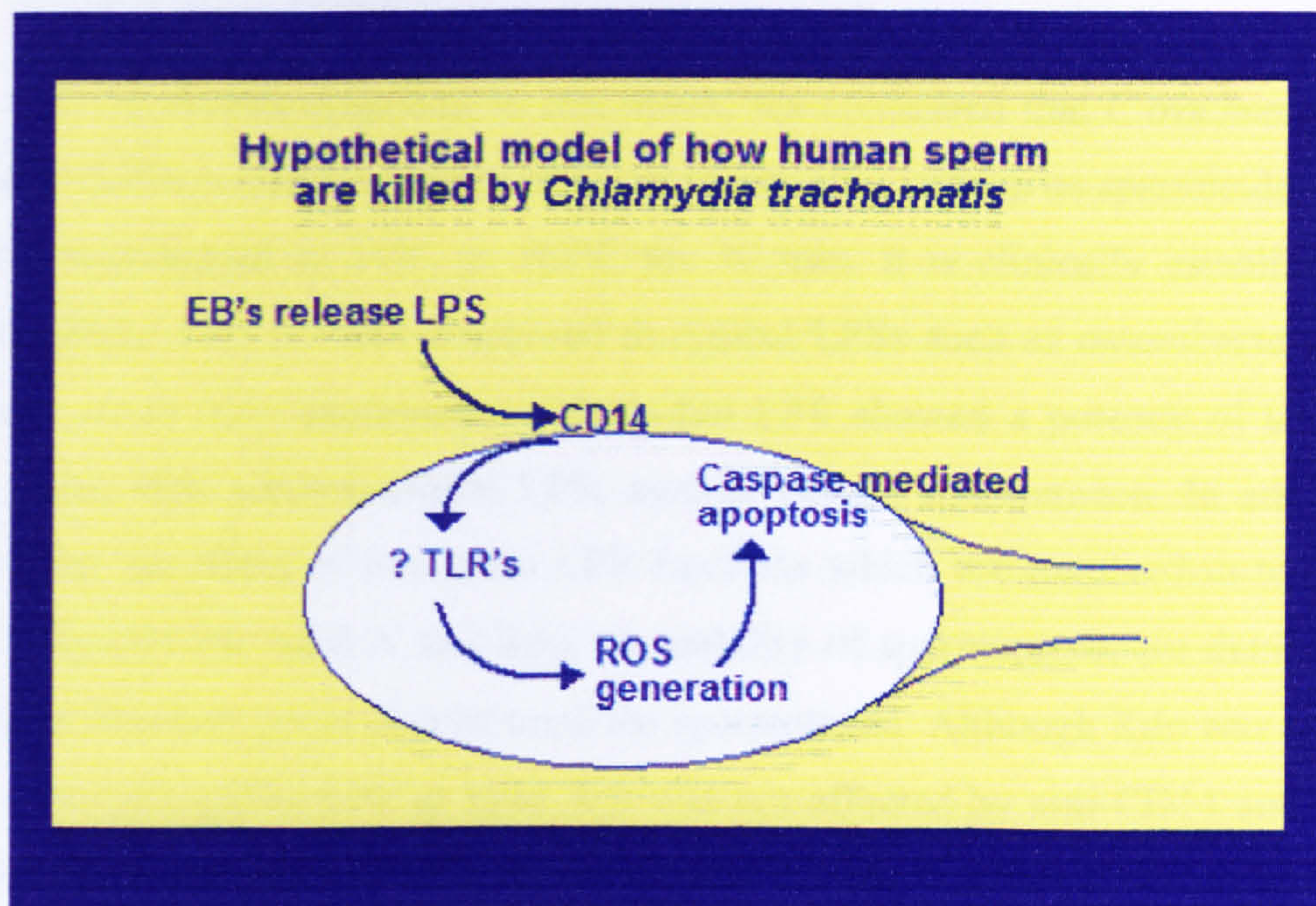


Figure 6. 1. Hypothetical model of how *C.trachomatis* LPS interacts with human spermatozoa.

6.4 Final conclusion

In the studies described in this thesis, we confirmed that *C.trachomatis* LPS like other LPSs is a heat-stable complex as it was able to keep its spermicidal activity when it was heated to 56°C or 100°C for 30 min. It is clinically established that *C.trachomatis* LPS is weak compared to typical LPSs such as enterobacterial LPS, however, in *in vitro* experiments chlamydial LPS showed a potency of about 500 times more than enterobacterial LPS, against human spermatozoa. In attempts to understand the effect of two main LPS fractions which are involved in toxicity or bioactivity of LPS, lipid A and Kdo, on viability of spermatozoa, we demonstrated that these fractions could also be toxic for spermatozoa. Although Kdo was shown to be spermicidal, unlike LPS or lipid A it was not affected by anti-CD14 antibody or polymyxin B. Chlamydial LPS and commercial lipid A from *E.coli* signalled via TLR2 and/or TLR4 in the transfected-HeLa cells whilst synthetic Kdo did not signal with either of these two TLRs. TNF-α production was also activated by chlamydial LPS or lipid A but not Kdo in IFN-γ-induced THP-1 cells. In spite of these differences that were observed for Kdo, similar to chlamydial LPS or lipid A, the spermicidal activity of Kdo was inhibited by ROS scavengers. Also, all three fractions used in our studies induced sperm apoptosis which was primarily caspase-mediated.

In conclusion, although Kdo induced apoptosis in spermatozoa since it did not interact via CD14, signal via TLRs, or activate TNF-α production it would appear that Kdo exerts its activites via a different route to chlamydial LPS or lipid A.

6.5 Future work

In the preceding chapters we tried to increase our understanding of pathogenicity of *C.trachomatis* LPS interacting with human spermatozoa. Despite the ever-increasing knowledge concerning different aspects of this organism which could threaten public health or even human life, there is still a need for better understanding of this pathogen. As diseases associated with chlamydial infection including infertility, genitourinary tract infections in male and females and ophthalmic disease continue to increase, the burden of such diseases is enormous. The complications of chlamydial infections are distressing and expensive, including infertility, ectopic pregnancy, and chronic pelvic pain.

Many investigators around the world are working on different facets of this organism such as genomic sequencing, development of chlamydial serology or diagnostic tests for genital infection. In recent years, including work in our laboratory, chlamydia research has succeeded in producing more information regarding the effect of either elementary bodies or LPS from *C.trachomatis* on standard parameters of human spermatozoa. We continued this work focusing mostly on the LPS fractions and their effects on spermatozoa, the role of LPS and its fractions in signalling via TLRs and apoptosis induction in spermatozoa.

Based on the present results, we believe this project deserves more attention and must be continued in the future with the following subjects:

1) Isolation of lipid A and Kdo from *C.trachomatis* LPS.

2) Challenge of spermatozoa with *C.trachomatis* lipid A and Kdo regarding the mechanism of spermatozoa death and whether it is via apoptosis.

3) Role of *C.trachomatis* LPS and its fractions in ROS production.

4) The role of TLRs in cell signalling of spermatozoa when exposed to LPS fractions especially Kdo.

Appendices

Appendix 1

Reference values of semen variables

Semen analysis is an integral part of the workup of couples consulting for infertility. The availability of semen renders possible direct examination of male germ cells, giving precious data that are not accessible for female germ cells. It should be mentioned that semen is an exception amongst biological fluids since its parameters display very wide intra and inter-individual variations. Therefore semen analysis should be repeated to decrease these variations and confirm abnormal parameters. The following reference values give the description of a semen sample analysed according to the methods described by WHO, 2000.

Volume: 2.0 ml or more

pH: 7.2 or more

Sperm concentration: 20×10^6 spermatozoa/ml or more

Total sperm count: 40×10^6 spermatozoa or more

Motility: 50% or more motile (grade a* +b**) or 25% or more with progressive motility (grade a) within 60 min after collection.

*, Rapid progressive motility. **, Slow or sluggish progressive motility.

Morphology: Has not been established yet by the WHO. Depends on the laboratory criteria, varies from 30% or more to 50% or more. Previous data suggest that the fertilization rate in vitro decreases if there are less than 15 percent normal-shaped sperm, as defined by the WHO manual.

Vitality:75% or more live

White blood cells: Less than 1×10^6 /ml

Appendix 2

The Makler counting chamber for rapid semen evaluation

Chamber description: The Makler counting chamber is only 10 microns deep: 1/10th of the depth of ordinary hemocytometers, making it the shallowest of known chambers. Constructed from two pieces of optically flat glass, the upper layer serves as a cover glass, with a 1 sq.mm fine grid in the centre subdivided into 100 squares of 0.1 x 0.1 mm each. Spacing is firmly secured by four quartz pins (see figure below).

Analysis technique: A small, uncalibrated drop from a well-mixed undiluted specimen is placed in the centre of the chamber by means of a simple rod and immediately covered. A microscopic objective of x 20 is required.

Motility evaluation: Non-motile sperm are counted within an area of nine or sixteen squares in the centre of the grid. Moving sperms are then counted, and graded if desired. The procedure is repeated in several areas. Percentage of motility and its quality are then calculated.

Figure: Makler counting chamber

Appendix 3

Chemiluminescence data

Measurement of ROS activity was carried out in spermatozoa incubated with 0.1 μg/ml of *C.trachomatis* LPS from serovar LGV with and without 1 mM of ascorbic acid using a luminometer as previously described by Urata *et al.*, (2001). Briefly, 0.075 μg/ml of phorbol 12-myristate acetate (PMA) (Sigma, UK) was added to the mixture of 5 x 10^6 spermatozoa/ml and 30 μg/ml of luminol (5-amino-2, 3dihydro-1, 4 phthalazinedione) (Sigma, Uk) as a probe, and the luminescence was recorded for 30 min. Earl's balanced salt solution (EBSS) alone was used as a background measurement. Data are shown in the following table.

	5 min	10 min	15 min	20 min	25 min	30 min
EBSS	36928	36638	36498	36420	36324	36304
Sperm	36744	36526	36206	35123	35993	36024
Sperm + L	37435	37158	37025	37036	36908	36797
Sperm + L + P	37908	37690	37475	37398	37311	37305
Sperm + Aa	36761	36533	36300	36164	37183	36174
Sperm + Aa + L	37533	37231	37001	37033	36925	36968
Sperm + Aa + L + P	37453	37321	37284	37087	37034	37069
Sperm + LPS	36533	36221	35945	35832	35847	35856
Sperm + LPS + L	37391	37054	36887	38901	36765	36645
Sperm + LPS + L + P	37880	37439	37195	37196	36931	37065
Sperm + LPS + Aa	36170	35997	35727	35367	35337	35270
Sperm + LPS + Aa + L	37461	37098	36931	36893	36870	36802
Sperm + LPS + Aa + L + P	37749	37445	37187	37084	37113	36911+

Time course of ROS production in human spermatozoa in response to LPS. Aa, ascorbic acid. L, luminol. P, phorbol.

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