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GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Male subfertility is multifactorial and entails more than the conventional sperm quality parameters

Promotor :
Prof. dr. Lena DE RYCK

Promotor :
dr. E. BOSMANS

Dorien Haesen

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

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List of abbreviations

AO: acridin orange

APC: allophycocyanin

ART: assisted reproductive techniques

ATP: adenosine triphosphate

BSA: bovine serum albumin

Caspases: cytosolic cysteine-containing aspartate-specific proteases

CMA3: chromomycin A3

CoA: coenzyme A

DGC: density gradient centrifugation

DMEM Ham F12: Dulbecco's Modified Eagle Medium Ham F12

DMF: dimethylformamide

DNA: deoxyribonucleic acid

FACS: fluorescence-activated cell sorting

FAD: flavin adenine dinucleotide

FITC: fluorescein isothiocyanate

HOS: hypo-osmotic swelling

ICSI: intracytoplasmic sperm injection

IMC: inseminating motile count

IUI: intrauterine insemination

IVF: in vitro fertilization

MACS: magnetic-activated cell sorting

NAD: nicotinamide adenine dinucleotide

PBS: phosphate buffered saline

PGCs: primordial germ cells

PI: propidium iodide

PS: phosphatidylserine

PSA: pisum sativum agglutinin

Q10: coenzyme Q

RCF: relative centrifugal force

TCA: tricarboxylic acid

WHO: World Health Organization

ZP3: zona pellucida protein 3

Preface

The senior practical training was the highlight of my five year education in Biomedical Sciences at the University of Hasselt. This senior internship project caught my eye the moment I read the abstract. I have always been fascinated by the field of reproductive medicine and the last 30 weeks have only strengthened my fascination.

Many people have helped and guided me throughout this project. Above all, I wish to extend my gratitude to Dr. Eugene Bosmans, my promoter, for providing me the opportunity to work in the field of male subfertility at Epsilon Biotech. Thank you of your teaching, guidance, support and inexhaustible source of ideas. I wish to extend my gratitude to Mark Marien and Melek Menese for preparing the sperm samples at Algemeen Medisch Laboratorium. In addition, a special thanks goes out to my internal promoter, Prof. Dr. Leen De Ryck, for her support and her good advice with regard to the writing of my thesis report. I thank Prof. Dr. Ivo Lambrichts for his advice. Furthermore, I wish to extend my gratitude to Prof. Dr. Marcel Ameloot and PhD student Kristof Notelaers for giving me the opportunity to work with the widefield microscope.

I would like to express some words of gratitude to my student colleagues, Stefanie Lemmens, Laura Visconti and Alicia Moreno Nicolás, and to Astrid Peters for their moral support and friendship.

I am extremely grateful to my parents and brother for giving me the opportunity to study, keeping me motivated and believing in me.

Abstract

Male subfertility accounts for 10 percent of subfertility in couples looking for reproductive help. The success rates of the subfertility treatments, intrauterine insemination (IUI) and in vitro fertilization (IVF), remain to be increased through the improvement in sperm quality. In order to define sperm quality, the conventional sperm parameters are routinely used, namely count, motility and morphology. However, hidden defects at the chromosomal, nuclear and membrane level may inefficiently or not be detected during a conventional sperm quality analysis.

We expect that male subfertility is multifactorial and that a conventional sperm quality analysis does not provide sufficient information to explore the fertility problem. During this project, the storage temperature of the sperm samples was optimized to increase cell survival. The potential of the hypo-osmotic swelling (HOS) test as a selection step for IUI and IVF protocols was evaluated. Different sperm parameters were studied to enhance our understanding of male subfertility and the current suboptimal successes of IUI and IVF, namely the protamination status, the deoxyribonucleic acid (DNA) fragmentation level, the acrosome reaction and the viability status. Moreover, a method based on magnetic-activated cell sorting (MACS) was performed to remove sperm cells expressing phosphatidylserine (PS). This method was evaluated in terms of the resulting sperm quality.

Improved survival of sperm cells is obtained when samples are stored at room temperature compared to 37°C. At a lower temperature, less motile cells are observed, indicating that spermatozoa adopt a resting state which presumably prolongs energy availability and survival. When placed at 37°C, sperm motility is restored. The HOS test proves to be unsuitable as a selection step for IUI and IVF protocols since it provides an excessively aggressive stress situation for the spermatozoa. Density gradient centrifugation (DGC) selects morphologically normal spermatozoa with less DNA fragmentation and a good protamination status. In addition, this sperm preparation protocol decreases the percentage of viable spermatozoa expressing PS. However, an increased percentage of acrosome-reacted spermatozoa, incapable of fertilizing an oocyte in IUI and IVF protocols, can be observed after DGC. This unwanted selection might be partially responsible for the suboptimal success rates of these subfertility treatments. Moreover, the high basal level of acrosome-reacted cells in the raw sample could be an important new characteristic of male subfertility. In addition, flow cytometric evaluation of CD46 positivity proves to be the superior acrosomal staining method. Furthermore, the acrosome reaction and PS surface expression are partially associated with one another. In final, major elimination of PS-expressing sperm cells through the promising separation technique, MACS, could not be confirmed during this thesis project.

This study illustrates that the acrosomal status of spermatozoa significantly contributes to sperm quality and the fertilizing potential of a sperm sample. Lack of information concerning the level of the acrosome reaction within an individual sperm sample might be partially responsible for the current suboptimal success rates of the subfertility treatments, IUI and IVF. Furthermore, the effect of temperature on spermatozoa should be taken into account when storing sperm samples in fertility laboratories. In our view, male subfertility is multifactorial and a conventional sperm quality analysis does not provide sufficient information regarding the fertility problem.

Samenvatting

Mannelijke subfertiliteit draagt voor 10 % bij tot subfertiliteit bij koppels die beroep doen op reproductieve geneeskunde. Er moet continue gestreefd worden naar vergroting van de slaagkansen van de subfertiliteitsbehandelingen, intra-uterine inseminatie (IUI) en in vitro fertilisatie (IVF), door verbetering van de spermakwaliteit. Spermakwaliteit wordt routinematig gedefinieerd aan de hand van de conventionele spermaparameters, namelijk concentratie, beweeglijkheid en morfologie. Echter, verborgen defecten op het niveau van de chromosomen, de kern en het membraan worden onvoldoende of niet gedetecteerd tijdens de conventionele analyse van de spermakwaliteit.

We verwachten dat mannelijke subfertiliteit multifactorieel is en dat een conventionele sperma-analyse onvoldoende informatie voorziet over het fertiliteitsprobleem. Tijdens dit project werd de bewaringstemperatuur van stalen geoptimaliseerd om een verbeterde overleving van spermacellen te verkrijgen. De mogelijkheid van de hypo-osmotische zwelling (HOS) test om te fungeren als selectiestap in IUI en IVF protocollen werd geëvalueerd. Ook werden meerdere spermaparameters bestudeerd om de kennis van mannelijke subfertiliteit te vergroten en de huidige suboptimale successen van IUI en IVF te begrijpen, namelijk de protaminatiestatus, deoxyribonucleïnezuur (DNA) fragmentatie, de acrosoom reactie en de viabiliteitstatus. Bijkomend werd een methode, gebaseerd op magnetic-activated cell sorting (MACS), uitgevoerd om zo spermacellen met fosfatidylserine (PS) oppervlakte-expressie te verwijderen uit een staal. De resulterende spermakwaliteit na selectie werd gedocumenteerd.

Een verbeterde overleving van spermacellen wordt verkregen bij bewaring op kamertemperatuur in vergelijking met bewaring op 37°C. Minder beweeglijke spermacellen worden gedetecteerd, met een verlengde beschikbaarheid van energie en een verlengde overleving tot gevolg. De beweeglijkheid kan gestimuleerd worden door de stalen op 37°C te plaatsen. Omdat de HOS test resulteert in een overmatig agressieve stress situatie voor spermacellen is deze ongeschikt als selectiestap in IUI en IVF. Density gradient centrifugation (DGC) selecteert morfologisch normale cellen met minder DNA fragmentatie en een goede protaminatiestatus. Verder vermindert deze voorbereidende selectiestap het percentage levende cellen met PS expressie. Echter, een verhoogd percentage acrosoom-gereageerde cellen, niet bruikbaar om een eicel te bevruchten tijdens IUI en IVF, wordt verkregen na DGC. Deze ongewenste selectie zou gedeeltelijk verantwoordelijk kunnen zijn voor de suboptimale slaagkansen van deze behandelingen. Bijkomend kan het hoge aantal acrosoom-gereageerde cellen in het onbehandelde staal een nieuwe eigenschap zijn van mannelijke subfertiliteit. Verder bewijst flow cytometrische analyse van CD46 positiviteit de meest betrouwbare acrosomale kleurmethode te zijn. De acrosoom reactie en PS expressie zijn gedeeltelijk geassocieerd. Ten slotte, de eliminatie van spermacellen met PS expressie via de veelbelovende scheidingstechniek, MACS, kon niet bevestigd worden tijdens dit thesisproject.

Deze studie illustreert dat de acrosomale status van spermacellen bijdraagt tot de spermakwaliteit en het bevruchtingspotentieel van een staal. Gebrek aan informatie hieromtrent binnen een individueel staal kan gedeeltelijk verantwoordelijk zijn voor de huidige suboptimale slaagkansen van IUI en IVF. Verder moet tijdens de bewaring van spermastalen in fertiliteitslaboratoria rekening gehouden worden met de bewaringstemperatuur. Onze resultaten tonen aan dat mannelijke subfertiliteit multifactorieel is en dat een conventionele sperma-analyse onvoldoende informatie verschaft over het fertiliteits-probleem.

1 Introduction

As many as 10 to 15 percent of couples in western countries experience subfertility, defined as all couples who are unable to conceive after one year of unprotected intercourse[1,2]. In 10 percent of these cases the problem is attributed to men[3]. Additionally, since the 1980s a decline has been observed in fertility and we must face the possibility of more subfertile couples in the future[4,5]. The decrease in male fertility may be caused by infections (e.g. chlamydia), environmental factors (e.g. occupational exposure to chemicals, heat, radiation and heavy metals, and exposure to environmental estrogens and pesticides) or lifestyle risk factors (e.g. cigarette smoking, alcohol consumption, recreational drugs, chronic stress and nutritional deficiencies)[6]. First line treatment, intrauterine insemination (IUI), and advanced treatments, in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), are assisted reproductive techniques (ART) recommended to treat subfertility[7]. IUI involves placing prepared sperm high within the uterine cavity and close to the site of fertilization via a catheter. This fertility treatment can be applied in association with a natural cycle or with ovulation induction. During the IVF protocol, oocytes obtained after ovarian stimulation are fertilized with prepared sperm to form an embryo outside the womb. When developed to an appropriate stage, the embryo is transferred back into the uterus where implantation takes place. The ICSI procedure involves the injection of a single, high quality sperm cell into an oocyte and transferring the embryo into the uterus when developed to the appropriate stage. Unfortunately, the success rate of IUI is still disappointing and the pregnancy rates of IUI and IVF remain to be improved (10-20 % in IUI[8] and 25-30 % in IVF[9]), despite great efforts in the field. Moreover, due to a relatively large number of early miscarriages, the life birth rate or take home baby rate is additionally reduced by 30 %. Hence, the improvement in sperm quality is an essential step to raise pregnancy and life birth rates and to reduce the number of ART cycles needed to achieve life birth. As a consequence, psychological and physical burdens encountered by patients will be alleviated and healthcare costs will be reduced.

During this project, storage conditions of the sperm samples will be optimized in terms of the temperature and the storage medium to increase the survival of spermatozoa. The potential of the hypo-osmotic swelling (HOS) test as a selection step for IUI and IVF protocols will be evaluated. Furthermore, different sperm parameters will be studied to enhance our understanding of male subfertility and the current suboptimal successes of IUI and IVF, namely the protamination status, the deoxyribonucleic acid (DNA) fragmentation level, the acrosome reaction and the viability status. Moreover, a method based on magnetic-activated cell sorting (MACS) will be performed to remove sperm cells expressing phosphatidylserine (PS). This method will be evaluated in terms of the resulting sperm quality.

1.1 Spermatogenesis and sperm morphology

The purpose of spermatogenesis is to establish and maintain daily output of 20 to 200 million fully differentiated spermatozoa, the haploid male gametes. This process begins in the seminiferous tubules of the testes after onset of puberty[10]. These tubules consist of a basal membrane, an active dividing germ epithelium, the Sertoli cells and a central lumen[11] (**Fig. 1**). The germ epithelium produces primordial germ cells (PGCs). During all steps of the spermatogenic process, the developing gametes are in intimate contact with the Sertoli cells, which provide nutrients, O_2 and growth factors. Moreover, the blood-testis barrier is shaped by cell-cell junctions between Sertoli cells and largely prevents immune

cells from infiltrating the lumen. The developing gametes are also interconnected via intercellular cytoplasmic bridges to allow synchronous development of large clusters of sperm cells[11,12]. Furthermore, testosterone-producing Leydig cells and blood vessels are present between the seminiferous tubules[11].

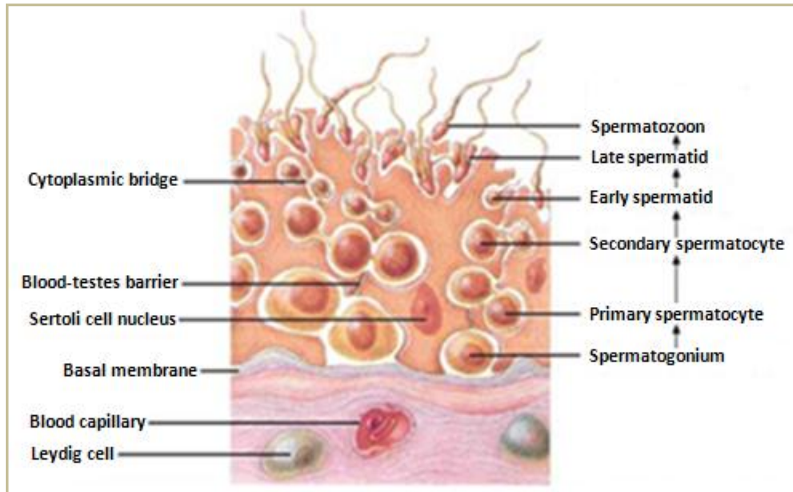

Figure 1: The anatomy of the seminiferous tubules and an overview of the spermatogenic process[13].

Mitotic arrest occurs at birth, whereby the PGCs become the non-proliferative A-type spermatogonia that are mitotically quiescent until the onset of puberty[11]. At that time, a massive mitotic proliferation of A-type spermatogonia is induced (**Fig. 1**). These stem cells can commit either to renewal to maintain sufficient numbers of spermatogonia, or to differentiation into B-type spermatogonia. The progeny of B-type spermatogonia, the primary spermatocytes, will penetrate the blood-testes barrier and will enter the first meiotic division. After completion, two secondary spermatocytes are formed and pass through the second meiotic division to produce four immature spermatids. From this moment on, these early spermatids will not divide but will undergo a series of profound changes that transform them into highly specialized spermatozoa, a process known as spermiogenesis. Many organelles and proteins are recycled to provide space and building blocks for the developing sperm cells. Reduction in the size of the nucleus and condensation of the chromosomal material are the major changes of the nucleus. Furthermore, profound reorganization of the cytoplasm occurs. Condensation of the Golgi apparatus gives rise to the acrosome and, at the far side of the nucleus, a prominent flagellum is formed. Approximately one half of the mitochondria is rejected while the other half is arranged in a spiral around the midpiece of the flagellum, called the helical mitochondrial sheath (**Fig. 2**). Finally, the intercellular cytoplasmic bridges between the late spermatids are disconnected and the remainder of the cytoplasm, namely the residual body, is shed and phagocytized through the Sertoli cells[10]. The highly specialized spermatozoa are detached from the epithelium and individual cells are released into the lumen of the seminiferous tubules[11].

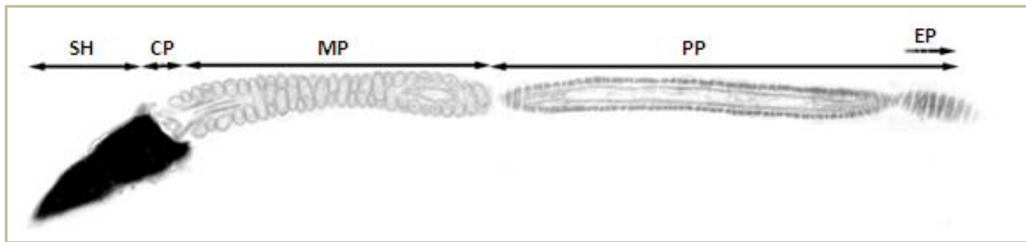

Figure 2: General structure of the spermatozoon, which consists of five major parts, namely the sperm head (SH), the sperm tail connecting piece (CP), the sperm tail midpiece (MP), the sperm tail principal piece (PP) and the sperm tail end piece (EP)[11].

A spermatozoon consists of a head and a tail, both covered by a plasma membrane, named the plasmalemma[11] (**Fig. 2**). The sperm head is composed of a compact nucleus, in which the DNA histones have been partially replaced by protamines during spermatogenesis. Protamines are positively charged DNA proteins that compact DNA very tightly through binding in the major groove. This completely neutralizes the negative charges of the double helix creating an uncharged DNA fiber. Moreover, during epididymal transit, the protamines establish intermolecular and intramolecular disulphide bonds to stabilize the nuclear chromatin structure. Zinc, secreted by the prostate gland, binds to protamines and stabilizes the final DNA-protamine interaction[14]. The compact sperm nucleus permits sperm motility and penetration and protects sperm DNA during the transit from the male to the female genital tract[11,12]. Furthermore, the sperm head contains the acrosome, which harbors glycoproteins, enzymes such as proteases and hyaluronidases, and receptors required for sperm interaction with the oocyte[10]. The sperm tail or flagellum provides the motile force for the spermatozoon. It consists of four parts, namely the connecting piece, the midpiece, the principal piece and the end piece[11] (**Fig. 2**). The midpiece is covered by the helical mitochondrial sheath containing 75 to 100 mitochondria, which generate adenosine triphosphate (ATP) for flagellar motion. However, the released spermatozoa are immotile. During the epididymal passage, spermatozoa become capable of initiating flagellar motility[15]. L-Carnitine is an important component of the epididymal fluid and a gradual increase is present along the length of the epididymis[15,16]. This component serves to accept short-, medium- and long-chain acetyl groups from acetyl-coenzyme A (CoA). Acetyl-L-carnitines are then transported across the inner mitochondrial membrane. Within the mitochondria the acetyl groups are transferred to generate acetyl-CoA, which is available for β-oxidation and the tricarboxylic acid (TCA) cycle or Krebs cycle to generate cellular energy[17]. The TCA cycle generates reduced electron carriers nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD(2H)). Consequently, ATP can be produced when NADH and FAD(2H) donate the electrons to O_2 via the mitochondrial electron transport chain. Free L-carnitine and acetyl-L-carnitine thus play a key role in sperm metabolism by providing readily available energy for the use by spermatozoa, which positively affects sperm motility, maturation and the spermatogenic process[18]. Moreover, acetyl-L-carnitine stimulates the motility of previously active spermatozoa that have become energy depleted, but no evidence is present for a role in the initiation of motility *in vitro*[15]. A high carnitine concentration also increases cellular viability[18]. Additionally, the secretions of the prostate gland (e.g. citrate, an intermediate of the TCA cycle) and the seminal vesicles (e.g. fructose, an energy source) provide the spermatozoa with an environment suited for sustained motility[2,10,12,19].

Morphologically abnormal spermatozoa generally have a lower fertilizing potential, depending on the types of anomalies, and may contain abnormal DNA, e.g. DNA fragmentation, structural chromosomal aberrations, immature chromatin and aneuploidy[2].

1.2 The acrosome reaction

A spermatozoon is a vesicle for the transmission of the paternal genome into the oocyte at fertilization[11]. However, sperm cells are incapable of fertilizing immediately after ejaculation, due to the inability to penetrate the zona pellucida, i.e. a surface layer of the oocyte. Spermatozoa must remain within the female tract for a period to undergo a series of maturation steps to obtain the ability to fertilize[12]. Capacitation, triggered by bicarbonate ions in the female reproductive tract, prepares the plasma membrane through lipid phase transition, cholesterol efflux and membrane hyperpolarization to enhance its fluidity[20]. Moreover, changes of intracellular ionic and metabolic contents occur. This step precedes the modification in the motility pattern, known as hyperactivated motility, and the acrosome reaction. The acrosome reaction is an exocytotic event, triggered by binding to the zona pellucida protein 3 (ZP3), in which the plasmalemma and acrosomal membrane fuse and vesiculate, leading to the release of various hydrolytic and proteolytic enzymes that digest the zona pellucida proteins. Therefore, the acrosome reaction is essential for sperm penetration through the oocyte envelope[20]. Moreover, modifications to plasma membrane proteins occur during the acrosome reaction, which allow fusion with the oocyte membrane. Furthermore, sperm cells can undergo spontaneous acrosome reaction *in vitro*, which might be due to self-aggregation of sperm receptors for the zona pellucida[21]. Additionally, the Na^+ and/or Ca^{2+}- pumping mechanisms might become less efficient with time, which could result in a gradual increase in intracellular Ca^{2+} and pH leading to spontaneous acrosome reaction[21].

In IUI and IVF, insemination with acrosome-reacted sperm is not successful since these spermatozoa are off-schedule for fertilization. The acrosome reaction must be precisely timed, with respect to sperm-zona pellucida binding, to ensure zona penetration and membrane fusion[22]. Consequently, selection of acrosome-unreacted spermatozoa is paramount to the success of these insemination protocols.

1.3 Apoptosis

Apoptosis is a pathway of cell death that is induced by a tightly regulated suicide program in which cells programmed to die activate enzymes, namely cytosolic cysteine-containing aspartate-specific proteases (caspases), to degrade their nuclear DNA and nuclear and cytoplasmic proteins[23]. Caspases-2, 8, 9 are initiator caspases, which activate effector caspases-3, 6, 7. Caspase-3 decides the fate of the cell and its activation marks the point of no return in the apoptotic cascade. Fragments of the apoptotic cells then break off. The plasmalemma of these dying cells remains intact, but the membrane is altered in such a way that the cell and its fragments become targets for phagocytes. The dead cell is rapidly cleared before the content can leak out. Therefore, apoptosis does not elicit an inflammatory reaction in the host[24]. This programmed cell death plays a key role in adjusting the appropriate number of proliferating PGCs via the Fas/FasL system[11]. Binding of Fas and its ligand triggers the recruitment of adaptor proteins, which in turn initiate the caspase cascade[24]. Furthermore, the integrity of the paternal genome is of paramount importance in the initiation and the maintenance of a viable pregnancy *in vivo* and *in vitro*. However, the genome can be compromised with nucleotide and/or DNA strand damage, generated during an oxidative attack, and with aneuploidy[25]. During spermatogenesis, damaged sperm cells and precursors, containing the mentioned defects, accumulate the p53 protein. This protein first arrests the cell cycle at the G_1 phase to allow time for repair. However, if the harm is too severe to be repaired successfully, p53 triggers apoptosis and the damaged sperm cells and precursors are eliminated by the Sertoli cells, a process known as abortive apoptosis[11]. Unfortunately, abortive apoptosis is not

always successful[26]. A small percentage of apoptotic spermatozoa may be released into the ejaculate. In addition, laboratory manipulation of sperm after ejaculation may contribute to the total amount of apoptotic cells present in the prepared sperm sample which will reach the oocyte via ART[11]. Fertilization of the oocyte with an apoptotic spermatozoon will jeopardize embryo survival[26].

Apoptosis is marked by chromatin condensation, plasma membrane blebbing, DNA fragmentation and cell fragmentation in apoptotic bodies[26]. Moreover, externalization of PS is an early characteristic of apoptosis, which is not routinely detected in sperm samples. Sperm cells expressing PS could be survivors of abortive apoptosis or could be generated by laboratory manipulation. In addition, PS expression could be the result of oxidative stress initiated during the transit or storage in the male genital tract or could be linked to the physiological processes of capacitation and the acrosome reaction. However, regardless of the underlying reason, PS surface expression has been proven to be detrimental to fertilization and embryo implantation and survival in ART[26].

1.4 Aim of the study

Male subfertility accounts for 10 percent of subfertility in couples looking for reproductive help. The pregnancy and life birth rates of first line treatment, IUI, and advanced treatment, IVF, remain to be improved, despite efforts in the field. Hence, improvement in sperm quality is a major step to raise the chances of pregnancy and life birth.

In order to define sperm quality, the conventional sperm parameters are used, namely count, motility and morphology. Male subfertility is associated with one or more subnormal parameters[27]. These parameters are also routinely used for predicting the fertility potential of spermatozoa. However, hidden defects at the chromosomal (aneuploidy), nuclear (DNA damage) and membrane (PS expression, acrosome reaction) level may inefficiently or not be detected during a conventional sperm quality analysis[28].

We expect that male subfertility is multifactorial and that a conventional sperm quality analysis does not provide sufficient information to explore the fertility problem. Lack of information concerning the quality of an individual sperm sample might be responsible for the current suboptimal success rates of the subfertility treatments, IUI and IVF.

Initially, storage conditions of the sperm samples will be optimized in terms of the temperature and the storage medium to increase the survival of spermatozoa.

During this thesis project, the potential of the HOS test as a selection step for IUI and IVF protocols will be evaluated since it is known that HOS is an excellent parameter to select viable cells for ICSI. The HOS test presumes that only viable cells with intact, active membranes (necessary in several physiological processes during fertilization) will swell at the level of the sperm tail in a hypo-osmotic solution. This viability test can be performed after an initial quality improvement through density gradient centrifugation (DGC) preparation. However, the use of the HOS test in IUI and IVF depends on whether sperm motility can be maintained after cells are restored back to their physiological status. Therefore, the mitochondrial energy carriers, L-carnitine and acetyl-L-carnitine, will be used during the reversal of tail swelling in an attempt to increase the final motility of sperm cells. Selection through HOS followed by the reversal of sperm swelling will act as a stress test for membrane integrity. Consequently, highly

motile sperm cells with superior membrane integrity can be selected through the swim up preparation technique, while sperm cells with inferior membrane integrity will lose their motility upon HOS and will be removed from the sperm sample. During swim up preparation, a layer of medium is placed on top of the sperm suspension. Only viable and highly motile spermatozoa are capable to swim towards the medium, which is collected.

DGC is routinely applied in fertility laboratories as an initial preparation method in ART protocols and sperm research. This technique uses centrifugation of a sperm sample over gradients, which separates cells on the basis of their density. A fraction of progressively motile and morphologically normal spermatozoa, free from debris, contaminating leukocytes, non-germ cells and degenerating germ cells is obtained[2]. The efficiency of DGC to recover sperm with normal protamine content will be evaluated using the chromomycin A3 (CMA3) staining. This fluorescent dye visualizes the protamination status of DNA[29]. Via the acridin orange (AO) staining, the ability of DGC to reduce the level of DNA fragmentation in sperm samples will be studied. AO staining determines single-stranded DNA[30]. Additionally, the effect of DGC selection on sperm morphology and motility will be examined using the Sperm Blue staining and the Makler Counting Chamber, respectively. Furthermore, the ability of DGC to improve the viability status of spermatozoa will be investigated using the annexin V-allophycocyanin (APC) conjugate, propidium iodide (PI) and the α-p53-Alexa Fluor 488 conjugate. Annexin V is an anticoagulant protein of 35.8 kDa which binds with a high affinity to outwardly exposed PS in a calcium-dependent manner[26].

Since acrosome-reacted spermatozoa are unable to fertilize oocytes, the degree of the acrosome reaction within the sperm sample before fertilization in IUI and IVF is important. The acrosome reaction will be evaluated through two techniques, namely the α-CD46-fluorescein isothiocyanate (FITC) staining and the pisum sativum agglutinin (PSA)-FITC staining. CD46 is present within the inner acrosomal membrane and is only exposed in acrosome-reacted cells[31]. PSA is a lectin, which binds to glycoproteins on the outer membrane of the acrosome. Acrosome-reacted cells lose their acrosomal cap and can be identified by the disappearance of fluorescence[31,32]. The most suitable acrosomal staining method will be identified. Moreover, the acrosomal status of sperm cells will be studied before and after DGC via the α-CD46-FITC conjugate.

In addition, selective removal of unwanted cells in a sperm sample will be performed using MACS columns. MACS employs the use of magnetic particles conjugated to annexin V to capture PS-positive spermatozoa. Before and after the MACS treatment, the viability and acrosomal states will be determined. Moreover, the protamination status and the level of DNA fragmentation will be evaluated.

Finally, it has been suggested that PS surface expression is partially associated with the physiological processes of capacitation and/or the acrosome reaction[26]. To evaluate this hypothesis, the percentage of acrosome-reacted and PS-expressing cells in a sperm sample will be determined using α-CD46-FITC and annexin V-APC staining.

2 Materials & methods

2.1 Sample collection of patients

Ejaculated sperm samples of subfertile men were obtained from the Algemeen Medisch Laboratorium in Antwerp, Belgium. Male subfertility is associated with one or more subnormal conventional sperm parameters, namely sperm concentration less than 20 million/ml, motility less than 50 %, or normal morphology, according to Kruger's strict criteria, less than 5 % in two consecutive semen analyses[2].

A fresh semen sample was acquired by masturbation into a clean sterile container after a recommended period of three to five days of abstinence to obtain an optimal sperm concentration. When incubated for 30 minutes at 37°C and 5 % CO_2, the ejaculate begins to liquefy, i.e. the semisolid coagulated mass becomes more homogeneous and watery. After the removal of seminal plasma, the spermatozoa were mixed with transport (storage) medium. To ensure optimal preservation of sperm quality during transport, the effect of two transport media on survival was compared, namely storage medium without L-carnitine and acetyl-L-carnitine and storage medium supplemented with these mitochondrial energy generators.

Survival was assessed through the evaluation of motility, since motile spermatozoa are essential in IUI and IVF protocols. Dulbecco's Modified Eagle Medium Ham F12 (DMEM Ham F12; Sigma-Aldrich, Bornem, Belgium) was supplemented with 1 mM sodium pyruvate (1/1000 dilution; Sigma-Aldrich), penicillin-streptomycin (3/2000 dilution; Invitrogen Corporation, Gibco, Merelbeke, Belgium), glutamax (1/100 dilution; Invitrogen Corporation, Gibco) and 1 % Solugel (1 g/100 ml; PB gelatins, Tessenderlo group, Brussels, Belgium). In order to optimize sperm vitality and functionality, L-carnitine tartrate (pharmacy Pol De Saedeleer, Lier, Belgium) and acetyl-L-carnitine HCl (pharmacy Pol De Saedeleer) were added to the transport medium up to a final concentration of 177 µM and 186 µM, respectively. All solutions were filtered and the manipulations occurred within the laminar flow cabinet to ensure sterile media. The media possessed a physiological pH.

Furthermore, the optimal storage temperature was selected by comparing the survival of spermatozoa at room temperature and at 37°C and 5 % CO_2 (New Brunswick scientific CO_2 8IR, New Brunswick scientific, Rotselaar, Belgium). The samples, stored at room temperature, were incubated for 1 hour at 37°C before evaluating the motility. Furthermore, to identify a potential different motility pattern during storage at room temperature, the motility of sperm cells was compared between storage at room temperature with and without an incubation period of 1 hour at 37°C.

2.2 Hypo-osmotic swelling test

100 µl of a sperm sample was added to 1 ml of HOS solution (a 1:1 solution of transport medium and distilled water) with an osmolality of approximately 160 mOsm. After an incubation period of approximately 25 minutes at 37°C and 5 % CO_2, tail swelling is stabilized. Viability, indicated by sperm tail swelling, was determined for 100 spermatozoa via the Makler Counting Chamber (Sefi Medical Instruments ltd, Haifa, Israel) and light microscopic evaluation (Wild Leitz GmbH; Leitz, Wetzlar, Germany) in an all-or-nothing approach.

2.3 Density gradient centrifugation

After centrifugation (400xg relative centrifugal force (rcf), 6 minutes) (Hettich zentrifugen Universal 16 R, Hettich, Tuttlingen, Germany), the cell pellet was resuspended in 2 ml of transport medium and loaded onto 2 ml of 75 % Sperm Filter gradient (Cryos International, Aarhus C, Denmark) and centrifuged. Next, the supernatant and the less dense fraction were removed. The dense spermatozoa were resuspended in transport medium and used for analysis.

2.4 Magnetic-activated cell sorting-annexin V treatment

MACS employs the use of magnetic particles conjugated to proteins or antibodies to tag cells of interest. These labeled cells are retained in the MACS column when exposed to the magnetic field of a MACS separator. MACS annexin V microbeads are superparamagnetic particles that are coupled to annexin V to separate the PS-positive, microbead-labeled fraction from the PS-negative spermatozoa[26].

After centrifugation (300xg rcf, 10 minutes) (Hettich zentrifugen Universal 16 R, Hettich), the supernatant was removed and the cell pellet was resuspended in 80 µl of 1 x Binding Buffer (Miltenyi Biotec, Leiden, The Nederlands) per 10^7 total cells. For fewer cells, the same volume was added. Afterwards, 20 µl of MACS annexin V MicroBeads (Miltenyi Biotec) was added per 10^7 total cells. The sample was mix well and incubated for 15 minutes at 6°C-12°C. Again, for fewer cells, the same volume of MACS annexin V MicroBeads (Miltenyi Biotec) was used. The labeled cells were then washed by added 10 to 20 times the labeling volume of 1 x Binding Buffer (Miltenyi Biotec) and centrifugation. After removal of the supernatant, the cell pellet was resuspended in the appropriate amount of 1 x Binding Buffer (500 µl per 10^8 total cells when using MS Columns and 1 to 2 ml per 10^8 total cells when using LX Columns; Miltenyi Biotec). Magnetic separation was performed by layering the labeled cell suspension onto an MS or LX Column (Miltenyi Biotec), which was first washed with 500 µl or 3 ml of 1 x Binding Buffer (Miltenyi Biotec), respectively. The microbead-labeled cells were retained in the column, while PS-negative spermatozoa passed through and were collected. Thereafter, the MS or LX column (Miltenyi Biotec) was rinsed four times with 500 µl or 3 ml of 1 x Binding Buffer (Miltenyi Biotec), respectively. The obtained sperm fraction was resuspended in transport medium and used for analysis.

2.5 Conventional sperm parameters

2.5.1 Sperm count

The sperm concentration was obtained via the Makler Counting Chamber (Sefi Medical Instruments ltd) and light microscopy (Wild Leitz GmbH; Leitz). This chamber contains a central grid, which is subdivided into 100 squares. The number of spermatozoa counted in any row of 10 squares of the grid indicates the sperm concentration in millions/ml. Only intact spermatozoa, consisting of a head and a tail, were included in the analysis. The reference value for normal sperm concentration is ≥ 20 million/ml, according to the World Health Organization (WHO) criteria[2]. However, reference values vary according to geography. Therefore, Ombelet et al. (1997) defined reference values which are suited for the subfertile population in the Limburg area of Belgium[33]. The normal values of the Antwerp patient population have been validated against the reference values used at Ziekenhuis Oost-Limburg. The reference value for normal sperm concentration is defined as ≥ 34 million/ml.

2.5.2 Sperm motility

The sperm motility was also evaluated through the Makler Counting Chamber (Sefi-Medical Instruments ltd) and light microscopy (Wild Leitz GmbH; Leitz). The motility of 100 spermatozoa was subdivided into four categories, namely progressively motile spermatozoa moving linear and fast (A), slow moving spermatozoa moving progressively or not (B), non-progressively motile, shaking spermatozoa (C) and immotile spermatozoa (D). Grade A+B spermatozoa are considered good motile spermatozoa, suitable for IUI and IVF protocols. The WHO reference value for normal sperm motility is ≥ 50 % A+B spermatozoa or ≥ 25 % A spermatozoa[2]. The reference value for normal sperm motility, introduced by Ombelet et al. (1997), is ≥ 45 % grade A+B spermatozoa or ≥ 8 % grade A spermatozoa[33].

2.5.3 Sperm morphology

Morphology, more specifically normal/abnormal subdivision, was assessed via light microscopic evaluation (Wild Leitz GmbH; Leitz) of sperm using Kruger's strict criteria (**Supplement 1**). Sperm smears were stained with Sperm Blue staining (Microptic, Barcelona, Spain). This dye differentiates the components of the spermatozoon (the acrosome, the head, the mid piece, the principle piece and the end piece) through different intensities of blue[34].

Dried sperm smears were submerged into a staining tray containing SpermBlue fixative (Microptic) during 10 minutes at room temperature. After draining the excess fixative, the fixed sperm smears were incubated with SpermBlue dye (Microptic) for 11 minutes at room temperature. The slides were washed twice into distilled water. After air-drying, the cover slip was mounted using clearium mounting medium (Surgipath, Richmond, United States of America) and light microscopic evaluation of 200 spermatozoa occurred. The reference value for normal sperm morphology, according to the current Kruger's strict criteria, is ≥ 5 % normal sperm cells[2].

2.6 Chromomycin A3 staining

CMA3 staining provides an indirect visualization of the protamination status of DNA. Competition occurs for sperm DNA between this guanine-cytosine specific dye and protamines at the molecular level. Spermatozoa with good protaminated DNA stain dull yellow, while low quality sperm cells with poorly protaminated DNA stain bright yellow[29].

A minimum of one million spermatozoa was washed twice in Dulbecco's Ca^{2+}-Mg^{2+}-free phosphate buffered saline (PBS) (8.7 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 148.8 mM NaCl; Sigma-Aldrich). The sample was centrifuged (1200xg rcf, 10 minutes) (Hettich zentrifugen Mikro 22, Hettich) and the sediment was fixed in 100 µl of a 3:1 methanol (Merck, Brussels, Belgium) and acetic acid (Merck) mixture for 5 minutes at 4°C. After homogenization, the spermatozoa were spread on glass slides. Staining was performed with 100 µl CMA3 solution (0.25 mg/ml (AG scientific, Marcq, Belgium) in McIlvaine buffer, pH 7.0, containing 10 mM $MgCl_2$ (VWR, Leuven, Belgium) (**Supplement 2**)) during 20 minutes at room temperature in the dark. Slides were rinsed with PBS (Sigma-Aldrich), air-dried and covered with a cover slip using a 1:1 solution of PBS (Sigma-Aldrich) and glycerol (Merck). The evaluation occurred immediately after staining on a Leitz fluorescence microscope (Wild Leitz GmbH; Leitz) with a mercury light source, using the appropriate objective (PL APO, 40x magnification, 0.75 numerical aperture, Leitz)

and an I2 filter (450-490 nm band pass filter and 515 nm long pass emission filter, Leitz). 200 sperm cells were examined in an all-or-nothing approach.

2.7 Acridin orange staining

The fluorescence pattern, generated by the AO staining, is determined by the thiol-disulfide status of the protamines. Moreover, disulfide bridges provide resistance of spermatozoa against the denaturation through acidic media, which are used during the AO staining protocol. AO generates a green fluorescent signal when it intercalates with native, double-stranded DNA, suggesting correct packaging. Red-, orange- or yellow-colored sperm cells possess incorrect packaging and single-stranded DNA[30].

Fixation of sperm smears occurred in a 3:1 solution of methanol (Merck) and acidic acid (Merck) for at least 2 hours. Afterwards, the slides were air-dried and stained with an AO solution (5 ml stock solution (1 g AO/L distilled water (Sigma-Aldrich)), 20 ml 0.1 M citric acid (Sigma-Aldrich) and 1.25 ml 0.3 M $Na_2HPO_4 \cdot 7\ H_2O$ solution (Merck)) for 10 minutes at room temperature in the dark. Then, the slides were washed twice in distilled water for 2 minutes. Immediately, the cover slip was mounted using distilled water and 200 sperm cells were evaluated via fluorescence microscopy (Wild Leitz GmbH microscope, PL APO objective, I2 filter; Leitz).

2.8 Evaluation of the acrosomal status of spermatozoa through α-CD46-fluorescein isothiocyanate

The acrosomal status of spermatozoa was determined using the α-CD46-FITC conjugate (BD biosciences, Erembodegem, Belgium). CD46 is present within the inner acrosomal membrane and, therefore, CD46 positivity suggests the presence of an acrosome-reacted cell[31]. CD46 positivity represents the complete acrosome-reacted cells[31].

A minimum of one million spermatozoa was washed in physiological water (0.9 % NaCl) and the pellet was resuspended in 100 μl physiological water. Staining took place with 5 μl of the α-CD46-FITC conjugate (BD biosciences) for 15 minutes at room temperature in the dark. After a final wash step, fluorescence-activated cell sorting (FACS) solution was added to the spermatozoa and the acrosome reaction was evaluated using flow cytometry (FACScanto II; BD biosciences). A gate was positioned on the forward scatter versus sideward scatter dot plot to minimize the interference of debris and clumps during the analysis of the spermatozoa. Fluorescence data were obtained via the 488 nm excitation laser and the green fluorescence channel (515-545 nm filter range) for a minimum of 10000 sperm cells using a medium flow rate.

2.9 Evaluation of the acrosomal status of spermatozoa through pisum sativum agglutinin-fluorescein isothiocyanate

The acrosomal status of spermatozoa was also assessed using the PSA-FITC conjugate (Invitrogen). PSA is a lectin, which binds to glycoproteins on the outer membrane of the acrosome. Consequently, acrosome-reacted cells lose their acrosomal cap and can be identified by the disappearance of green fluorescence. PSA-positive spermatozoa display bright, homogeneous fluorescence indicating acrosome-intact sperm, whereas a decrease in fluorescence occurs in acrosome-reacted, PSA-negative sperm[32]. PSA staining visualizes both the complete and partial acrosome-reacted cells[31].

A minimum of one million spermatozoa was centrifuged (400xg rcf, 5 minutes) (Hettich zentrifugen Mikro 22, Hettich) and the pellet was fixed in 500 μl ice cold methanol for 10 minutes. Afterwards, the cells were washed twice with distilled water. Thereafter, the cells were resuspended in 50 μl PBS (Sigma-Aldrich), pH 7.4, supplemented with 7.5 % bovine serum albumin (BSA; Sigma-Aldrich), and incubated for 20 minutes at room temperature. Then, 50 μl of PSA-FITC (100 μg/ml PSA-FITC (Invitrogen) in PBS (Sigma-Aldrich)) was added and incubation took place during 15 minutes at room temperature in the dark. The cells were washed twice in distilled water. Sperm smears were made and a coverglass was mounted using distilled water. Slides were examined immediately after staining via fluorescence microscopy (Wild Leitz GmbH, PL APO objective, I2 filter, Leitz). 200 cells were examined in an all-or-nothing approach.

2.10 Evaluation of the viability status of spermatozoa through annexin V-allophycocyanin, propidium iodide and α-p53-Alexa Fluor 488

The viability status of spermatozoa was studied using a combination of the annexin V-APC conjugate, PI and the α-p53-Alexa Fluor 488 conjugate. PI distinguishes between viable and non-viable cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. The sperm population, which stains positive for annexin V and negative for PI, is heterogeneous, since PS surface expression might be linked to oxidative stress, to the physiological processes of capacitation and the acrosome reaction or to apoptosis[26]. In addition, sperm cells positive for both annexin V and p53 are undergoing DNA repair or are apoptotic. Annexin V-positive and PI-positive cells are either in the end stage of apoptosis, are undergoing necrosis, or are already dead.

First, α-p53 clone 1801 (1 mg, 2.91 mg/ml, Epsilon Biotech, Zonhoven, Belgium) was conjugated to the fluorescent label Alexa Fluor 488 (Invitrogen). Conjugation is accomplished through amine bond between sulfodichlorophenol esters of the fluorescent label and NH_2-groups located on the proteins or antibodies, according to manual instructions. Briefly, incubation of Alexa Fluor 488 (Invitrogen), dissolved in dimethylformamide (DMF; Sigma-Aldrich), and α-p53 clone 1801 (Epsilon Biotech) took place for 1 hour in the dark using a ratio of 4 to 1 molecules, respectively. The reaction was terminated with 1 M hydroxylamine (Sigma-Aldrich, Fluka). To remove the unbound fluorescent label, dialysis was performed in PBS (Sigma-Aldrich) via Centriprep YM-30 Centrifugal Filter Devices (Amicon Bioseparations, Millipore Corporation, Bedford, United States of America), according to manual instructions. Fractions of the conjugate (0.5 mg/ml) were stored at 4°C in the dark in a 1:1 solution of PBS/7.5 % BSA (Sigma-Aldrich) and 7.5 % Solugel (PB gelatins, Tessenderlo group).

The viability status was measured as follows. After the spermatozoa were washed twice with cold PBS (Sigma-Aldrich) (400xg rcf, 5 minutes) (Hettich zentrifugen Mikro 22, Hettich), the cells were resuspended in 100 µl of 1 x Binding Buffer (10 mM Hepes/NaOH (pH 7.4; Sigma-Aldrich), 140 mM NaCl (Merck), 2.5 mM $CaCl_2$ (Sigma-Aldrich)). Next, 5 µl of annexin V-APC (BD biosciences), 5 µl of PI (2 µl/ml in PBS; Invitrogen) and 5 µl of α-p53-Alexa Fluor 488 (10 µg/ml, Epsilon Biotech) was added. The solution was gently vortexed. After incubation during 15 minutes at room temperature in the dark, the cells were washed, diluted in FACS solution and analyzed via flow cytometry (FACScanto II; BD biosciences). A gate was positioned on the forward scatter versus sideward scatter dot plot to minimize the interference of debris and clumps during the analysis of the spermatozoa. Fluorescence data were collected for a minimum of 10000 sperm cells using a medium flow rate. The APC signal was obtained via the 633 nm

excitation laser and the red fluorescence channel (650-670 nm filter range), whereas the Alexa Fluor 488 and PI signals were obtained via the 488 nm excitation laser and the green (515-545 nm filter range) and yellow (566-606 nm filter range) fluorescence channel, respectively.

2.11 Widefield microscopy

Fluorescence microscopic illustrations of the different fluorescent dyes used during this study were made via an inverted epifluorescence microscope Zeiss Axiovert 100 (Zeiss, Jena, Germany). Fluorescence was elicited by illumination with a XBO 75 W/2 xenon light source (Osram, Berlin, Germany) and the images were captured by a CoolSnap HQ2 camera (Photometrics, Tucson, Arizona, United States of America). Fluorescence generated by the PSA-FITC conjugate was collected using the Zeiss Plan-APOCHROMAT 63X/1,4 Oil DIC objective (Zeiss), while the Zeiss LD Achroplan 20X/0,4 objective (Zeiss) was used to collect the fluorescence generated by the remaining fluorescent dyes and conjugates (**Supplement 3**). All optical filters (**Supplement 3**) and the dichroic mirror were obtained from Semrock Inc (HC-Sedat Quaband set DAPI/FITC:TexasRed/Cy5; Semrock, Rochester, New York, United States of America). The images were edited using µManager 1.3 software (open-source microscopy software initiative at University of California San Francisco; www.micro-manager.org).

2.12 Statistical analysis

The statistical analysis of the data was performed using MedCalc Version 11.5 (MedCalc Software, Mariakerke, Belgium). After checking for normal distribution of the data, statistical calculations were performed using the appropriate tests. To test the null hypothesis (i.e. the average of the differences between a series of paired observations is zero), the paired samples t-test was used. The observations are paired since each data set comes from the same subject. If the calculated P-value is less than 0.05, it can be concluded that the mean difference between the paired observations is significantly different from zero. However, if the sample data are not normally distributed, the non-parametric equivalent of the paired samples t-test, namely the Wilcoxon test, was used. If the resulting P-value is less than 0.05, it can be accepted that the median of the differences between the paired observations is significantly different from zero.

Furthermore, the degree of relationship between the two acrosomal staining methods, α-CD46-FITC staining and PSA-FITC staining, was evaluated using rank correlation since the distribution of variables was not normal. If the resulting P-value is less than 0.05, a significant relationship between the two methods is present.

3 Results & discussion

3.1 Storage at room temperature significantly improves the survival of spermatozoa

To optimize the storage and transport temperature of sperm samples, the survival of sperm cells was compared between samples, stored at room temperature and at 37°C. When stored at room temperature, sperm cells were incubated for one hour at 37°C before evaluating the survival.

Storage of spermatozoa at room temperature significantly improves survival up to 120 hours after ejaculation compared to storage at 37°C ($p<0.05$, n=44; **Fig. 3A**). Moreover, at a lower temperature, viable sperm cells are observed up until 192 hours after ejaculation (**Fig. 3A**). The improved survival and preservation may be due to a decrease in energy consumption, whereby the depletion of the energy supplies is delayed. This hypothesis was evaluated by comparing the motility of sperm cells, stored at room temperature without and with an incubation period of one hour at 37°C at 24 hours after ejaculation. Compared to cells, which were incubated for one hour at 37°C, the motility of spermatozoa is significantly decreased when stored at room temperature without an incubation period (28.25 ± 16.04 vs. 20.45 ± 14.82, $p<0.0001$, n=56; **Fig. 3B**).

Hence, during transport and storage at room temperature, the spermatozoa adopt a resting state, which presumably prolongs energy availability and survival. Moreover, the motility of the resting spermatozoa can be restored upon incubation at 37°C (**Fig. 3B**).

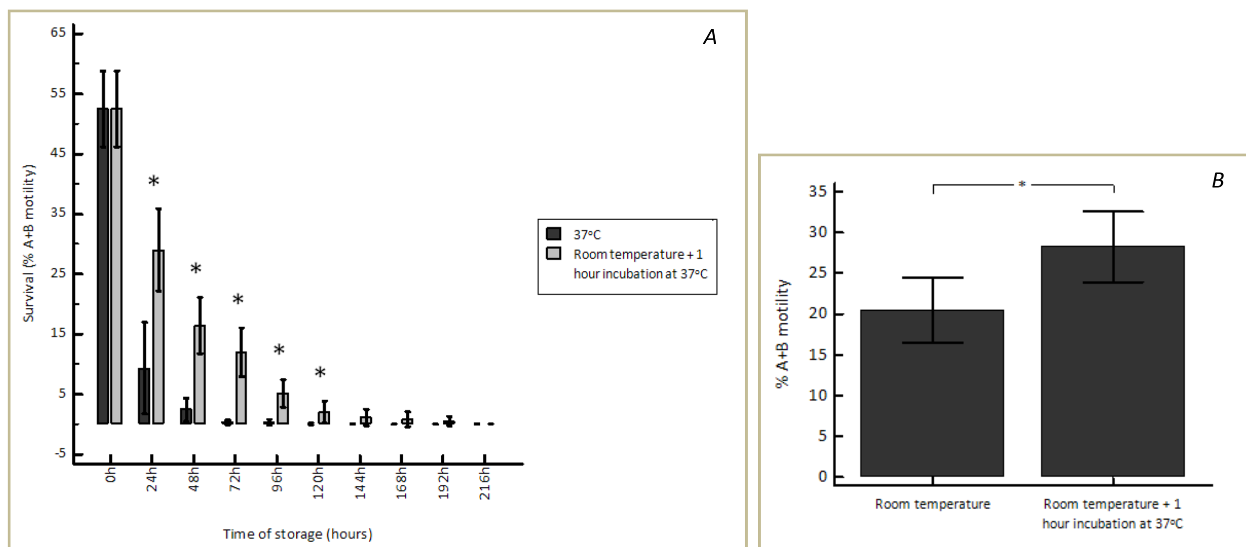

Figure 3: The effect of temperature on the survival of spermatozoa. Data are represented as mean value with standard deviation. *A*. Comparison of the survival (% A+B motility) of spermatozoa, stored at room temperature (▢) or at 37°C (■). When stored at room temperature, sperm cells were incubated for 1 hour at 37°C before evaluating the survival. *B*. Identification of a different motility pattern during storage at room temperature by comparing the motility of sperm cells at room temperature without and with an incubation period of 1 hour at 37°C at 24 hours after ejaculation. Evaluation of the motility of spermatozoa occurred via the Makler Counting Chamber and light microscopy. A= progressively motile spermatozoa moving linear and fast, B= slow moving spermatozoa moving progressively or not. * Significantly different at $p<0.05$.

3.2 In vitro addition of L-carnitine and acetyl-L-carnitine to spermatozoa does not improve survival

In an attempt to optimize sperm vitality and functionality, the survival was compared between spermatozoa, stored in transport (storage) medium without and with L-carnitine and acetyl-L-carnitine.

Sperm samples were stored at room temperature and were incubated for one hour at 37°C before evaluating the survival.

No significant difference in sperm cell survival is observed between the two conditions at all time points (n=35; **Fig. 4**). As mentioned before, L-carnitine serves to accept acetyl groups from acetyl-CoA. Acetyl-L-carnitines then enter the mitochondria, where the acetyl groups are transferred to generate acetyl-CoA. Acetyl-CoA is now available for β-oxidation and the TCA cycle to generate cellular energy[18]. Moreover, acetyl-L-carnitine has been suggested to stimulate the motility of previously active spermatozoa that have become energy depleted[15]. These observations could not be reproduced in this study given that the motility is not increased and the survival is not prolonged when sufficient amounts of L-carnitine and acetyl-L-carnitine are available for sperm cells. A possible explanation might be that these compounds cannot enter sperm cells *in vitro* when added through the transport medium. Moreover, other components, which are necessary for carnitine metabolism, may be deficient (e.g. coenzyme Q (Q10)). Therefore, L-carnitine and acetyl-L-carnitine may not be able to exert their energy promoting function.

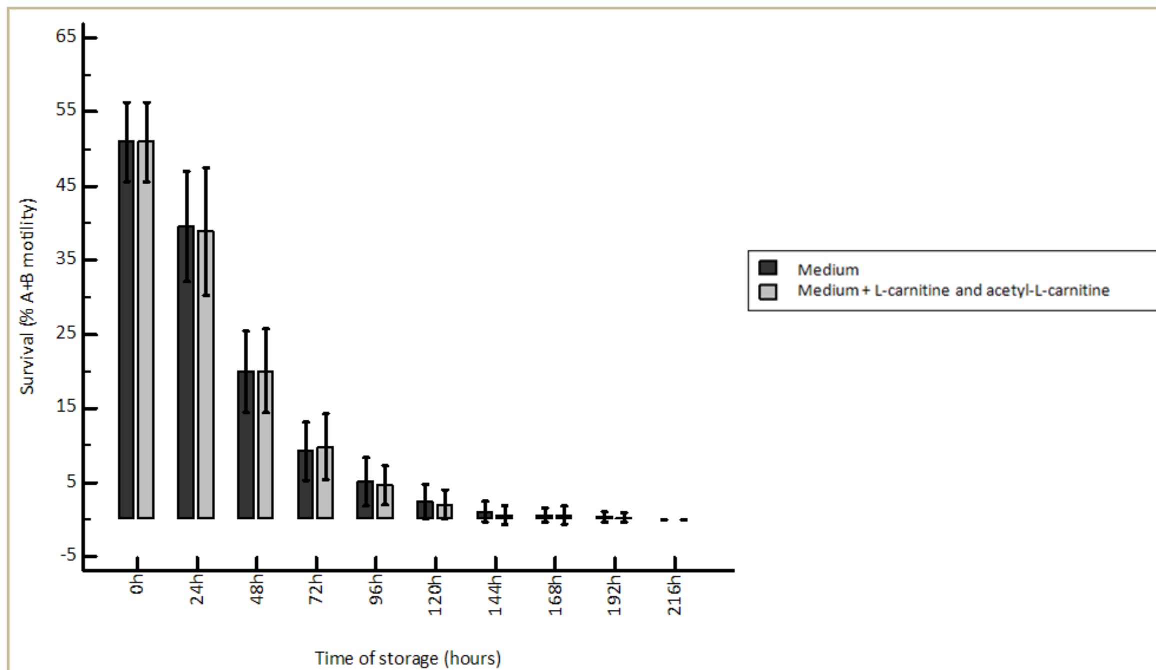

Figure 4: The effect of L-carnitine and acetyl-L-carnitine availability on the survival of spermatozoa. Data are represented as mean value with standard deviation. Comparison of the survival (% A+B motility) of sperm cells, stored in transport (storage) medium without L-carnitine and acetyl-L-carnitine (■) and in transport medium supplemented with these mitochondrial energy promoters (□). The sperm samples were stored at room temperature and were incubated for 1 hour at 37°C before evaluating the survival. Evaluation of the motility of spermatozoa occurred via the Makler Counting Chamber and light microscopy. A= progressively motile cells moving linear and fast, B= slow moving cells moving progressively or not.

3.3 Motility cannot be restored after hypo-osmotic swelling and reversal of the tail swelling

The potential of the HOS test as a selection step for IUI and IVF protocols was evaluated since it is known that HOS is an excellent parameter to select viable cells for ICSI. First, the HOS test was performed before and after DGC to evaluate whether this initial sperm preparation technique increases the percentage of viable cells, displaying tail swelling. Next, the motility was assessed via the Makler Counting Chamber before and after HOS and the reversal of the tail swelling, an osmotic treatment which acts as a stress test for membrane integrity. Moreover, a hypertonic medium, containing L-

carnitine and acetyl-L-carnitine, was used to return the swollen spermatozoa back to their physiological status. This, in an attempt to increase the final motility of the selected cells by stimulating the motility of viable, previously active spermatozoa with good membrane integrity that have become energy depleted[15]. Consequently, provided that motility can be restored or improved after osmotic treatment, the highly motile sperm cells with superior membrane integrity can be selected through the swim up preparation technique, while sperm cells with inferior membrane integrity will lose their motility upon HOS and will be removed from the sperm sample.

Although DGC significantly ameliorates the percentage of viable spermatozoa demonstrating tail swelling compared to the raw sample (26.71 ± 12.27 vs. 31.71 ± 15.39, p=0.0122, n=31; **Table 1**), motility is significantly reduced after HOS and reversal of sperm tail swelling was performed (30.43 ± 14.32 vs. 10.76 ± 11.54, p=0.0001, n=21; **Table 2**). The reversal of tail swelling is confirmed via light microscopy. The possibility exists that osmotic treatment provides an excessively aggressive stress situation for the spermatozoa to recover their motility. Therefore, the reduced motility might be caused by the interference of sperm tail swelling with the functionality of the mitochondria, which are located within the tail. Moreover, introducing the HOS test as a selection step, subsequently to DGC, would result in a greatly decreased availability of highly motile sperm cells for IUI and IVF protocols. Regarding IVF, it is common practice to use a minimum of 50000 progressively motile spermatozoa to fertilize oocytes. In IUI protocols, the inseminating motile count (IMC) must exceed one million progressively motile spermatozoa and should, preferentially, be more than five million progressively motile sperm cells.

Consequently, the HOS test and reversal of the tail swelling is unsuitable as a selection step for IUI and IVF protocols, since this osmotic treatment provides an excessively aggressive stress situation for the spermatozoa.

Table 1: Evaluation of sperm tail swelling induced by HOS before and after DGC preparation.

Test	Raw sample	DGC	P-value
% sperm tail swelling	**26.71** (± 12.27; 7-59)	**31.71** (± 15.39; 10-74)	0.0122

Mean values are shown with in between brackets the standard deviation, the minimum value and the maximum value, respectively. Sperm tail swelling was visualized through light microscopy. HOS: hypo-osmotic swelling, DGC: density gradient centrifugation.

Table 2: Comparison of motility before and after performing the HOS test and returning the swollen spermatozoa to their physiological status.

Test	Raw sample	HOS + reversal of swelling	P-value
% A+B motility	**30.43** (± 14.32; 7-57)	**10.76** (± 11.54; 0-44)	0.0001

Mean values are shown with in between brackets the standard deviation, the minimum value and the maximum value, respectively. Evaluation of the motility of spermatozoa occurred via the Makler Counting Chamber and light microscopy. A= progressively motile cells moving linear and fast, B= slow moving cells moving progressively or not. HOS: hypo-osmotic swelling.

3.4 Density gradient centrifugation improves the morphology, the protamination status and the level of DNA fragmentation within a sperm sample and reduces the amount of phosphatidylserine-expressing viable cells

The efficiency of DGC to recover morphologically normal and motile sperm was evaluated using respectively the Sperm Blue staining and the Makler Counting Chamber. The protamination status and the level of DNA fragmentation was studied before and after sperm preparation via the CMA3 staining and the AO staining, respectively. In addition, the effect of DGC preparation on the viability status of

spermatozoa was evaluated through the combination of the annexin V-APC conjugate, PI and the α-p53-Alexa Fluor 488 conjugate.

Regarding the conventional sperm parameters, the percentage motile sperm cells is slightly, but non-significantly, increased after DGC selection (21.88 ± 16.81 vs. 24.56 ± 17.07, p=0.1414, n=43; **Table 3**). This result is in contrast with the general characteristic of DGC, namely that this technique significantly increases the percentage of progressively motile spermatozoa[2]. This discrepancy may be explained by the fact that analyses took place 24 hours after ejaculation. Thus, at a time point that motility is already decreased in the raw sample and energy supplies are also reduced. Furthermore, the percentage morphologically normal sperm cells is significantly enhanced through DGC (5.24 ± 2.98 vs. 7.07 ± 3.72, p<0.0001, n=35; **Table 3**), as expected[2].

Comparison of the mean values of CMA3 positivity before and after selection shows that the percentage of cells with poorly protaminated DNA is significantly decreased following DGC (41.90 ± 17.29 vs. 30.44 ± 15.58, p<0.0001, n=26; **Table 3**, **Fig. 5A**). Good protaminated spermatozoa are less vulnerable to DNA damage, which can be induced during transit from the male to the female genital tract. Moreover, DNA-protamine interactions compact the sperm nucleus, which permits sperm motility and penetration[11,12]. A similar result was obtained during a study, conducted by Kheirollahi-Kouhestani et al. (2009)[35]. This research group published a significantly reduced percentage of CMA3-positive sperm following DGC preparation (41.37 ± 11.03 vs. 32.49 ± 10.34, p<0.05), which corresponds to the results presented here.

DGC also significantly reduces the percentage of spermatozoa possessing DNA fragmentation compared to unprocessed samples (21.12 ± 8.05 vs. 13.84 ± 7.03, p=0.0001, n=25; **Table 3**), as analyzed through the AO staining (**Fig. 5B**). The research group of Kheirollahi-Kouhestani et al. (2009) also reported a significant reduction in the level of DNA fragmentation following DGC (49.65 ± 15.1 vs. 32.65 ± 14.38, p<0.05)[35]. We have to take into account that the patient samples in this latter study originate from men with lower sperm quality, which are recommended to undergo ICSI. Therefore, the level of DNA fragmentation in this patient population is higher compared to samples of subfertile men used in this study. In addition, the amount of sperm cells possessing DNA fragmentation remains constant at different time points (Time point 1: 09 p.m. day 0 (immediately after ejaculation), Time point 2: 09 a.m. day 1, Time point 3: 12 a.m. day 1; n= 25; **Fig. 6**). Hence, the results are not compromised through analysis at 24 hours past ejaculation and are representative for subfertile men.

Table 3: Conventional sperm parameters, protamination and DNA fragmentation in unprocessed sperm samples and DGC preparations.

Test	Raw sample	DGC	P-value
% A+B motility	**21.88** (± 16.81; 0-69)	**24.56** (± 17.07; 0-59)	0.1414
% morphologically normal sperm	**5.24** (± 2.98; 1-12)	**7.07** (± 3.72; 2-16.5)	<0.0001
% CMA3 positivity	**41.90** (± 17.29; 17-74)	**30.44** (± 15.58; 7-61)	<0.0001
% DNA fragmentation	**21.12** (± 8.05; 5.5-36)	**13.84** (± 7.03; 5-32.5)	0.0001

Mean values are shown with in between brackets the standard deviation, the minimum value and the maximum value, respectively. The conventional sperm parameters, motility and morphology, were studied via light microscopy using the Makler Counting Chamber and the Sperm Blue staining, respectively. The protamination status of spermatozoa was visualized through the CMA3 staining and fluorescence microscopy, whereby CMA3-positive sperm cells possess poorly protaminated DNA. The level of DNA fragmentation was determined by fluorescence microscopic evaluation of the AO staining. A= progressively motile cells moving linear and fast, B= slow moving cells moving progressively or not. DNA: deoxyribonucleic acid, DGC: density gradient centrifugation, CMA3: chromomycin A3, AO: acridin orange.

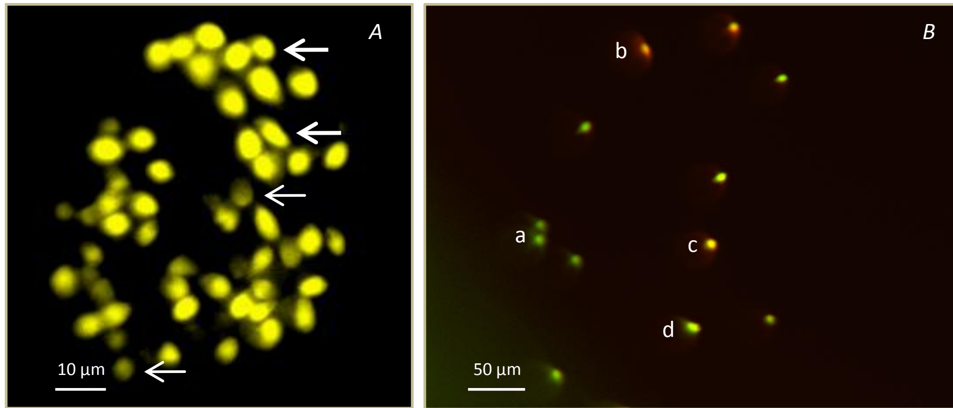

Figure 5: Fluorescence microscopic illustrations of the CMA3 staining (A) and the AO staining (B). *A.* The CMA3 dye provides an indirect visualization of the protamination status of DNA since competition occurs for sperm DNA between this guanine-cytosine specific dye and protamine at the molecular level. Spermatozoa with protaminated DNA stain dull yellow (→), while sperm cells with poorly protaminated DNA stain bright yellow (→) (scale bar 10 µm)[28]. *B.* The AO staining generates a fluorescent pattern depending on the efficiency of DNA packaging. AO generates a green (a) fluorescent signal when it intercalates with native, double-stranded DNA, suggesting correct packaging. Red- (b), orange- (c) or yellow- (d) colored sperm cells possess incorrect packaging and single-stranded DNA (scale bar 50 µm)[29]. Images were taken with an inverted epifluorescence microscope Zeiss Axiovert 100. CMA3: chromomycin A3, AO: acridin orange, DNA: deoxyribonucleic acid.

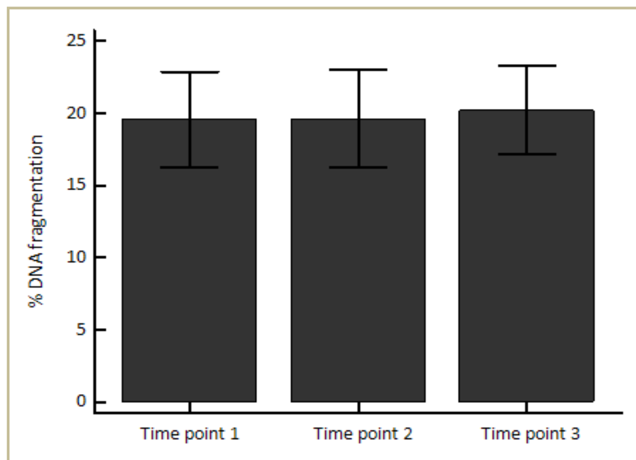

Figure 6: The level of DNA fragmentation in time. Data are represented as mean value with standard deviation. The level of DNA fragmentation within sperm samples was measured at three time points via the AO staining and fluorescence microscopy (Time point 1: 09 p.m. day 0 (immediately after ejaculation), Time point 2: 09 a.m. day 1, Time point 3: 12 a.m. day 1). DNA: deoxyribonucleic acid, AO: acridin orange.

In addition, DGC is able to significantly reduce the percentage of viable spermatozoa expressing PS (annexin V^+ PI^- cells, 5.74 ± 2.80 vs. 2.14 ± 1.35, p=0.0001, n=16; **Table 4**, **Fig. 7**). This population of annexin V-positive sperm cells is heterogeneous, since PS surface expression might be linked to oxidative stress, to the physiological processes of capacitation and the acrosome reaction or to apoptosis[25]. Furthermore, compared to unprocessed samples, the percentages of annexin V^+ $p53^+$ cells and annexin V^+ PI^+ sperm cells remain unchanged after sperm preparation (0.14 ± 0.18 vs. 0.08 ± 0.12, p=0.4609, n=16 and 24.01 ± 10.75 vs. 24.41 ± 12.04, p=0.8752, n=16, respectively; **Table 4**, **Fig. 7**). These results confirm the observations of de Vantéry Arrighi et al. (2009) that DGC preparation decreases the percentage of annexin V-positive spermatozoa, but does not eliminate all cells expressing PS[26]. However, a significant decrease of annexin V^+ PI^+ cells would be expected after DGC since these necrotic, dead cells will first swell and become less dense and, eventually, cell fragmentation will occur[24]. Both these swollen cells and cell fragments should partially appear in the less dense fraction, which is removed during DGC preparation. One possible explanation might be that necrotic, dead sperm cells become sticky and incorporate into aggregates, either among each other or in combination with the silica

particles of the DGC gradient. Therefore, these cells will not be removed from the sperm sample. Via light microscopic evaluation, aggregates of silica particles were identified and might be partially responsible for the unchanged percentage of necrotic cells when performing DGC preparation. Furthermore, flow cytometric analysis shows that aggregates of silica particles are present within the spermatozoa gate (2.9 %, **Fig. 8**) and that these aggregates bind PI (24.5 % PI positivity, **Fig. 8**). The contribution of the stained aggregates to the final percentage of annexin V$^+$ PI$^+$ cells after DGC is just 0.71 %.

Table 4: The viability status in unprocessed sperm samples and DGC preparations.

Test	Raw sample	DGC	P-value
% annexin V+ PI- cells	**5.74** (± 2.80; 2.3-13.8)	**2.14** (± 1.35; 0.3-4.5)	0.0001
% annexin V+ p53+ cells	**0.14** (± 0.18; 0-0.6)	**0.08** (± 0.12; 0-0.4)	0.4609
% annexin V+ PI+ cells	**24.01** (± 10.75; 4.6-39.5)	**24.41** (± 12.04; 5.2-40.2)	0.8752

Mean values are shown with in between brackets the standard deviation, the minimum value and the maximum value, respectively. Evaluation of the viability status of spermatozoa occurred via the combination of annexin V-APC, PI and α-p53-Alexa Fluor 488 staining and flow cytometry (FACScanto II). DGC: density gradient centrifugation, PI: propidium iodide, APC: allophycocyanin.

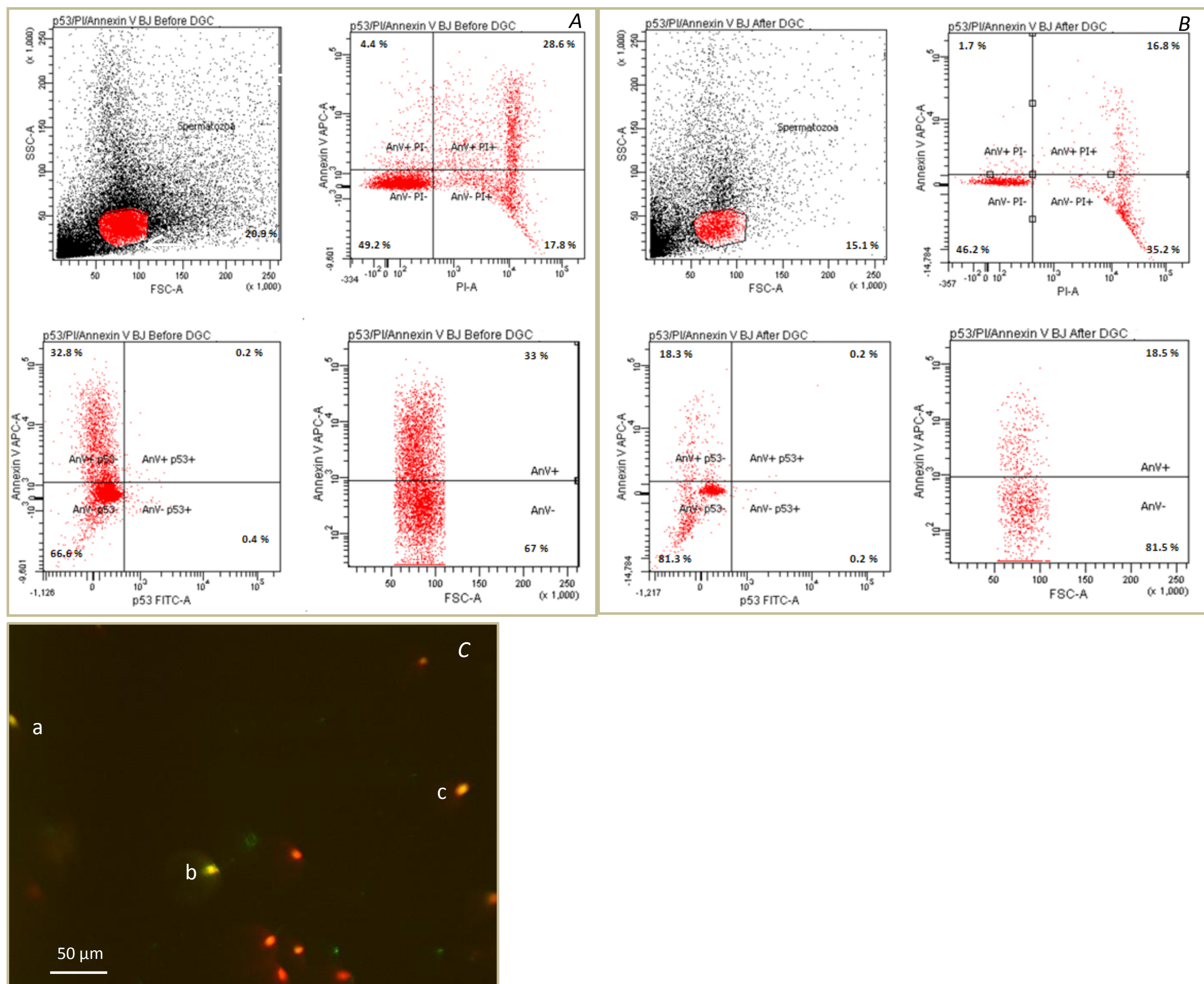

Figure 7: Evaluation of the viability status of spermatozoa. *A,B*. Flow cytometric analysis (FACScanto II) of the viability status of sperm cells before (A) and after (B) DGC through annexin V-APC, PI and α-p53-Alexa Fluor 488 staining. *C.* A fluorescence microscopic illustration of annexin V$^+$ PI$^-$ (a), annexin V$^+$ p53$^+$ (b) and annexin V$^+$ PI$^+$ (c) spermatozoa (scale bar 50 µm). The image was taken with an inverted epifluorescence microscope Zeiss Axiovert 100. DGC: density gradient centrifugation, AnV: annexin V, PI: propidium iodide, APC: allophycocyanin.

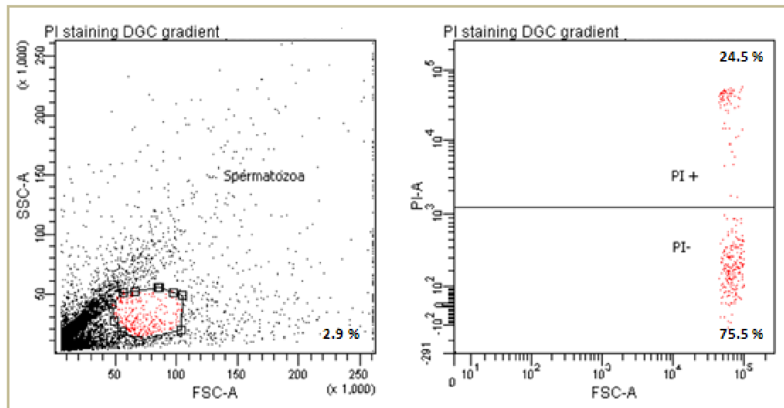

Figure 8: Flow cytometric analysis of DGC gradient stained with PI. To evaluate the contribution of aggregates of silica particles to the percentage of annexin V^+ PI^+ sperm cells, the DGC gradient was stained with PI and analysis occurred by FACScanto II. PI: propidium iodide, DGC: density gradient centrifugation.

3.5 Evaluation of the acrosome reaction through flow cytometric analysis of CD46 positivity is more reliable than fluorescence microscopic evaluation of PSA negativity

To identify the most suitable acrosomal staining method, α-CD46-FITC staining was compared to PSA-FITC staining through the evaluation of the level of the acrosome reaction within raw samples.

The mean percentage of CD46-positive cells within the raw sample is 30.98 % (30.98 ± 13.05, n=15), while the mean percentage of PSA-negative sperm cells is 85.37 % (85.37 ± 12.54, n=15). Moreover, no significant relationship was observed between the two staining methods (p=0.4307). These observations show that PSA-FITC staining results in an unrealistic overestimation of the number of acrosome-reacted cells within a sperm sample. This can be explained by the fact that CD46 positivity was objectively evaluated using flow cytometry, while PSA negativity was evaluated visually via fluorescence microscopy, a more subjective evaluation method. Moreover, the high amount of debris in unprocessed sperm samples, which is extensively stained with PSA-FITC, makes the differentiation between PSA positivity and PSA negativity via light microscopy extremely difficult (**Fig. 9A**). On the contrary, the difference between CD46 positivity and CD46 negativity is more pronounced, as demonstrated in **Figure 9B** and **Figure 10C,D**.

Consequently, evaluation of the acrosome reaction through flow cytometric analysis of CD46 positivity is more reliable than fluorescence microscopic evaluation of PSA negativity.

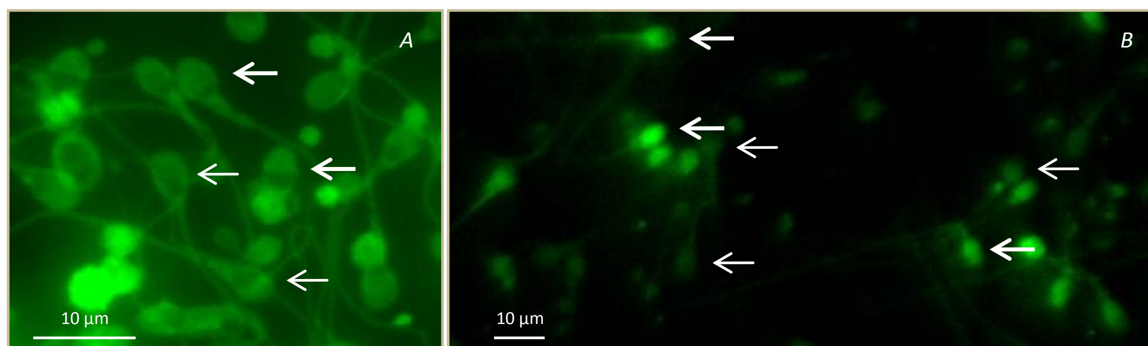

Figure 9: Fluorescence microscopic evaluation of the acrosome reaction using the PSA-FITC staining (*A*) and the α-CD46-FITC staining (*B*). Images of sperm cells, stained with PSA-FITC (scale bar 10 µm) and α-CD46-FITC (scale bar 10 µm), were taken with an inverted epifluorescence microscope Zeiss Axiovert 100. PSA: pisum sativum agglutinin, FITC: fluorescein isothiocyanate.

3.6 Density gradient centrifugation selects acrosome-reacted spermatozoa

The percentage acrosome-reacted sperm cells was determined using the α-CD46-FITC conjugate before and after DGC preparation. Moreover, the acrosomal status of sperm cells was evaluated at 24 hours and 48 hours after ejaculation to evaluate the effect of storage time on the level of acrosome reaction. Also, sperm storage in transport medium and in physiological water was compared at 24 hours after ejaculation in order to exclude the presence of a component within the transport (storage) medium, which can induce the acrosome reaction.

Compared to the raw sample, a significantly increased percentage of acrosome-reacted cells (28.82 ± 12.65 vs. 40.98 ± 18.07, p=0.0009, n=19; **Fig. 10A,C,D**) was observed after DGC selection, with a minimum value of 18.8 % and a maximum value of 79.1 % acrosome-reacted cells. The DGC gradient contains capacitation medium. Therefore, to exclude that the gradient itself induces the acrosome reaction, spermatozoa were incubated with gradient during six minutes and the level of acrosome reaction was determined. The percentage of acrosome-reacted cells is not affected by incubation with DGC gradient (33.48 ± 19.12 vs. 34.81 ± 21.42, p=0.6240, n=8; **Fig. 10B**). Consequently, DGC selects acrosome-reacted cells during sperm preparation. Spermatozoa possess condensed DNA and an acrosomal content with a low density. Upon the acrosome reaction, loss of this less dense acrosomal content occurs and, therefore, the dense acrosome-reacted cells are selected through DGC.

No significant difference in the percentage of acrosome-reacted spermatozoa was observed at 48 hours compared to 24 hours after ejaculation (38.97 ± 15.77 vs. 38.04 ± 15.60, p=0.5973, n=11; **Fig. 11A**).

Furthermore, when comparing storage of sperm cells in transport medium to storage in physiological water, sperm samples possess a significantly lower percentage of acrosome-reacted cells when stored in physiological water (34.99 ± 12.87 vs. 24.18 ± 12.49, p=0.0046, n=17; **Fig. 11B**). The only protein component with a potential to induce the acrosome reaction in the transport medium is Solugel since it is the only protein added to the medium. Solugel is a soluble, hydrophilic hydrolyzed gelatin and functions to stabilize the oncotic pressure within the medium. It is known that this matrix protein does not stimulate capacitation. However, a possibility exists that Solugel shows homology with the ZP3, which triggers the acrosome reaction *in vivo* upon binding. Furthermore, Solugel might be able to facilitate the self-aggregation of sperm receptors and, therefore, the acrosome reaction. Another possible explanation for the increased level of acrosome reaction in the transport medium may be the presence of energy carriers (e.g. pyruvate), which are absent in physiological water. Spontaneous acrosome reaction in the medium could then be explained as an energy requiring process.

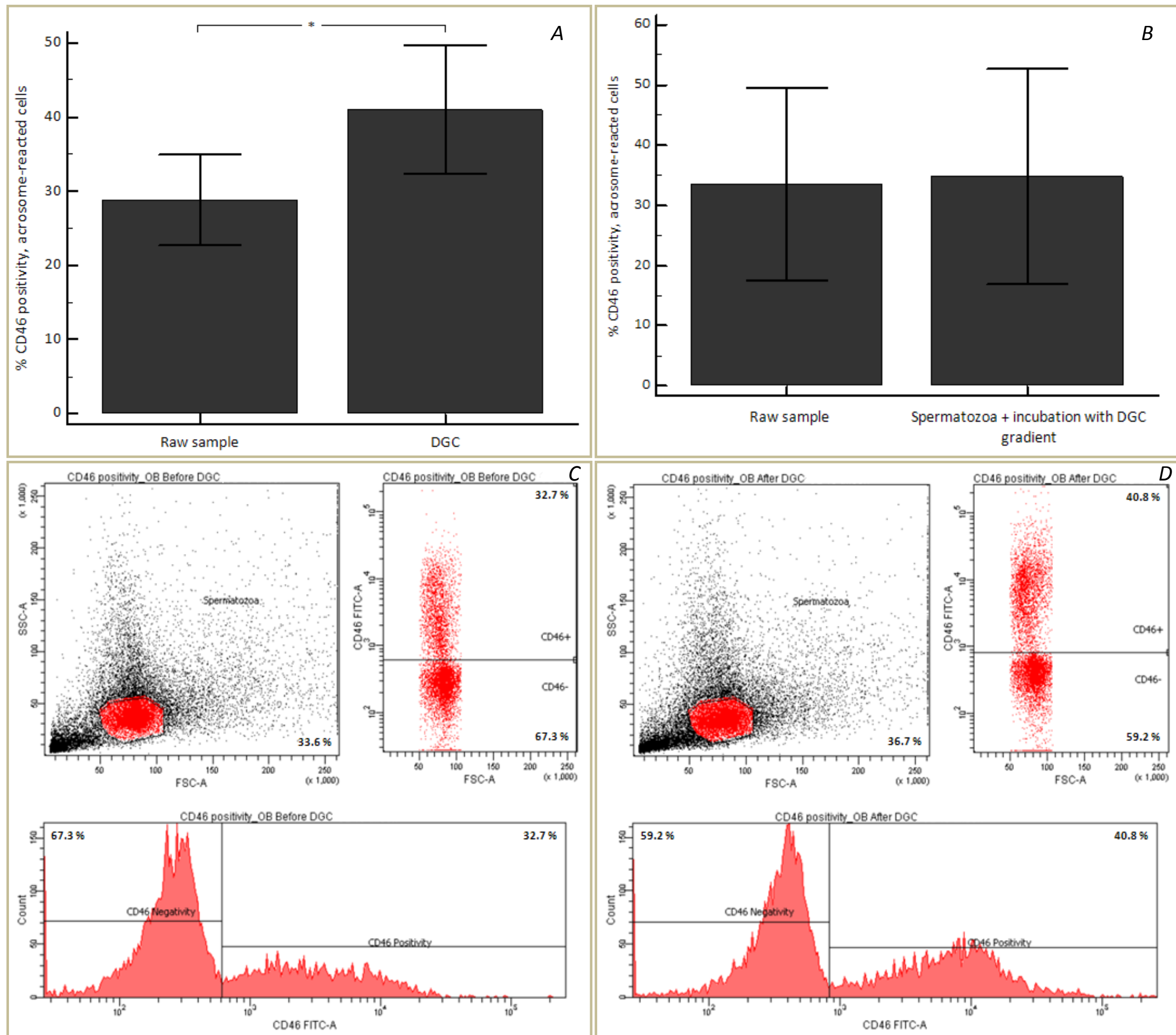

Figure 10: The effect of DGC selection on the percentage of acrosome-reacted spermatozoa. *A,B.* Data are represented as mean value with standard deviation. *A.* The percentage of acrosome-reacted sperm cells in unprocessed sperm samples and DGC preparations was determined by α-CD46-FITC staining and flow cytometry (FACScanto II). CD46 positivity indicates the occurrence of the acrosome reaction. *B.* To determine whether the DGC method selects acrosome reacted cells or the DGC gradient induces this phenomenon, spermatozoa were incubated with DGC gradient during 6 minutes and the sperm cells were stained with α-CD46-FITC. *C,D.* Flow cytometric analysis of the acrosome reaction through α-CD46-FITC staining before (C) and after (D) DGC. DGC: density gradient centrifugation, FITC: fluorescein isothiocyanate. * Significantly different at $p<0.05$.

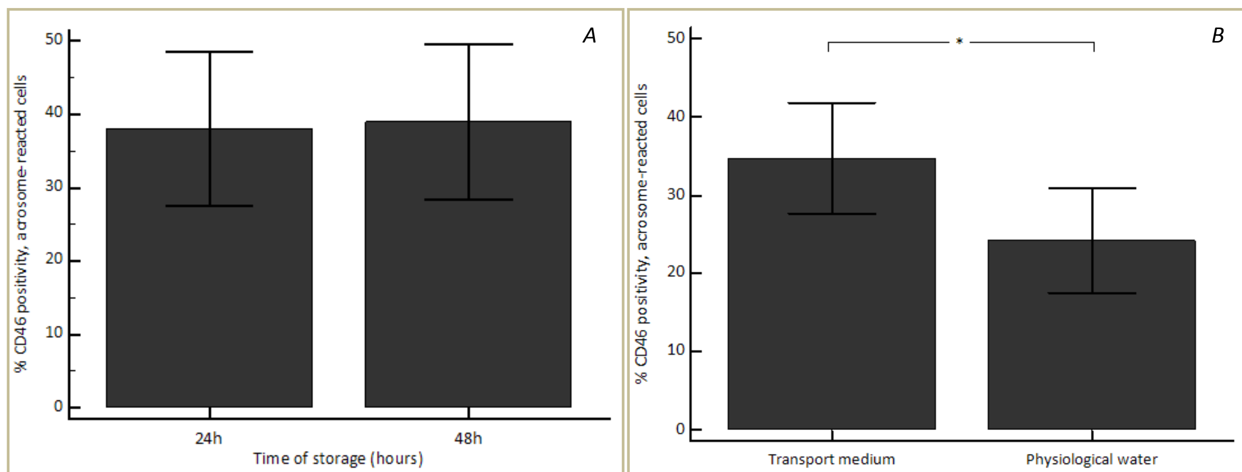

Figure 11: The effect of sperm storage time and medium on the percentage acrosome-reacted spermatozoa. Data are represented as mean value with standard deviation. *A.* The percentage of acrosome-reacted cells at 24 hours and 48 hours after ejaculation was evaluated using α-CD46-FITC and flow cytometry. CD46 positivity indicates the occurrence of the acrosome reaction. *B.* Comparison of the level of the acrosome reaction between spermatozoa, stored in transport medium and in physiological water, was performed through α-CD46-FITC staining at 24 hours after ejaculation. Flow cytometric analyses occurred via FACScanto II. FITC: fluorescein isothiocyanate. * Significantly different at $p<0.05$.

3.7 Magnetic-activated cell sorting-annexin V treatment decreases the level of DNA fragmentation within a sperm sample, but does not affect the protamination, acrosomal and viability states of spermatozoa

The efficiency of MACS-annexin V treatment to recover spermatozoa with normal protamine content and a reduced level of DNA fragmentation was determined via the CMA3 and AO fluorescent dyes, respectively. The acrosome reaction in unprocessed and prepared samples was compared using the α-CD46-FITC conjugate. In addition, the effect of cell sorting on the viability status of spermatozoa was evaluated via the combination of the annexin V-APC conjugate, PI and the α-p53-Alexa Fluor 488 conjugate.

Comparison of the mean values of CMA3 positivity before and after treatment shows that MACS-annexin V treatment does not affect the protamination status of spermatozoa (37.72 ± 9.36 vs. 37.73 ± 13.23, p=0.9983; **Table 5**). However, the level of DNA fragmentation within a sperm sample is significantly reduced after cell sorting (23.61 ± 12.08 vs. 18.18 ± 9.72, p=0.0034; **Table 5**). Since both PS surface expression and DNA fragmentation are characteristics of apoptosis, it was expected that a reduced level of DNA fragmentation would result from this treatment. To our knowledge, no other research group evaluated the efficiency of MACS-annexin V treatment to recover spermatozoa with normal protamine content and a reduced level of DNA fragmentation.

Compared to the raw sample, the percentage of acrosome-reacted cells remains unchanged after MACS-annexin V treatment (32.27 ± 15.82 vs. 29.31 ± 15.28, p=0.0608, n=14; **Table 5**, **Fig. 12A,B**). In addition, treatment results in a slight, but non-significant, decrease of the heterogeneous population of spermatozoa expressing PS (annexin V^+ PI^- cells, 3.26 ± 2.47 vs. 2.10 ± 1.52, p=0.0658, n=14; **Table 5**, **Fig. 12C,D**). Also, the percentage of annexin V^+ $p53^+$ cells remains unchanged after MACS-annexin V treatment (0.36 ± 0.77 vs. 0.37 ± 077, p=0.8438, n=14; **Table 5**, **Fig. 12C,D**), as well as the percentage of annexin V^+ PI^+ sperm cells (15.68 ± 15.03 vs. 13.94 ± 13.54, p=0.3769, n=14; **Table 5**, **Fig. 12C,D**). These results contradict the observations of the research group of de Vantéry Arrighi et al. (2009), which prove that MACS-annexin V treatment significantly decreases the total amount of annexin V-positive cells,

compared to the unprocessed sample (13.1 ± 2.0 vs. 6.7 ± 1.2, $p<0.05$)[26]. Analysis of annexin V positivity in the raw sample and after cell sorting in the current study cannot reproduce this result (20.45 ± 16.33 vs. 16.67 ± 14.27, p=0.1047, n=13; **Table 5**). The contradicting observations might be due to an insufficient concentration of MACS annexin V microbeads (20 µl microbeads per 10^7 total cells) and, consequently, an ineffective separation of the PS-positive and PS-negative fraction. Therefore, the concentration of MACS annexin V microbeads was adjusted to 100 µl microbeads per 10^7 total cells. However, the adjusted separation protocol does not result in a dramatic improvement when analyzing six sperm samples. Compared to the raw sample, the percentages of CD46-positive, annexin V^+ PI^-, annexin V^+ $p53^+$ and annexin V^+ PI^+ cells remain unchanged (46.58 ± 17.34 vs. 55.95 ± 25.43, 0.58 ± 0.48 vs. 2.28 ± 2.51, 1.30 ± 2.60 vs. 0.25 ± 0.44, 10.00 ± 11.72 vs. 9.23 ± 10.67, respectively). Due to lack of improvement, this experiment was interrupted. In addition, the contradicting results might be explained by the fact that flow cytometry does not take into account fluorescence background signals in the spermatozoa gate, generated by debris particles present in semen. Moreover, DGC, performed prior to the MACS-annexin V treatment by the research group of de Vantéry Arrighi et al. (2009)[26], reduces to a large extent the amount of debris in the sperm sample. **Figure 13** shows a flow cytometric analysis of α-CD46, annexin V-, PI- and α-p53-fluorescence staining, performed on a sperm sample of a vasectomized patient, containing debris but no spermatozoa. This figure demonstrates background of debris in the spermatozoa gate (approximately 10 % of all debris), which accounts for 15.6 % CD46 positivity, 1.9 % annexin V positivity, 1.5 % PI positivity and 0.4% p53 positivity.

Table 5: The protamination, acrosomal and viability states, and the level of DNA fragmentation in unprocessed sperm samples and after MACS-annexin V separation.

Test	Raw sample	MACS	P-value
% CMA3 positivity	**37.72** (± 9.36; 23-57)	**37.46** (± 13.23; 19.5-64.33)	0.8596
% DNA fragmentation	**23.61** (± 12.08; 6.75-58)	**18.18** (± 9.72; 5.5-25.5)	0.0034
% acrosome-reacted cells	**32.27** (± 15.82; 8.4-67.9)	**29.31** (± 14.80; 8.9-62.5)	0.0608
% annexin V^+ PI^- cells	**3.26** (± 2.47; 0.4-8.4)	**2.10** (± 1.52; 0.3-5.5)	0.0658
% annexin V^+ $p53^+$ cells	**0.36** (± 0.77; 0-3)	**0.37** (± 0.77; 0-3)	0.8438
% annexin V^+ PI^+ cells	**15.68** (± 15.03; 0.6-46.4)	**13.94** (± 13.54; 0.4-41.4)	0.3769
% annexin V^+ cells	**20.45** (± 16.33; 1-48.6)	**16.67** (± 14.27; 0.4-42.8)	0.1047

Mean values are shown with in between brackets the standard deviation, the minimum value and the maximum value, respectively. The percentage of poorly protaminated (CMA3-positive) cells and the level of DNA fragmentation were determined via fluorescence microscopic evaluation of the CMA3 staining and the AO staining, respectively. The percentage acrosome-reacted sperm cells was compared by α-CD46-FITC staining and flow cytometry. Via the combination of annexin V-APC, PI and α-p53-Alexa Fluor 488 staining, the viability status was evaluated through flow cytometry. Flow cytometric analyses occurred via FACScanto II. DNA: deoxyribonucleic acid, MACS: magnetic-activated cell sorting, CMA3: chromomycin A3, PI: propidium iodide, AO: acridin orange, FITC: fluorescein isothiocyanate, APC: Allophycocyanin.

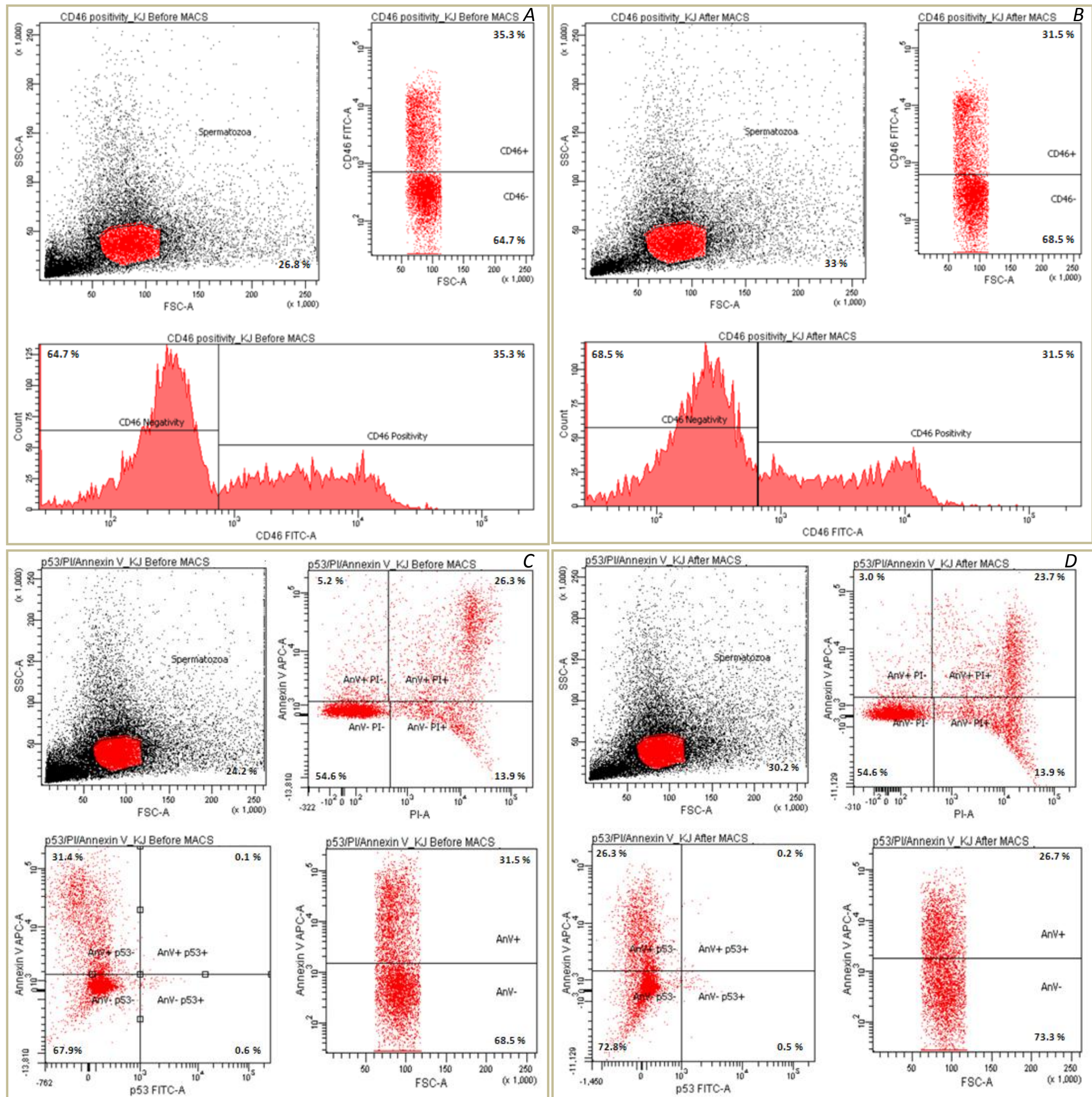

Figure 12: The effect of MACS-annexin V treatment on the percentage acrosome-reacted spermatozoa and the viability status of sperm cells. *A,B*. Flow cytometric analysis (FACScanto II) of the level of acrosome reaction through α-CD46-FITC staining before (A) and after (B) cell sorting. *C,D.* Analysis of the viability status using the combination of annexin V-APC, PI and α-p53-Alexa Fluor 488 staining before (C) and after (D) MACS-annexin V treatment. MACS: magnetic-activated cell sorting, AnV: annexin V, FITC: fluorescein isothiocyanate, APC: allophycocyanin, PI: propidium iodide.

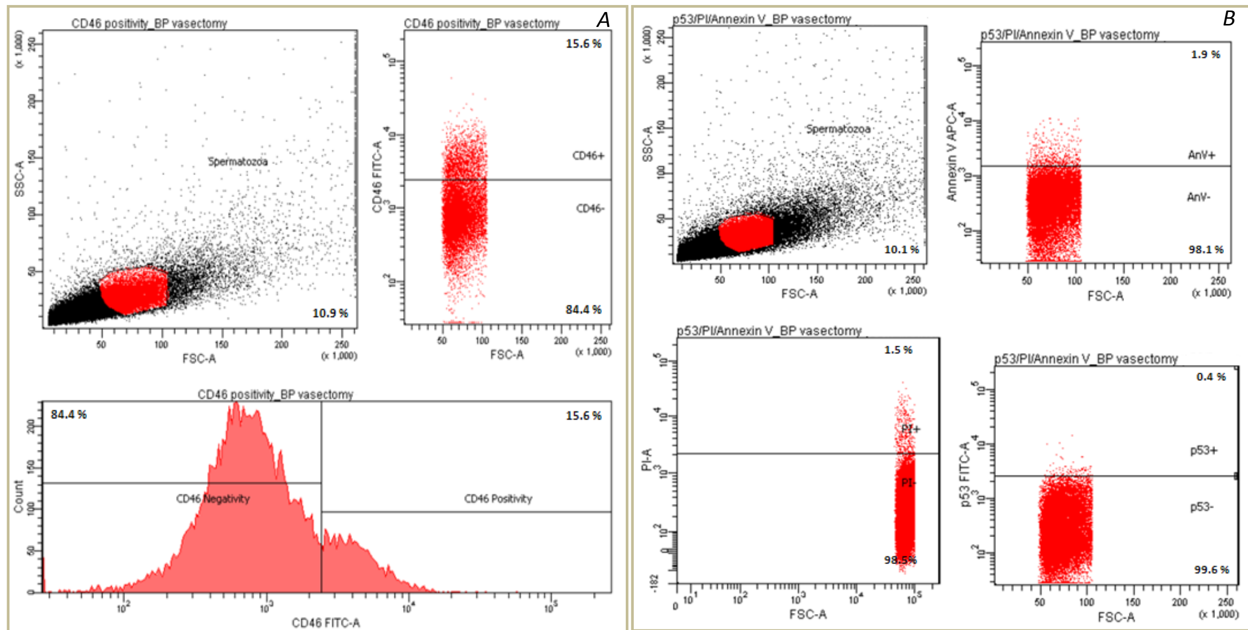

Figure 13: Flow cytometric analysis of α-CD46, annexin V, PI and α-p53 fluorecence staining, performed on a sperm sample of a vasectomized patient, containing debris but no spermatozoa. Flow cytometric analysis occurred via FACScanto II. AnV: Annexin V, PI: propidium iodide.

3.8 Phosphatidylserine-expression is partially associated with the acrosome reaction

The theory that PS surface expression is partially associated with the physiological processes of capacitation and/or the acrosome reaction was evaluated via staining of raw samples with the α-CD46-FITC and annexin V-APC conjugates.

The percentage of acrosome-reacted cells varies from 8.4 % as the minimum value and 70 % as the maximum value. The percentage of $CD46^+$ annexin V^+ spermatozoa varies from 1.6 % to 15.2 % as the minimum and maximum values, respectively. In addition, this experiment shows that 20.66 % of the CD46-positive spermatozoa also express PS (n=10; **Table 6**, **Fig. 14**) and, consequently, the two phenomenon's are partially associated with one another.

Table 6: The percentage of CD46-positive and annexin V-positive spermatozoa on the total population of acrosome-reacted sperm cells.

CD46-positive cells	CD46-positive, annexin V-positive cells	% annexin V-positive cells on total amount of CD46-positive cells
31.22 (± 19.02; 8.4-70)	**6.45** (± 4.72; 1.6-15.2)	20.66

Mean values are shown with in between brackets the standard deviation, the minimum value and the maximum value, respectively. The theory that PS surface expression is partially associated with the physiological processes of capacitation and/or the acrosome reaction was evaluated using the α-CD46-FITC staining and annexin V-APC staining of raw sperm samples and flow cytometric analysis (FACScanto II). PS: phosphatidylserine, FITC: fluorescein isothiocyanate, APC: allophycocyanin.

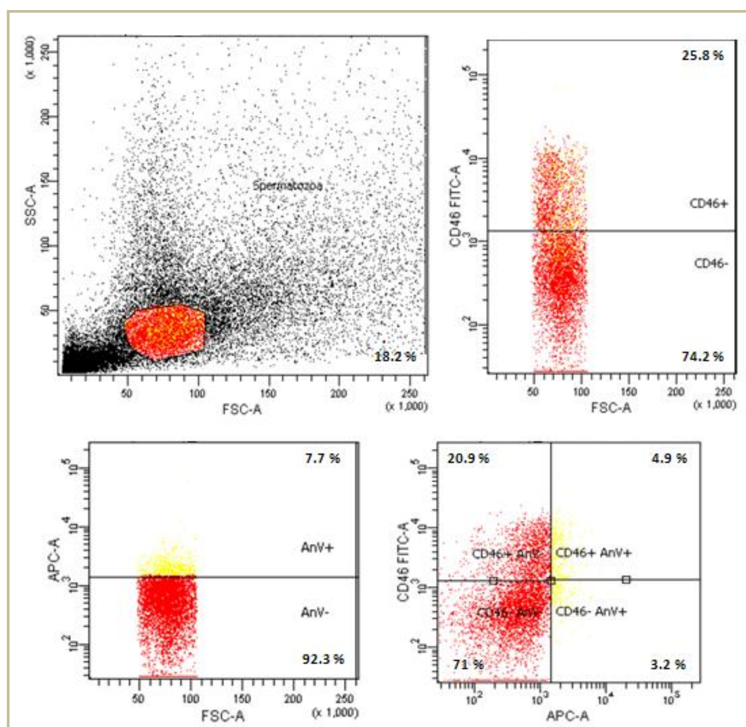

Figure 14: Flow cytometric analysis of the relationship between the acrosome reaction and PS surface expression through the combination of the α-CD46-FITC staining and annexin V-APC staining. Flow cytometric analysis was performed via FACScanto II. AnV: annexin V, PS: phosphatidylserine, FITC: fluorescein isothiocyanate, APC: allophycocyanin.

4 Conclusion & synthesis

During this senior internship project, the storage temperature of sperm samples was optimized. The HOS test was evaluated as a potential selection step in IUI and IVF protocols. Different sperm parameters were studied to enhance our understanding of male subfertility and the current suboptimal success rates of IUI and IVF treatments, namely the protamination status, the level of DNA fragmentation, the acrosome reaction and the viability status. Moreover, a method based on MACS was performed to remove sperm cells expressing PS. This method was evaluated in terms of the resulting sperm quality.

In conclusion, storage of the sperm samples at room temperature significantly improves survival of spermatozoa, due to a temporal decrease in motility and, therefore, the energy consumption whereby the depletion of the energy supply is delayed. When incubated at 37°C, the motility of resting spermatozoa can be restored. Nowadays, fertility laboratories routinely store sperm samples at 37°C, which may result in a faster decrease in the quality of stored spermatozoa, prior to their use during IUI and IVF.

HOS and reversal of the tail swelling provides an excessively aggressive stress situation for the spermatozoa to recover their motility. By applying this treatment, the motility of the original preparation is only partially recovered. Consequently, the HOS test is not a suitable selection step for IUI and IVF protocols, due to practical considerations regarding the number of progressively motile spermatozoa required after sperm preparation.

DGC selects morphologically normal sperm cells with a good protamination status and reduces the amount of DNA fragmentation and the percentage of PS-expressing viable cells within sperm samples. However, this sperm preparation technique increases the percentage of acrosome-reacted cells. These spermatozoa are incapable of fertilizing an oocyte during the insemination protocols because they are off-schedule for fertilization. However, the acrosomal status is not routinely evaluated in fertility laboratories. This unwanted selection of acrosome-reacted sperm cells may be partially responsible for unexplained pregnancy failures since the conventional sperm quality parameters do not entirely reflect the real fertilizing potential of the sperm cells, prior to their use in IUI and IVF. Moreover, the high basal level of acrosome-reacted cells in the raw sample (24.18 % when spermatozoa are stored in physiological water) is surprising and could be an important new characteristic of male subfertility. Lack of information concerning the acrosomal status of an individual sperm sample might be responsible for the current suboptimal success rates of the subfertility treatments, IUI and IVF. When a high percentage of acrosome-reacted cells is detected in the sample, ICSI can be performed with the best quality spermatozoon. The correlation of the zona pellucida-induced acrosome reaction with sperm morphology, sperm-zona pellucida binding and IVF strengthens the need for more extensive exploration of the acrosomal status of sperm before its use in IUI and IVF[22]. Furthermore, flow cytometric evaluation of CD46 positivity proves to be the most reliable acrosomal staining method.

In addition, an increased amount of acrosome-reacted cells can be observed when spermatozoa are stored in transport medium, compared to storage in physiological water. To identify Solugel as a potential acrosomal stimulator, media with and without Solugel can be compared regarding the percentage acrosome-reacted cells after overnight incubation at room temperature. Moreover, the

sequences of ZP3 and gelatin, the source protein of the Solugel hydrolysate, can be compared to evaluate the hypothesis that Solugel and ZP 3 show homology. Furthermore, Solugel might be able to facilitate the self-aggregation of sperm receptors and, therefore, the acrosome reaction. A potential interaction might be visualized by fluorescent labeling of Solugel and spermatozoa differentially. Finally, the effect of energy carriers (e.g. pyruvate) within the transport medium on the acrosome reaction can be evaluated through comparison of physiological water with and without the energy carrier(s).

MACS-annexin V treatment does not significantly affect the percentage of PS-expressing sperm cells. Moreover, the protamination and acrosomal states of spermatozoa remain unchanged after MACS-annexin V separation. Although a slight, but non-significant, decrease of annexin V-positive cells is obtained after separation, the level of DNA fragmentation is significantly reduced. Altogether, DGC proves to be a more effective separation method to reduce the percentage of spermatozoa with poorly protaminated DNA, the level of DNA fragmentation and the percentage of the heterogeneous population of PS-expressing cells compared to MACS-annexin V.

The hypothesis stated that male subfertility is multifactorial and that a conventional sperm quality analysis does not provide sufficient information to explore the fertility problem. This study illustrates that the acrosomal status of spermatozoa contributes significantly to sperm quality and the fertility potential of a sperm sample. Furthermore, the effect of temperature on spermatozoa should be taken into consideration when storing sperm samples in fertility laboratories and research institutes.

In the future, the effect of temperature on the survival of spermatozoa can be studied more elaborate. Optimization of the storage temperature should be established through the evaluation of different temperatures in a more controlled manner with the use of special containers. Moreover, survival should be evaluated through other methods in addition to the evaluation of motility. Further optimization of the MACS-annexin V columns is necessary for this separation technique to result in a significant decrease in PS-expressing cells. To verify that the high percentage of acrosome-reacted cells in sperm samples is a true characteristic of male subfertility, CD46 positivity should be compared between fertile and subfertile/infertile men. Additionally, a threshold could be set regarding the percentage of acrosome-reacted sperm cells within a sample of a male patient. ICSI might be recommended when the threshold is surpassed. Also, CD46 staining is the most interesting acrosomal staining method, given that negative selection of CD46-positive spermatozoa via MACS columns might be possible to remove acrosome-reacted cells from a sample. Moreover, α-CD46 will only stain and therefore select the completely acrosome-reacted cells[31]. Consequently, when performing MACS-α-CD46 selection, the partially acrosome-reacted cells are not removed from the sample and can still contribute to the fertilization of an oocyte in IUI and IVF protocols. If proven successful, the MACS-α-CD46 columns should be studied with regard to several parameters in addition to the acrosome reaction, namely the conventional sperm parameters (count, motility and morphology), DNA fragmentation, protamination, the viability status, aneuploidy, etc. In final, the efficiency of the swim up preparation technique to select acrosome-unreacted sperm cells must be evaluated and compared to DGC preparation to identify the most suitable preparation method regarding this parameter.

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Supplement

Supplement 1: Strict criteria concerning normal and abnormal morphology

For a spermatozoon to be considered normal, both the head and the tail must be normal. All borderline forms should be considered abnormal. The sperm head must be oval in shape with smooth contours. A head, which is slightly smaller, tapered and/or narrower is considered normal. A well-defined acrosomal region should be present, comprising 40-70 % of the head area. The head should not contain large vacuoles or more than two small vacuoles. Neck and mid-piece regions should not have abnormalities and a cytoplasmic droplet, if present, must not be larger than half the size of the sperm head. The tail must not be coiled or bent and should not possess a droplet at the end.

The morphological defects are usually mixed. The following categories should be noted:

- Head defects: large or small, tapered, one sided oval, irregularity, pyriform, round, flat at tail implantation side, vacuolated (more than two vacuoles or >20 % of the head area occupied by unstained vacuolar areas), small or large acrosomal areas, double heads.
- Tail defects: asymmetrical insertion of the midpiece into the head, midpiece detached from sperm head, thick midpiece, sharply bent, coiled, multiple.
- Cytoplasmic droplet

Supplement 2: Chromomycin A3 staining solution

The CMA3 stock solution was dissolved in McIlvaine buffer. Briefly, 18.2 ml of a 0.1 M citric acid solution (Sigma-Aldrich) and 81.8 ml of a 0.2 M Na_2HPO_4 solution (Merck) were combined to obtain 100 ml of the McIlvaine buffer (pH 7.0). Furthermore, 10 mM $MgCl_2$ (VWR) was dissolved in this buffer. 5 mg CMA3 (AG scientific), dissolved in methanol, was added to 20 ml of McIlvaine buffer. This volume of the finale work solution (0.25 mg/ml) was stored at -20°C until use.

Supplement 3: Widefield microscopy: Overview of the fluorescent channels

Table 6: Overview of the fluorescent channels, which were used during widefield microscopy.

Fluorescent dye, fluorochrome	Excitation filter (nm)	Emission filter (nm)
CMA3 staining	440/10	535/40
AO staining	485/20	535/40 and 607/36
APC	650/13	684/24
FITC, Alexa Fluor 488	485/20	525/30
PI	540/25	607/36

A fluorescent channel represents the combination of an excitation filter and an emission filter. CMA3: chromomycin A3, AO: acridin orange, APC: allophycocyanin, FITC: fluorescein isothiocyanate, PI: propidium iodide.

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