Potential alternative applications of magnetic activated cell sorting in assisted reproduction

Doctoral (PhD) thesis

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LIST OF ABBREVIATIONS

ART:	assisted reproductive techniques
BPA:	bisphenol A
BSA:	bovine serum albumin
CASA:	computer assisted sperm analysis
CD:	Cluster of Differentiation
DFI:	DNA-fragmentation index
DGC:	density gradient centrifugation
EDTA:	ethylenediaminetetraacetic acid
ESHRE:	Europeran Society of Human Reproduction and Embryology
FITC:	fluorescein isothiocyanate
GPA:	glycophorin A
HBA:	hyaluronic acid binding assay
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOST:	hypo-osmotic swelling test
ICSI:	intracytoplasmic sperm injection
IL-6:	interleukin-6
IL-8:	interleukin-8
IMSI:	intracytoplasmic morphologically selected sperm injection
IUI:	intrauterine insemination
IVF:	in vitro fertilisation
LAISS:	laser-assisted immotile sperm selection
LBR:	live birth rate
MACS:	magnetic activated cell sorting
MALDI:	matrix-assisted laser desorption/ionization
MDA:	malondialdehyd
MSOME:	motile sperm organelle morphology examination
PBS:	phosphate-buffered saline
PICSI:	physiological intracytoplasmic sperm injection
PMN:	polimorphonuclear
PS:	phosphatidyl-serin
RCT:	randomised controlled trial
ROS:	reactive oxygen species
SCD:	sperm chromatin dispersion
SU:	swim up test
TESE:	testicular sperm extraction
TNF-α:	tumor necrosis factor-α
TUNEL:	terminal deoxynucleotidyl transferase dUTP nick end labeling
WHO:	World Health Organisation
ZEA:	zearalenone

1. INTRODUCTION

1.1. The epidemiology and etiology of infertility

Since the mid-20th century, a decline in human fertility has been observed, which, according to data from the *World Health Organization (WHO*), affects one in six people globally (Cox et al., 2022). The *total fertility rate* is defined as the number of live births per woman over her lifetime, without considering mortality. Despite the typically high birth rates in developing countries, a global decline in fertility has been noted as a consequence of the fertility crisis affecting the human species. Approximately two-thirds of the world's population live in areas (mainly in developed countries) where the fertility rate has fallen below the critical replacement level of 2.1, posing a long-term threat to the local population's survival. This may also be influenced by the worldwide deterioration in male sperm parameters (volume, total sperm count, concentration, motility, morphology) (Luo et al., 2023; Pakmanesh et al., 2024; Garcia-Grau et al., 2022; Li et al., 2023), which has been observed since the second half of the 20th century. According to a meta-analysis conducted by Levine et al. in 2017, a 50-60% decrease in sperm count was measured between 1973 and 2011 among the male populations of North America, Europe, and Australia.

The underlying causes are diverse, among which various civilization-related harms are certainly noteworthy: the effects stemming from a Western lifestyle (sedentary lifestyle, reduced physical activity, consequent obesity, smoking, alcohol consumption, delayed childbearing for both genders, psychological distress, etc.) and environmental harms linked to industrialization and globalization (extensive use of fossil fuels and their derivatives, such as plastics, smog, etc.) (Skakkebæk et al., 2022). Of particular importance in relation to the latter are the so-called *endocrine disruptors*. These are chemical agents (pesticides, plastics (e.g., bisphenol A (BPA)), phytoestrogens (e.g., in soybeans), mycotoxins (e.g., zearalenone (ZEA)), etc.) that, when entering the body, act as *xenoestrogens*, exerting estrogenic and antiandrogenic effects that disrupt the homeostasis of hormonal regulation. They may also act as oxidative stressors, thus impairing fertility in both genders (Czarnywojtek et al., 2021).

Infertility is defined as the inability of a couple to achieve a spontaneous pregnancy after at least one year of regular intercourse without contraception. For these couples, *assisted reproductive technologies (ART)* have provided an opportunity to have their own biological offspring since 1978, the year of the birth of the first "test-tube baby" (Kamel et al., 2013). Despite decades of development and worldwide application, their efficiency remains relatively

low: in 2021, only 37.3% of cycles in the USA resulted in a live birth. In approximately 30-50% of cases, a male factor is involved (Winters and Walsh, 2014), the investigation and treatment of which fall within the scope of andrology.

1.2. Sperm selection

In assisted reproduction, particularly during intracytoplasmic sperm injection (ICSI), the natural selection mechanisms that act on sperm within the female genital tract are bypassed. These selection mechanisms include the acidic pH of the vagina, changes in cervical mucus consistency, cellular immune response, sperm-epithelium interaction in the isthmus of the fallopian tube, and sperm-zona pellucida interaction (Suarez and Pacey, 2006; Sánchez-Calabuig et al., 2014). As a result, a subpopulation of sperm with the highest fertilization potential is naturally selected. Theoretically, the artificial selection and subsequent use of these sperm could improve the success rates of ART. Several sperm selection techniques have been developed for this purpose, but so far, none have proven to be more effective than the others in terms of clinical outcomes, particularly regarding the live birth rate (LBR) (Vaughan and Sakkas, 2019; Baldini et al., 2021).

Conventional sperm selection techniques include the swim-up (SU) test and density gradient centrifugation (DGC), which are simple to perform, do not require special knowledge or equipment, and are therefore commonly used in fertility centers for semen sample preparation. However, in specific cases, such as with extremely low cell counts (severe oligozoospermia, cryptozoospermia) or high viscosity, these conventional methods are unfortunately ineffective. In such instances, *advanced* sperm selection procedures can be applied.

The advanced techniques include: zeta potential-based selection, motile sperm organelle morphology examination (MSOME→intracytoplasmic morphologically selected sperm injection (IMSI)), hyaluronan binding assay (HBA→physiological intracytoplasmic sperm injection (PICSI)), magnetic activated cell sorting (MACS), microfluidics, hypo-osmotic swelling test (HOST), laser-assisted selection of immotile sperm (LAISS), polarization microscopy-based selection, etc. (Pinto et al., 2021). Detailing the functioning of all these methods exceeds the scope of this writing; therefore, given the focus of this dissertation, the basics of the MACS technique will be discussed in the followings.

1.2.1. Magnetic activated cell sorting (MACS)

During magnetic cell separation, various separation strategies exist, with the most common techniques selecting cells based on specific surface markers that are conjugated to special ligands. These ligands are mostly antibodies but can also include synthetic molecules such as peptides or aptamers. The advantages of MACS include its ease of use (it does not require specialized expertise or highly trained operators), the precise adjustability of the magnetic field strength, its optimization for specific target cells, its integration into complex separation systems, and its high selectivity. The main challenge in using this method is separating target cells from the microbeads without damaging their viability or function. This can be achieved in numerous ways (saturated protein solution, enzymes, changes in temperature/pH/electrical charge/light, aptamers, hydrogel, etc.) (Plouffe et al., 2015). The simplest approach would be to eliminate all unwanted cells, allowing only the target cells to be collected at the bottom of the separation column (*negative selection*). However, due to the diversity of contaminating cells in biological samples, this approach can present significant challenges.

The method was first applied in reproductive medicine by Grunewald et al. in 2001 to eliminate apoptotic sperm from cryopreserved and subsequently thawed semen samples. During apoptosis, a series of molecular changes occur both in the cell membrane and intracellularly. One important aspect of this process is the externalization of phosphatidylserine (PS), a phospholipid molecule that is normally located on the inner surface of the cell membrane (Fadok et al., 1992). Unfortunately, sperm carrying early apoptotic signals can function without significant damage to motility or morphological alterations, allowing them to fertilize the oocyte while potentially avoiding the later stages of programmed cell death (Garrido and Gil Juliá, 2024).

In a study published by Hichri et al. in 2018, various apoptotic changes in sperm (such as the ratio of activated caspases, externalized PS and DNA-fragmentation) were shown to be independent predictors of ART outcomes, regardless of classical semen parameters. Among these, DNA-fragmentation exhibited the best predictive value. The original method of MACS is based on the high-affinity binding of PS to a protein called annexin V. Annexin V molecules are conjugated to the surface of magnetic microbeads, which are then incubated with the sperm cells for an appropriate duration to label them. Following this, the sample is loaded onto a separation column placed in a magnetic field. The apoptotic sperm bind to annexin V and remain on the column (*positive fraction*), while healthy sperm pass through without binding, allowing their separation and extraction (*negative fraction*) (Said et al., 2006).

Numerous studies have previously demonstrated that MACS effectively reduces DNA-fragmentation and the occurrence of structural chromosomal abnormalities in treated semen samples, thereby allowing for the isolation of sperm with higher DNA integrity, which are of higher quality for assisted reproductive methods (Said et al., 2006; Aziz et al., 2007; de Vantéry Arrighi et al., 2009; Lee et al., 2010; Zahedi et al., 2013; Bucar et al., 2015; Degheidy et al., 2015; Chi et al., 2016; Martínez et al., 2018; El Fekih et al., 2020). Despite this, the actual positive clinical impact of the technique on ART outcomes has not yet been proven in large-scale randomized controlled trials (RCT), and the current European assisted reproduction guidelines (*European Society of Human Reproduction and Embryology (ESHRE)*) do not recommend its use at this time (Lundin et al., 2023).

Although high-level evidence and professional recommendations supporting the application of the method are lacking, it is often suggested in fertility centers for couples who have experienced two unsuccessful ICSI attempts or unexplained miscarriages alongside high sperm DNA-fragmentation. This recommendation should always follow an individual assessment, along with thorough information and consultation (Garrido and Gil Juliá, 2024).

1.3. Absolute asthenozoospermia

Physiologically, the maturation of sperm occurs during their transit through the epididymal ducts, during which sperm acquire the ability to undergo capacitation and gain progressive motility (Gervasi and Visconti, 2017). This latter capability is particularly important for reaching the fallopian tube from the vagina and for penetrating the cumulus oophorus, establishing a clear connection between sperm motility and fertilization capacity (Beauchamp et al., 1984).

Absolute asthenozoospermia is a rare disorder (occurrence: 1 in 5000 men) characterized by a complete lack of motility in fresh ejaculate. Its causes include intrinsic factors, such as ultrastructural defects of the flagellum (e.g., Kartagener syndrome), mitochondrial disorders, and extrinsic factors, including necrozoospermia as a consequence of infections, oxidative stress, anti-sperm antibodies, environmental exposure to pollutants (e.g., pesticides), cryopreservation, and prolonged abstinence. In many cases, however, it is considered an idiopathic condition (Ortega et al., 2011; Chen et al., 2022). On the other hand, the immotility of testicular sperm is a physiological phenomenon resulting from their metabolic immaturity and potential residual connections with Sertoli cells.

In 1995, Nagy et al. conducted a retrospective analysis of 966 cases, demonstrating that in cases of ICSI, even patients with severely compromised classical sperm parameters (e.g., cryptozoospermia, severe astheno-/teratozoospermia) could achieve high fertilization and pregnancy rates. From the male side, the only factor showing a strong negative predictive value was the fertilization using immotile (presumably non-viable) sperm.

While sperm *vitality* refers to the proportion of live sperm indirectly assessed based on intact cell membranes, *viability* is a more sophisticated concept that considers functional integrity (Hecht and Jeyendran, 2022). However, in laboratory practice, these terms are often used interchangeably as if they held identical meanings.

The vitality of immotile sperm can be easily assessed using simple eosin-nigrosin staining, which allows for the differentiation of samples with 100% immotile, living sperm from necrozoospermic samples. However, sperm visualized as viable through this method are unsuitable for further use in the process of assisted reproduction. Several methods are mentioned in the literature for determining viability while preserving sperm usability, such as examining the elasticity of the sperm tail, HOST, diode laser pulse application to the sperm tail (LAISS), the use of phosphodiesterase inhibitors (e.g., pentoxifylline, theophylline, 2-deoxyadenosine), and birefringence microscopy (Yovich et al., 1988; Liu et al., 1997; Soares et al., 2003; Aktan et al., 2004; Baccetti, 2004; Ortega et al., 2011; Ebner et al., 2014). However, these methods can be problematic due to issues of inaccuracy, invasiveness, and potential toxicity.

1.4. Leukocytospermia and its importance

An ideal semen sample for ART is free of contaminating cells (e.g., white blood cells, red blood cells, epithelial cells). However, white blood cells are physiologically present in the male genital system and the seminal tract, where they play important roles in various processes that contribute to maintaining homeostasis, such as phagocytosis of abnormal or senescent sperm by macrophages in the epididymis and pathogen elimination (*"immunosurveillance"*) (Khodamoradi et al., 2020; Henkel, 2024). In routine clinical practice, nearly all samples tested show some degree of contamination with white blood cells, primarily polymorphonuclear (PMN) cells (granulocytes, 50-60%) (Wolff, 1995). According to the current WHO guidelines (*WHO laboratory manual for the examination and processing of human semen, 6th ed., 2021*), only concentrations exceeding 1.0×10^6/ml are considered clinically significant and abnormal.

The prevalence of leukocytospermia in infertile men is estimated at approximately 10-20% (Wolff, 1995).

A review of the literature reveals numerous data supporting the potential relationship between leukocytospermia and reduced sperm parameters, consequently linking them to male subfertility or infertility. Infiltration by white blood cells is most commonly a result of infections in the seminal tract (such as orchitis, epididymitis, and prostatitis); however, it can also arise from other non-infectious conditions and factors that elevate cytokine levels, such as varicocele, smoking, and the consumption of marijuana and alcohol (Close et al., 1990; Mongioì et al., 2020).

PMN cells are activated by inflammatory cytokines, leading to the production of reactive oxygen species (ROS) during an *„oxidative burst"*, which subsequently results in oxidative stress (Saleh et al., 2002). If oxidative effects become predominant and exceed the capacity of the seminal plasma antioxidant enzyme system, lipid peroxidation may occur, leading to loss of membrane integrity. This can result in damage and fragmentation of both mitochondrial and nuclear DNA (Derbel et al., 2021), ultimately leading to sperm apoptosis. Clinically, all these changes manifest as a deterioration in classic semen parameters (volume, concentration, progressive/total motility) and impaired sperm-oocyte fusion (Aitken et al., 1994; Plante et al., 1994; Fraczek et al., 2016; Fan et al., 2023).

1.5. Importance of haemospermia and red blood cells contaminating testicular tissue samples

Haemospermia refers to the presence of blood in semen, which can occur as a result of various pathological processes, such as infections of the seminal tract, urogenital malignancies (including prostate, bladder, and testicular tumors), and prostatic/seminal vesicle cysts; however, in approximately 30-70% of cases, the process is considered idiopathic (Mathers et al., 2017). The testicular tissue sample obtained during testicular sperm extraction (TESE) not only contains spermatozoa but also their precursors and cells of other origin (white blood cells, red blood cells, epithelial cells).

In the aforementioned cases, the presence of red blood cells in the biological sample intended for assisted reproduction may have potentially negative effects on sperm. In samples containing only a few sperm, the abundance of red blood cells can make isolating them extremely challenging and time-consuming, particularly when the sperm cells are immotile, as in the case of testicular samples. To address this issue, several methods have been developed, such as

erythrocyte lysis buffer solutions and the use of pentoxifylline to stimulate motility; however, the safety of these methods remains questionable (Tournaye et al., 1994; Popal and Nagy, 2013; Soygur et al., 2018; Yazdinejad et al., 2020).

Cryopreservation of sperm for later use (e.g., before radical surgery or the initiation of oncotherapy in patients with testicular cancer) has become routine practice in fertility centers. A significant aspect in this context is the potentially harmful effects caused by the hemolysis of red blood cells, which may lead to combined oxidative and direct cytotoxic effects on thawed sperm due to the release of hem and iron molecules (Rijsselaere et al., 2004).

1.6. Cluster of differentiation

The basic concept of the *Cluster of Differentiation (CD)* molecular identification system were established in Paris in 1982 (Bernard and Boumsell, 1984). This system relies on *clusters* of similar cell surface antigens recognized by the same antibody, allowing for the differentiation of various cell populations based on their immunophenotype. To date, over 400 distinct proteins have been classified as CD markers (Tian et al., 2022).

1.6.1. CD45

The CD45 marker is also known as the *common leukocyte antigen*, as it is a genetically highly conserved membrane glycoprotein found on the surface of all mature nucleated hematopoietic cells, except mature red blood cells and platelets. It plays a key role in regulating immune responses (Rheinländer et al., 2018).

1.6.2. CD235a

CD235a denotes a transmembrane glycoprotein called *glycophorin A (GPA)*, which is present on red blood cells and their precursors. It is known for its structural role in forming the shape of red blood cells (Karsten et al., 2010). Additionally, it participates in pathological processes by serving as a receptor for various pathogens, including *Plasmodium falciparum*, the protozoon responsible for malaria (Ridgwell et al., 1983).

2. OBJECTIVES

In addition to the original annexin V-based separation method, the MACS technique holds several possibilities: antibodies targeting any desired molecule can be attached to the surface of the applied microbeads (even multiple types simultaneously). Theoretically, this allows for the superselection of sperm with high integrity genetic material and high fertilization potential based on their characteristic cell surface markers. Furthermore, it could enable the extraction of contaminating cells in the biological sample that complicate processing, such as leukocytes and red blood cells present in semen or testicular tissue samples obtained during surgical sperm retrieval.

In the first part of my doctoral research, I investigated a specific application of the original method, while in the second part, I focused on the possibilities of the extraction of contaminating cells. We examined the following questions:

1. Can the DNA-fragmentation index (DFI) of semen samples exhibiting elevated DNA-fragmentation be significantly reduced using annexin V-based MACS? (This positive effect has been described in several previous studies, which we aimed to verify.)

2. Examination of the correlation between sperm vitality and DFI. Does a decrease in DFI manifest as increased vitality, thus can vitality be significantly improved in cases of absolute asthenozoospermia using annexin V-based MACS? (Due to the rare occurrence of this condition, this has been investigated through a case study, in a retrospective manner.)

3. Can the proportion of contaminating white blood cells be significantly reduced in leukocytospermic semen samples through magnetic separation targeting the CD45 molecule?

4. Can the proportion of contaminating red blood cells be significantly reduced in testicular tissue samples obtained during TESE through magnetic separation targeting the CD235a molecule?

3. MATERIALS AND METHODS

3.1. Sample collections and preliminary investigations

Semen samples were collected through masturbation following a three-day period of sexual abstinence. After incubation at 37°C and liquefaction, the semen analysis was conducted within one hour using CASA system (SCA SCOPE, manufacturer: Microptic S.L., Barcelona, Spain) and flow cytometer (MACSQuant Analyzer 16, manufacturer: Miltenyi Biotec, Bergisch Gladbach, Germany). In our daily clinical practice, every male patient presenting for andrological evaluation undergoes sperm analysis based on classical semen parameters (sperm count, concentration, motility, progressive motility, morphology, vitality, and the number of round and peroxidase-positive cells), as well as DNA-fragmentation assessment.

In the routine examination of 17 patients, semen samples with a high DFI (>30%) were revealed, so we performed annexin V labeling and magnetic separation for scientific purposes.

In 13 patients, leukocytospermia was detected during routine examination, and we also performed magnetic separation in these cases for research purposes.

For 12 patients, testicular sperm extraction (TESE) was performed as a part of infertility treatment. Following the mechanical processing of the testicular tissue sample, density gradient centrifugation was carried out to remove unprocessed tissue scraps. After centrifugation, the sediment obtained was used for further magnetic separation. Inclusion criteria were confirmed sperm hit in the biopsy and signed research consent form.

In a retrospective analysis of data from an additional 205 patients undergoing routine examinations, we established correlations between vitality and DNA-fragmentation. During the analysis of the 205 samples, absolute asthenozoospermia was observed in one case, we perfomed annexin V labeling and magnetic separation of this sample for scientific purposes.

In all of the above cases, sample collection was carried out on a voluntary basis with a signed research consent form at the Dunamenti REK IVF Centre's Pannon Facility (formerly the Pannon Reproduction Institute, Tapolca) and at the Urology Clinic of the University of Pécs (Pécs). Our studies were conducted with prior approval from the Regional Research Ethics Committee of the University of Pécs.

3.2. Magnetic separation of apoptotic sperm cells

After the determination of cell count, DGC of the liquified semen sample was carried out at 300×*g* for 12 min. For DGC, sperm samples were processed using 40% and 80% PureCeption^{TM} (PureCeption^{TM} is a sterile colloidal suspension of silica particles stabilized with covalently bound hydrophilic silane formulated in HEPES-buffered human tubal fluid) gradient layers. Briefly, 1 mL of the lower phase gradient (80%) was moved into a conical bottom tube. A second 1 mL layer of upper phase (40%) was then slowly placed over the lower phase. PureCeption^{TM} was diluted with sperm preparation medium. A proper volume of liquified semen was gently placed over of the upper phase. The prepared tube was then centrifuged as described above. The supernatant was discarded, cells were resuspended in MACS ART binding buffer and centrifugated at 300×*g* for 4 min. The supernatant was discarded, 200 µL of MACS ART annexin V reagent was added to cells and the final volume was completed to 500 µL with the MACS ART binding buffer. This solution was incubated for 15 min at room temperature. A MACS ART MS separator column was rinsed with MACS ART binding buffer and the labeled cells were loaded onto the column. Unlabeled cells that passed through were collected. Sperm DNA-fragmentation measurement with TUNEL assay was performed before and after separations based on the method described by Sharma et al. in 2016. DFI was calculated by dividing the TUNEL-positive sperm count by the total number of sperm cells.

In the case of absolute asthenozoospermia, sperm vitality was assessed after eosin staining, DNA-fragmentation was assessed using the sperm chromatin dispersion (SCD) (Fernández et al. 2003) following the manufacturer's instructions and the WHO guidelines. Otherwise, the sample collection and the basic examinations were carried out as detailed above. To asses sperm vitality, one drop of semen sample (5 µL) was mixed with equal volume of 1% eosin-Y solution, a smear was made on a glass side, covered with a coverslip, incubated for 30 s at 37°C, and 200 spermatozoa were evaluated. For the SCD test, semen was diluted with phosphate buffer saline to 5–10 million mL^{-1}. A 30 µL sample was mixed with 1% agarose at 37°C. A total of 14 µL of the above mixture was pipetted onto a prepared agarose glass slide and covered with a glass coverslip (18 × 18 mm). The slide was cooled for 5 min at 4°C. The coverslip was removed and the glass slide was immersed horizontally into a denaturation solution (0.08 M HCl) for 7 min at room temperature. After this, the slide was transferred to a lysis buffer (0.4 M Tris, 0.4 M DTT, 50 mM EDTA, 0.3% SDS and 1% Triton Xysis) and incubated for 25 min at room temperature. The slide was thoroughly washed with distilled water, dehydrated for 2 min in each of 70, 90 and 100% ethanol and subsequently air dried. Then, the slide was stained with

Giemsa and analyzed by an SCA Scope system. During the analysis, so-called „*halos*" around the head of sperm cells were observed. Healthy cells show extended or moderate halos, while sperms with fragmented DNA show small or no halos (the phenomenon is based on the light dispersion of intact DNA loops present in the denatured, thus deproteinized nucleoid). A minimum of 200 spermatozoa per sample were evaluated.

The annexin V-based MACS separation was performed identically to the aforementioned method, the assessment of vitality and DFI was carried out both before and after the separation. The DFI was calculated by dividing the number of cells with fragmented DNA by the total cell count. For the DFI threshold, we applied the commonly accepted 30% cut-off for the SCD test.

3.3. Magnetic separations of leukocytes

Separations were carried out based on the recommendations of the manufacturer. Namely, semen samples were centrifuged at 300×*g* for 10 min, the plasma were discarded and the cell pellets were resuspended in 80 µL of buffer (PBS, containing 0.5% BSA and 2 mM EDTA, pH 7,2) per 10^7 total cells. 20 µL of CD45 (clone REA747) magnetic microbeads were added to the samples per 10^7 total cells. After mixing, this solution was incubated for 15 min on room temperature (the manufacturer recommends 4–8°C, but for spermatozoa it is not an optimal temperature). After the incubation, 2 mL of PBS was added and a centrifugation was performed as described earlier. The supernatant was aspired completely and up to 10^8 cells were resuspended in 500 µL of PBS. LS separator columns were rinsed with PBS and the labelled cell suspensions were loaded onto the columns. Unlabeled cells that passed through were collected. The unlabeled cell fraction was labeled with CD45-FITC stain to ensure complete removal of CD45+ cells. Flow cytometric analyses were performed. Ratios of CD45+ cells were determined before and after magnetic separations.

3.4. Magnetic separation of erythrocytes

Separations were carried out based on the recommendations of the manufacturer. Namely, prepared testicular biopsy samples were centrifuged at 300×*g* for 10 min, the supernatants were discarded and the cell pellets were resuspended in 80 µL of PBS buffer (the composition is the same as described above) per 10^7 total cells. 20 µL of CD235a (clone REA175) magnetic microbeads were added to the samples per 10^7 total cells. After mixing, this solution was incubated for 15 min on room temperature. After the incubation, 2 mL of PBS was added and centrifugation was performed as described earlier. The supernatant was aspired completely and

up to 10^8 cells were resuspended in 500 µL of PBS. LS separator columns were rinsed with PBS and the labelled cell suspensions were loaded onto the columns. Unlabeled cells that passed through were collected. Number of erythrocytes before and after magnetic separation was observed and counted with light microscopy. Erythrocytes were identified based on their colour and morphological features.

3.5. Chemicals

The annexin V, CD45, and CD235a microbeads used for magnetic separation, as well as the CD45-FITC fluorophore for fluorescent labeling were purchased from Miltenyi Biotec. The buffer solutions used for sample preparation were products manufactured by Sigma Aldrich (Sigma Aldrich, St. Louis, Missouri, USA). The TUNEL assay used for DNA-fragmentation analysis was obtained from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the reagents for the SCD test were purchased as a kit from Microptic S.L. The PureCeption™ used for density gradient centrifugation and sperm media were produced by Origio (CooperSurgical, Trumbull, Connecticut, USA).

3.6. Statistical analysis

Data were presented with their mean and standard deviation, and for box plot diagrams, we additionally included the median, the lower and upper quartiles. The Shapiro-Wilk test was used to assess data distribution. Variables with normal distribution were analyzed with a parametric two-sample t-test, while non-normally distributed variables were analyzed using the non-parametric Kruskal-Wallis test. We used GraphPad InStat 7.0 software (Dotmatics, GraphPad Software Inc., Boston, Massachusetts, USA) to perform the tests.

4. RESULTS

4.1. Magnetic separation of sperm cells with high DNA-fragmentation

Since the inclusion criterion was high DFI, the average DFI of the spermatozoa before separation was 42,04 ± 9,34%. After separation, a reduction of 2,19× was observed, lowering the DFI to 19,11 ± 5,90% (p<0,01; Fig. 1.).

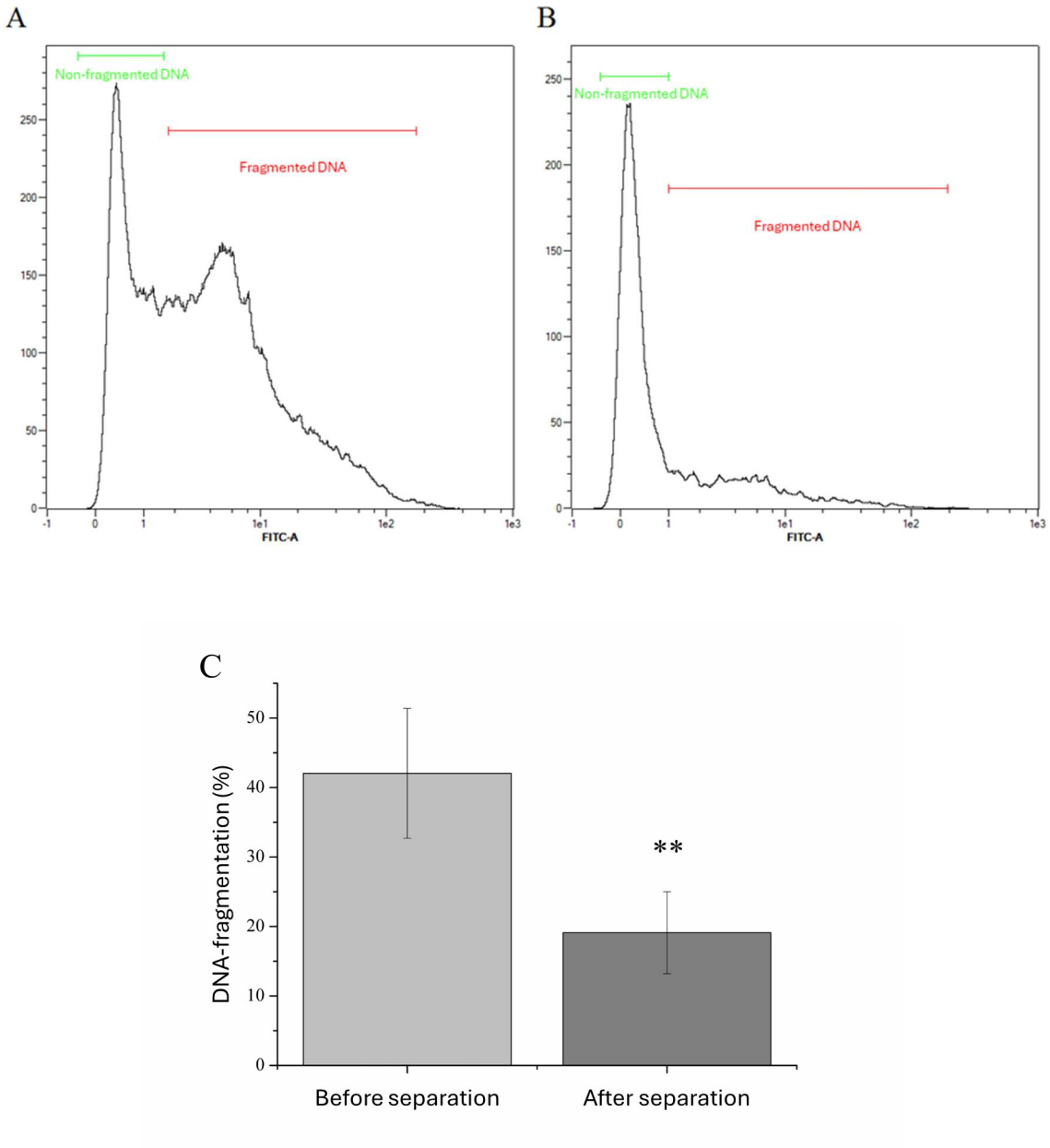

*Fig. 1. The effect of annexin V labeling-based magnetic separation on sperm DNA-fragmentation. DNA-fragmentation measured by the TUNEL assay before (A) and after (B) separation. DFI before and after separation (C). **p<0,01; two-sample t-test.*

4.2. Examination of the possible correlations between sperm vitality, DNA-fragmentation, and classical semen parameters

Among the 205 samples examined retrospectively, 170 showed a low DFI (<30%), while 35 fell into the high DFI (>30%) group. Normal vitality (>58%) was observed in 151 cases, while necrozoospermia was evident in 54 cases.

The DFI was 1,36× higher in the necrozoospermia group (vitality: <58%) compared to the samples with normal vitality (vitality: >58%) (17,38 ± 10,38% vs. 23,71 ± 16,17%; p=0,0261). The concentration, motility, and progressive motility were as follows in the two groups: 30,07 ± 29,50 M/ml vs. 13,58 ± 16,81 M/ml, 31,86 ± 16,65% vs. 18,31 ± 11,94%, and 24,31 ± 14,11 vs. 12,67 ± 9,95%, respectively (Fig. 2/A.).

From the perspective of DFI, even more significant differences were observed (p<0,001): the vitality in the low DFI group was 66,91 ± 14,08%, while it was 56,25 ± 16,44% in the high DFI group. The concentration, motility, and progressive motility were as follows in the two groups: 26,52 ± 28,51 M/ml vs. 21,85 ± 23,22 M/ml, 30,51 ± 16,67% vs. 17,51 ± 11,53%, and 23,01 ± 14,23% vs. 12,68 ± 9,68%, respectively (Fig. 2/B.).

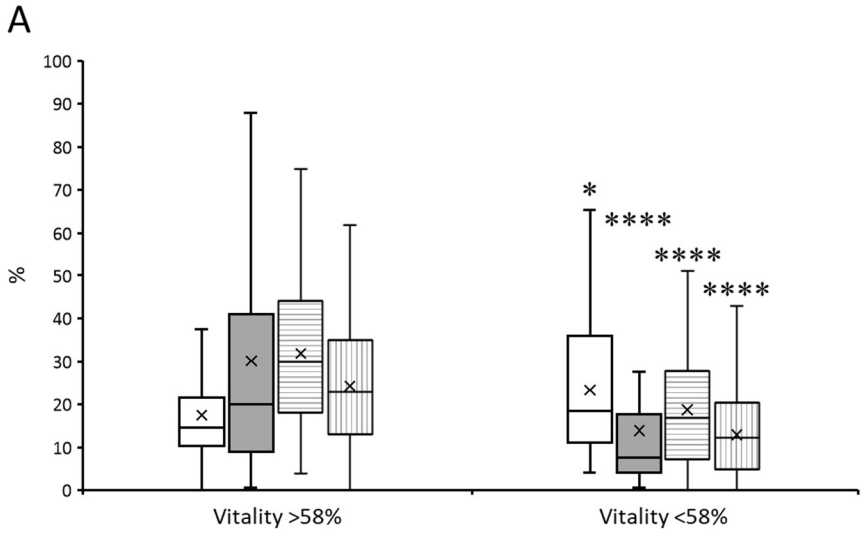

Fig. 2/A. Correlation between sperm vitality and DNA-fragmentation. DNA-fragmentation (white box), sperm concentration (gray box), motility (horizontally striped box), and progressive motility (vertically striped box) in cases of normal vitality vs. necrozoospermia.

**p<0,05, ****p<0,0001, Kruskal-Wallis test.*

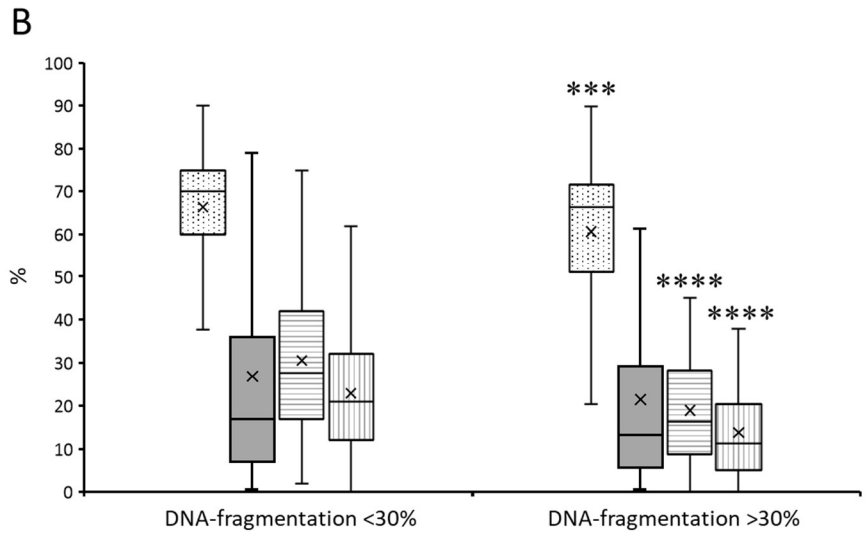

Fig. 2/B. Correlation between sperm vitality and DNA-fragmentation. Vitality (dotted box), sperm concentration (gray box), motility (horizontally striped box), and progressive motility (vertically striped box) in cases of low vs. high DNA-fragmentation.
****p<0,001; ****p<0,0001; Kruskal-Wallis test.*

4.3. Magnetic separation of absolute asthenozoospermic semen sample

In our case study, the concentration of the examined semen sample was 8 M/ml with 0% motility, 10% vitality, and 77,6% DFI. Following MACS separation, the vitality increased to 73%, while the DFI decreased to 28,2%.

4.4. Magnetic separation of leukocytes

In cases of leukocytospermia, the initial ratio of CD45+ cells compared to the total cell count was 6,92 ± 3,71%. Following separation, their proportion significantly decreased to 0,44 ± 0,39% (p<0,001; Fig. 3.).

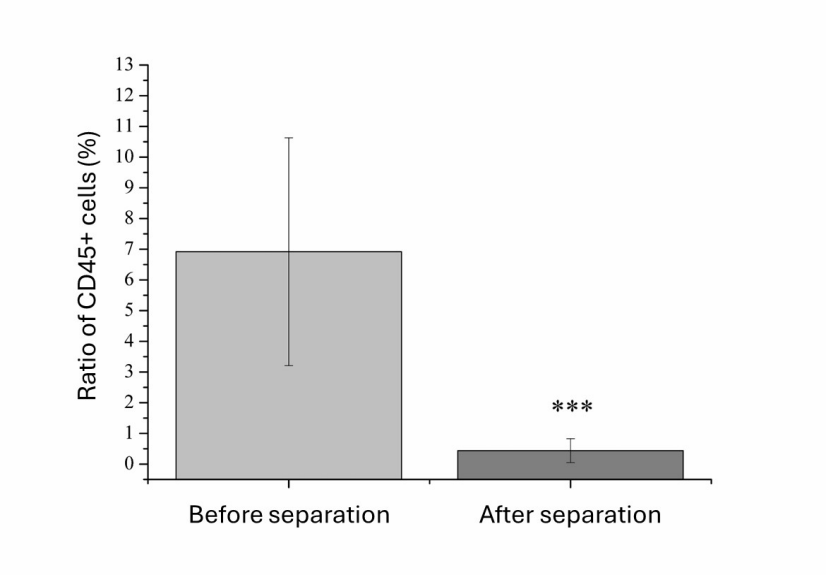

Fig. 3. Ratio of CD45+ cells before and after magnetic separation.
****p<0,001; two-sample t-test.*

4.5. Magnetic separation of erythrocytes

The processed testicular tissue samples contained an average of 73,71 ± 39,85 M/ml of red blood cells. Following magnetic separation, the number of CD235a+ cells significantly decreased to 2,39 ± 2,04 M/ml in the sample (p<0,001; Fig. 4.).

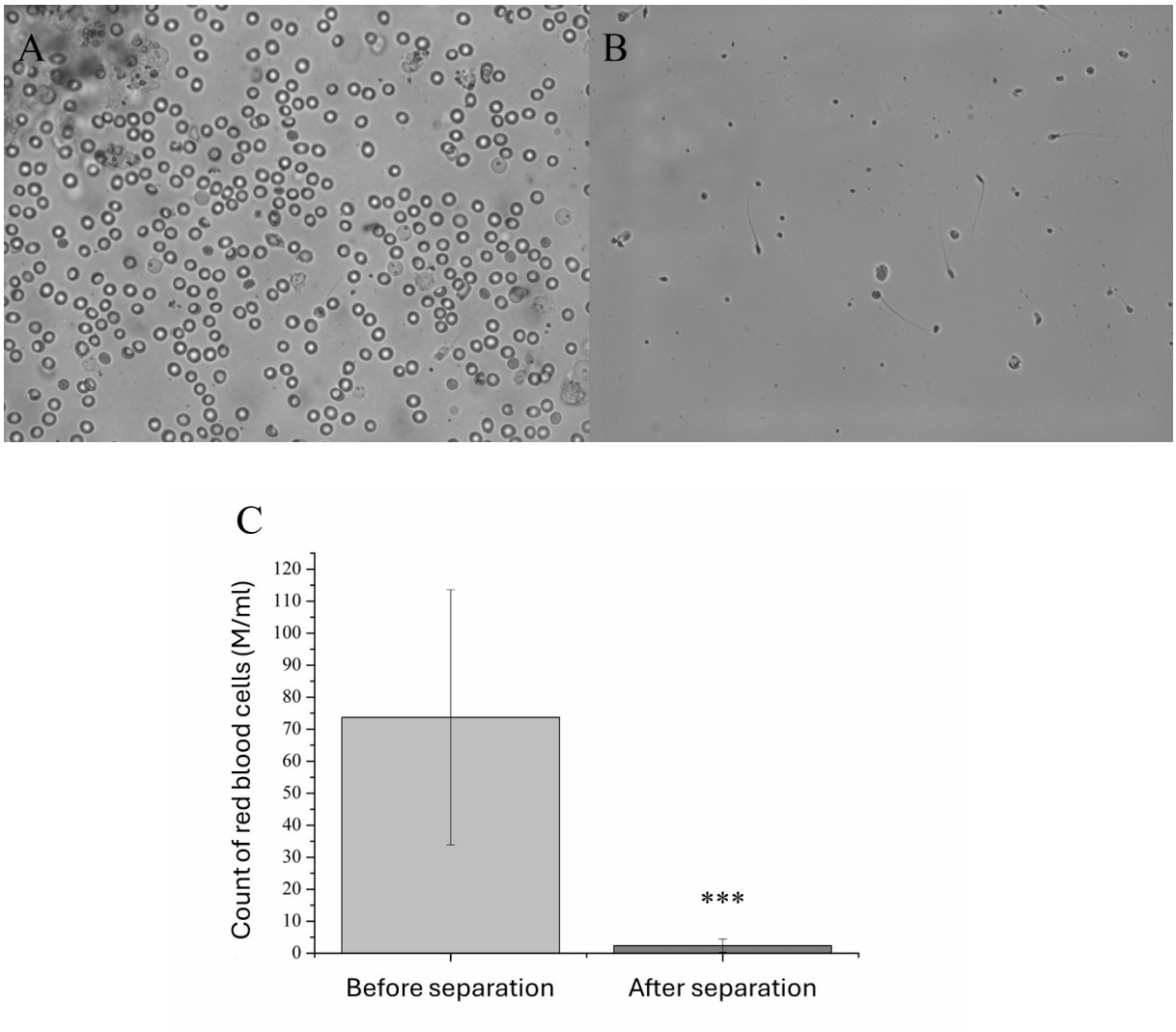

*Fig. 4. Light microscopic image illustrating the number of red blood cells before (A) and after (B) magnetic separation of CD235a+ cells. The number of red blood cells before and after separation (C). ***p<0,001; two-sample t-test.*

5. DISCUSSION AND CONCLUSIONS

To compensate the global fertility crisis affecting the human species, it is essential to increase the effectiveness of the currently relatively low-efficiency assisted reproductive techniques. One crucial aspect is to ensure the highest possible quality of the biological samples and gametes used. From the male perspective, this can be achieved both by selecting sperm with high DNA integrity and by removing contaminating cells from the ejaculate or testicular tissue sample. None of the sperm selection procedures developed so far have proven to be more effective than others concerning clinical endpoints, thus the need for further development of these methods remains.

In this dissertation, I aimed to detail the potential alternative applications of advanced sperm selection methods, particularly focusing on the possibilities offered by MACS. The original method is based on the binding of annexin V on magnetic microbeads to PS expressed on the membrane surface of spermatozoa exhibiting apoptotic signals. *Apoptosis* refers to the physiological process of programmed cell death, during which the cell systematically dismantles its own structures through active, energy-dependent mechanisms. In this process, the cell begins to shrink, its chromatin condenses, and then fragments internucleosomally. These DNA-fragments, along with cell organelles, eventually become enclosed in so-called *apoptotic bodies*, which are surrounded by an intact cell membrane and then detached from the cell. Subsequently, they are phagocytosed by surrounding phagocytes. The basis of this latter step is the externalization of PS, which functions as a non-inflammatory phagocytosis signal and plays an essential role in the complete execution of apoptosis (Elmore, 2007; Szeberényi, 2011). The proportion of spermatozoa exhibiting apoptotic signals (such as activated caspases, externalized PS, and DNA-fragmentation) is higher in the ejaculates of infertile men (Weng et al., 2002; Hichri et al., 2018). The activation of apoptotic signaling pathways has been shown to have markedly negative effects on sperm fertilization potential, with caspase-3 activation resulting in reduced motility, impaired capacitation, disrupted acrosome reaction, compromised oocyte penetration, and ultimately a lower fertilization rate (Aziz et al., 2007; Grunewald et al., 2008; Grunewald et al., 2009a; Grunewald et al., 2009b; Grunewald et al., 2017).

The DNA-fragmentation-reducing effect of MACS on spermatozoa has already been demonstrated in numerous studies (Said et al., 2006; Aziz et al., 2007; de Vantéry Arrighi et al., 2009; Lee et al., 2010; Zahedi et al., 2013; Bucar et al., 2015; Degheidy et al., 2015; Chi et al., 2016; Martínez et al., 2018). As a starting point for our investigations, we verified this effect:

using annexin V-based separation of the samples, a significant 2,19× reduction in DFI could be achieved (Fig. 1.), consistent with literature data—a result that is, in fact, unsurprising. As discussed above, DNA-fragmentation is a natural phenomenon during the apoptotic process. The elimination of apoptotic cells from the system logically results in a reduction of DFI as well.

Severe or absolute asthenozoospermia presents a major challenge in assisted reproductive techniques, as isolating immotile yet viable immotile sperm from dead sperm without causing damage is highly difficult. The applicability, safety, and success of previously mentioned techniques (e.g., HOST, LAISS) remain uncertain. An alternative solution could be obtaining testicular sperm with low DNA-fragmentation, but this is achievable only via surgical procedure (TESE), which patients are often reluctant to undergo (Agarwal et al., 2022). MACS is well-known for its DNA-fragmentation-reducing effect, and beyond that, numerous studies report its vitality-enhancing effect when isolating sperm with high DNA integrity (Aitken et al., 2010). In line with previously reported results, our study found a negative correlation between sperm vitality and the extent of DNA-fragmentation (Fig. 2.). We also examined the impact of changes in these two characteristic on classical semen parameters. Both reduced vitality and high DFI led to significant changes in the parameters examined. As vitality decreased, DFI significantly increased, and concentration, motility, and progressive motility decreased. The increase in DFI resulted in similar changes, except for a significant change in concentration. Furthermore, it was observed that changes in DFI had a more substantial impact on vitality than vice versa. The correlation between DFI and vitality can also be traced back to the process of apoptosis. DNA fragmentation is part of the apoptotic process, ultimately resulting in the death of the cell. Thus, a high DFI value in a semen sample essentially indicates fragmented genetic material as a consequence of programmed cell death, with the clear consequence being reduced vitality. Nothing illustrates the above relationship better than the reduction in the DFI following the SU test, a selection technique fundamentally based on the migration of live, healthy, motile sperm cells. During this procedure, the motile/live fraction separates from the immotile/potentially dead fraction, greatly enhancing vitality and, as mentioned, reducing DNA-fragmentation (Jayaraman et al., 2012; Oguz et al., 2018; Muratori et al., 2019; Viswambharan and Murugan, 2021). This phenomenon is utilized by microfluidic separation chips (e.g. ZyMōt™), which reduce DFI, increase motility, and consequently improve vitality (Parrella et al., 2019). Unfortunately, these methods are ineffective in cases with predominantly immotile sperm populations. Based on the above, a further critical conclusion can be drawn: when interpreting the results of current DNA-fragmentation tests, it must be considered that they reflect a

cumulative value, as they include data from non-viable sperm, which can be misleading. Thus, while DF tests provide valuable information about natural fertility, they may be inaccurate in predicting outcomes of intrauterine insemination (IUI)/in vitro fertilisation (IVF)/ICSI. In IUI/IVF/ICSI cycles, sperm are prepared routinely using a conventional selection technique (SU/DGC), which itself reduces DFI and improves vitality and motility (Jayaraman et al., 2012; Oguz et al., 2018; Muratori et al., 2019; Viswambharan and Murugan, 2021). Consequently, based on DF test results and IVF/ICSI cycle outcomes, the impact of DNA-fragmentation on clinical pregnancy rate, miscarriage rate, and LBR cannot be clearly determined. In summary, the effect of DNA-fragmentation on clinical endpoints remains unclear, and further RCTs and meta-analyses are needed (Chen et al., 2020). To date, MACS has been considered a safe method (Gil Juliá et al., 2023); however, the potential effects of the magnetic field on sperm and their role in assisted reproduction outcomes require further investigation.

Based on the strong correlation between DNA-fragmentation and vitality detailed above, a fragmentation-reducing procedure could potentially enhance vitality. MACS separation has proven suitable for this purpose: in our study, the vitality of an absolute asthenozoospermic sample increased by 7,3× following annexin V-based magnetic separation. Although vitality could not be raised to 100%, and the sperm remained immotile after separation, the method statistically significantly enhances the chance of fertilization with viable sperm. Simultaneously, in line with previously mentioned literature data, we observed a substantial decrease in the sample's DFI (from 77,6% to 28,2%). The method we employed could also be effective for TESE samples, where, due to the immature nature of testicular sperm, motile sperm are also absent. The study’s limitation lies in the case-study nature due to the extremely low incidence of absolute asthenozoospermia.

By modifying the original annexin V-based method and using other molecules (antibodies) bound to the surface of the microbeads, it is possible not only to enrich target cells (high-quality sperm) based on additional cell surface markers but also to eliminate undesirable, contaminating cells in the sample.

The CD45 molecule, also known as a common leukocyte antigen, is a membrane glycoprotein found on the surface of all mature nucleated hematopoietic cells, making it suitable for removing contaminating white blood cells from leukocytospermic ejaculates. These cells have detrimental effects on fertility: in their study from 2016, Fraczek et al. observed lower sperm concentration, total and progressive motility, and elevated malondialdehyde (MDA) levels in isolated leukocytospermic semen samples compared to controls, with MDA being a biochemical marker of lipid peroxidation. It appears that while bacterial infections in semen

activate the mitochondrial pathway of apoptosis and increase intracellular ROS levels, leukocytospermia is associated rather with direct cellular damage and lipid peroxidation caused by ROS generated by white blood cells (primarily neutrophils). (In case of a seminal tract infection, leukocytospermia and bacteriospermia are generally present simultaneously and their effects combine.) Given that the cell membrane of sperm cells contain an extremely high (approximately 50%) proportion of highly reactive polyunsaturated fatty acids, they are particularly vulnerable to lipid peroxidation, which reduces membrane fluidity in both the membrane of the cell and of the intracellular organelles. This fundamentally impairs key functions, such as capacitation, acrosome reaction, sperm-oocyte fusion, and normal operation of various receptor-mediated signaling pathways. Moreover, lipid peroxidation by-products (e.g. MDA) form DNA adducts, indirectly exerting genotoxic effects in addition to direct DNA-damaging effects of ROS (Henkel, 2024). Activated white blood cells also express proinflammatory cytokines (interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α)), which negatively correlate with sperm concentration, total and progressive motility, vitality, and DNA integrity (Eggert-Kruse et al., 2001; Koçak et al., 2002; Martínez et al., 2007). Therefore, eliminating contaminating white blood cells from the ejaculate intended for further use is clearly beneficial for the outcome of reproductive procedures.

In our study, CD45-based separation achieved a significant 15,73-fold reduction in the percentage of leukocytes in leukocytospermic semen samples (Fig. 3.). A limitation of this method may be potential activation and ROS production induced by antibody binding, with possible adverse effects on sperm that warrant further investigation.

The presence of red blood cells in semen or testicular tissue samples used for reproductive procedures is unfavorable for several reasons. First, they make sperm isolation more challenging for the embryologist. Secondly, any potential cytolysis (e.g. due to application of erythrocyte-lysis buffer or thawing of frozen samples) can release free radicals, contributing to oxidative stress and direct cytotoxicity (Rijsselaere et al., 2004; Yazdinejad et al., 2020). Furthermore, erythrocyte-lysis buffers themselves have been shown to be potentially toxic to sperm: the Band 3 anion channel involved in lysis of red blood cells is also present in sperm's cell membrane, and if the osmolarity of the buffer differs significantly from the medium, osmotic shock may occur (Yazdinejad et al., 2020).

Using CD235a-based magnetic separation, we achieved a substantial (30,8-fold) and successful removal of red blood cells contaminating testicular tissue samples obtained via TESE (Fig. 4.). According to our literature review, our research group is the first to report the elimination of red blood cells by magnetic separation in semen/TESE samples.

If we would have access to a molecular marker specific to the highest quality sperm subpopulation (with highest DNA integrity), this could serve as a target to further enhance the MACS technology to positively select this group of cells. Mass spectrometry imaging (MALDI) could be a valuable tool for identifying such molecules; however, it is limited by its high costs and the need for advanced training and expertise. In the future, an effective, routine positive selection technique based on similar technology could, in our opinion, improve ART outcomes by selecting sperm ideal for fertilization. Adams et al. in 2008 described and validated a multitarget MACS method that combines magnetic separation with microfluidic technology on a chip-based platform, allowing for highly selective bacterial cell separation based on multiple surface markers. Similarly, it may be possible to separate sperm with high fertilization potential and contaminant cells even in a single-step process based on this principle.

6. SUMMARY AND NOVEL FINDINGS

1. We have confirmed once again and thus supported the well-known effect of the MACS-annexin V system in reducing sperm DNA-fragmentation as documented in the literature.

2. We have supported the strong negative correlation between DNA-fragmentation and viability with our results and confirmed their effects on classical semen parameters (sperm concentration, motility, progressive motility).

3. Based on the current literature, we were the first to apply annexin V-based MACS separation to enhance the viability of semen samples (and thus reduce DFI) in the context of rare absolute asthenozoospermia.

4. By using magnetic microbeads targeting the CD45 molecule, we achieved a significant reduction in the proportion of contaminating white blood cells in leukocytospermic semen samples.

5. By using magnetic microbeads targeting the CD235a molecule, we achieved a significant reduction in the proportion of contaminating red blood cells in testicular tissue samples obtained during TESE, applying this technique for the first time to the best of our knowledge.

7. PUBLICATIONS

Publications related to the dissertation:

Máté G, Balló A, Márk L, **Czétány P**, Szántó Á, Török A. Magnetic-Activated Cell Sorting as a Method to Improve Necrozoospermia-Related Asthenozoospermic Samples. J Clin Med. 2022 May 21;11(10):2914. **Q1, IF: 3,0**

Czétány P, Balló A, Márk L, Török A, Szántó Á, Máté G. An Alternative Application of Magnetic-Activated Cell Sorting: CD45 and CD235a Based Purification of Semen and Testicular Tissue Samples. Int J Mol Sci. 2024 Mar 24;25(7):3627. **Q1, IF: 4,9**

Aggregated impact factor of the publications forming the basis of the thesis: **7,9**

Cumulative impact factor: **35,2**

Citations: **41**

Other publications not related to the dissertation:

Illés A, Opper B, Reglődi D, Kerényi M, **Czétány P**, Boronkai Á, Schäfer E, Tóth G, Fábián E, Horváth G. Effects of pituitary adenylate cyclase activating polypeptide on small intestinal INT 407 cells. Neuropeptides. 2017 Oct; 65:106-113. **Q2, IF: 2,5**

Sarlós DP, **Czétány P**. Development of a laparoscopic partialnephrectomy training model. Magyar Urológia. 2018; 30(1): 8-11.

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Bányai D, Sarlós DP, Belák M, **Czétány P**, Szántó Á. Our initial experience with robot-assisted partial nephrectomy. Magy Onkol. 2024 Sep 19; 68(3):243-247.

Czétány P, Balló A, Szántó Á. Introduction and current results of testis sparing surgery in Pécs. Magyar Urológia. 2024; 36(3): 139-142.

8. PRESENTATIONS

Czétány P, Balló A, Kádár Zs, Damásdi M, Kenyeres B, Szántó Á. Acut management and reconstruction of Fournier gangrene in young fertile male.

24th annual congress of the Hungarian Urological Society, Eger, Hungary, 2019.10.11. (poster)

12th annual congress of the Hungarian Andrological Society, Zalakaros, Hungary, 2019.12.05.

Czétány P, Pusztai Cs, Szántó Á. Laparoscopic ureterolysis in case of ureteral obstruction caused by retroperitoneal fibrosis. 25th annual congress of the Hungarian Urological Society, 2020.10.08.

Czétány P, Pusztai Cs, Szántó Á. Laparoscopic postchemotherapy retroperitoneal lymph node dissection – case presentation. 34th FUN, 2021.02.20.

Czétány P, Balló A, Pytel Á, Szántó Á. Diagnostic „enigma”: a case of segmental testicular infarction.

14th annual congress of the Hungarian Andrological Society, Kecskemét, Hungary, 2022.09.22.

27th annual congress of the Hungarian Urological Society, Siófok, Hungary, 2022.10.07.

Central European Meeting 23, Kraków, Poland, 2023.03.24-25 (poster)

Czétány P. Case presentation at the European School of Urology, Siófok, Hungary, 2022.10.08.

Czétány P, Bányai D. Laparoscopic treatment of prostatic cystadenoma, 28th annual congress of the Hungarian Urological Society, Budapest, Hungary, 2023.10.12.

Czétány P, Balló A. Testis sparing surgery: introduction of the technique and its results between 2018-2013 in Pécs:

15th annual congress of the Hungarian Andrological Society, Kecskemét, Hungary, 2023.11.23.

Central European Meeting 24, Vienna, Austria, 2024.04.26-27. (poster)

Czétány P. Management of locally advanced prostate cancer, 14th congress of „Minimal invasive procedures in urology”, Budapest, Hungary, 2024.02.22.

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I dedicate my work to the unborn children.