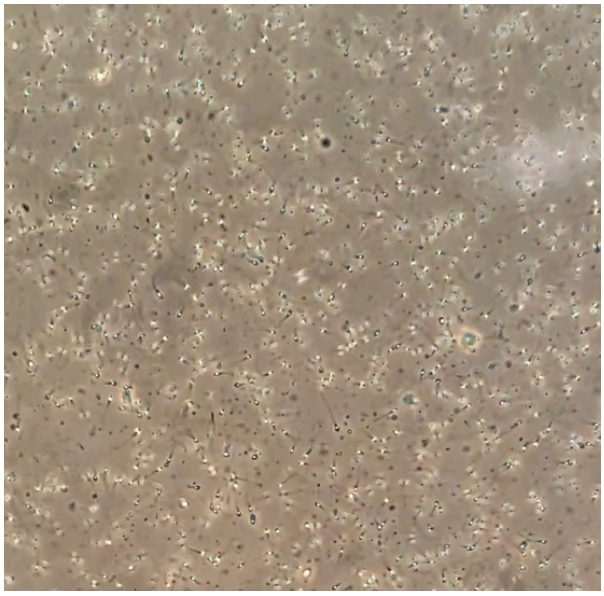
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

MICROFLUIDIC SORTING OF SPERM FOR ASSISTED REPRODUCTION

An Evaluation of ZyMōt Multi Sperm Separation Device



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Picture showing spermatozoa through brightfield microscopy 40x, by Emma Olofsson

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Abbreviations

ART – Assisted reproduction technology

WHO – World Health Organization

IVF – *In vitro* Fertilization

ICSI – Intracytoplasmic sperm injection

DGC – Density-gradient centrifugation

ROS – Reactive oxygen species

OS – Oxidative stress

SDF – Sperm DNA Fragmentation

TSPC – Total sperm count

LS – Lysis solution

IQR – Interquartile ranges

Abstract

Infertility is a worldwide problem and the main reason for the use of assisted reproductive technology (ART). *In-vitro* fertilization (IVF) is one ART treatment normally used, where the fertilization of oocytes occurs *in vitro*. The quality of the spermatozoa has a great impact on the outcome of IVF, highlighting the importance of obtaining spermatozoa with the highest fertilization potential. For this, different sperm preparation techniques are used where the density-gradient centrifugation (DGC) method is generally preferred. However, it is discussed whether the centrifugation step in this method affects the spermatozoa negatively, and new methods using microfluidics have recently appeared on the market, one being the ZyMōt Multi Sperm Separation Device. In this study, 25 semen samples were split and prepared with either DGC or ZyMōt. The aim was then to compare the prepared samples regarding 4 main parameters. (1) The proportion of motile spermatozoa, (2) the degree of motility, (3) the yield, and (4) the amount of sperm DNA fragmentation (SDF) was assessed by microscopic examination. An evaluation of the level of oxidative stress (OS) in the native semen samples was also performed. Significant differences were observed for SDF ($p < 0,0001$) and the proportion of motile spermatozoa ($p < 0,0001$) where samples prepared with ZyMōt displayed better results. Moderate evidence was observed for a difference regarding the yield between the methods ($p = 0,04647$), where DGC generated a higher total amount of spermatozoa. No difference in the degree of motility was observed. No correlation between the level of OS in the origin semen sample and SDF in the prepared samples was observed. The results of this study indicate that ZyMōt generates good-quality samples containing a high number of motile spermatozoa with almost no DNA fragmentation. ZyMōt also proved to be a simpler method to perform, improving laboratory efficiency.

Keywords: Assisted reproduction technologies / Spermatozoa / DNA fragmentation / Density-gradient centrifugation / Microfluidics / ZyMōt Multi

Sammanfattning

Infertilitet är ett världsomfattande problem och det främsta motivet för tillämpning av assisterad befruktning. En vanlig behandling är *in vitro*-fertilisering (IVF) där förening av ägg och spermie sker i labb. Spermiekvalitén har visat sig ha en stor påverkan på resultaten inom IVF vilket styrker vikten av att få fram de spermier med högst befruktningspotential. För detta används olika spermiepreppareringsmetoder där gradientcentrifugering är en av de vanligast förekommande. Det diskuteras dock huruvida centrifugeringssteget i denna metod har en negativ påverkan på spermiekvaliteten, och nya metoder som i stället använder sig av mikroflödestekniker har dykt upp på marknaden där en av dom är ZyMōt Multi Sperm Separation Device. I den här studien delades 25 prover upp och preparerades med antingen gradientcentrifugering eller ZyMōt. Syftet var sedan att jämföra de preparerade proverna med avseende på 4 huvudparametrar. (1) Andelen rörliga spermier, (2) rörlighetsgrad, (3) utbytet, och (4) mängden DNA fragmentering bedömdes genom mikroskopisk undersökning. Även test av nivån för oxidativ stress utfördes på ursprungsprovet. Signifikanta skillnader observerades för DNA fragmentering ($p < 0,0001$) och andelen rörliga spermier ($p < 0,0001$) där prov preparerade med ZyMōt uppvisade ett bättre resultat. För utbytet observerades en måttlig signifikant skillnad mellan metoderna ($p = 0,04647$) där densitetsgradientcentrifugering gav det högre utbytet. Ingen skillnad i rörlighetsgrad observerades. Ingen korrelation mellan nivån på oxidativ stress i ursprungsprovet och graden av DNA fragmentering i de preparerade proven observerades. Resultatet av studien indikerar på att prover preparerade med ZyMōt är av mycket god kvalitet bestående av ett högt antal rörliga spermier med nästan fullständig avsaknad av DNA-fragmentering. ZyMōt visade sig också vara en enklare metod att genomföra som även ökar effektiviteten av det laborativa arbetet.

Nyckelord: Assisterad befruktning / Spermier / DNA fragmentering / Gradientcentrifugering / Mikroflödestekniker / ZyMōt Multi

1. Introduction

Infertility affects 8-12% of couples around the world (Kumar and Singh, 2015) and is defined as the failure to become pregnant after 12 months or more of unprotected intercourse on a regular basis (Q-IVF, 2022). Infertility is the main reason for the use of assisted reproduction technology (ART) along with a growing social acceptance for single and same-sex parents (Kushnir et al., 2022). The prioritization of education and a career, the ambition to have a stable income and more effective contraceptives are some reasons why many couples choose to postpone their childbearing (Kushnir et al., 2022, Mills et al., 2011). This could be one reason for increased female infertility since the risk for aneuploidies in oocytes increases with age, thus also generating an increased demand for ART (Kushnir et al., 2022, van Kooij et al., 1996). Male infertility is often due to sperm defects which can be caused by life factors such as smoking, alcohol and drugs, obesity, and psychological stress (Durairajanayagam, 2018), although a large proportion of male infertility cases are considered idiopathic (Pinto et al., 2021). The factors of greatest importance regarding male infertility are low sperm concentration (oligospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia) (Harris et al., 2011), but other parameters also matter. The World Health Organisation (WHO) has developed a manual with guidelines for the analysis of sperm which amongst other things describe essential parameters for male infertility and their reference values. Here, selected values of interest are presented in **Table 1** where values above the threshold value are classified as normal (World Health, 2010). Around 50% of infertility cases are, completely or partly, due to male factors. Thus, sperm analysis becomes a very fundamental part of fertility investigations, both for the evaluation of male fertility, but also in the decision of what ART treatment should be used (Hreinsson et al., 2005). Also recently, a new health crisis has been revealed, a global decline in sperm count. Studies display strong evidence that all around the world the concentration of spermatozoa is declining and that the pace has accelerated since the year 2000. In 1973 the mean concentration of spermatozoa was around 100 million/ml and in 2018 these numbers had decreased to around 50 million/ml (Levine et al., 2023). Environmental influences, chemicals, pesticides, heat, and lifestyle factors such as diet, stress, smoking, and high BMI are believed to be causes of this negative trend which is seen as a serious health concern (Levine et al., 2017).

***Table 1**. Important parameters for sperm analysis with their respective limiting values. The analyzed value must exceed the threshold value to be classified as normal (World Health 2010).*

Parameters	**Limit Value**
Semen volume	1,5 mL
Sperm concentration	15×10^6 sperm/mL
Progressive motile sperm	32%
Total number of sperm	39×10^6 sperm/ejaculate

1.1 Assisted Reproductive Technology

ART is the collective term for all procedures performed on oocytes, spermatozoa, and embryos outside of the human body. Some treatments to fall under the concept are *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), meaning that the fertilization of the oocyte occurs outside of the uterus in a lab (De Geyter, 2019).

1.1.1 *In vitro* fertilization

The first child in the world to be born using IVF was Louise Brown in 1978 and here in Sweden, the first IVF child was born in 1982 (Q-IVF, 2022). Since this starting point the use of ART has

only been, and still is, increasing. In Sweden, around 20 000 IVF treatments are performed yearly, and in 2020 5046 children were born using IVF, corresponding to 4% of all children born annually (Q-IVF, 2022). In the natural female cycle, normally only one egg matures at a time and detaches from the ovary with the possibility of fertilization (Hreinsson et al., 2005). To promote the chances of getting pregnant during IVF, hormone stimulation is used to increase the number of eggs that will mature, which are then aspirated directly from the ovary. This is accomplished with a thin needle that through the vaginal wall is inserted into the ovarian follicles, and follicular fluid containing the oocytes is aspirated into a test tube. The oocytes are then placed together with spermatozoa in a petri dish and are incubated overnight allowing fertilization to occur on its own. Afterward, the eggs are analyzed for the presence of two pronuclei that indicate successful fertilization. The transfer of the blastocyst back into the uterus is performed after 2-5 days of incubation (Hreinsson et al., 2005). Of all the fresh IVF treatments with own gametes performed in 2020 that led to embryo transfer, 28% resulted in a live birth (Q-IVF, 2022).

1.1.2 Intracytoplasmic sperm injection

ICSI is another type of ART treatment that is performed when the semen sample shows reduced parameters, such as motility or sperm count. If less than 1 million spermatozoa are obtained after preparation this usually indicates that ICSI should be used. For this treatment, one single spermatozoon is selected and then injected into the mature egg (Hreinsson et al., 2005).

1.2 The preparation of sperm

Before the semen sample can be used for ART, the spermatozoa with the highest fertilization potential need to be isolated and separated from other parts of the ejaculate. The seminal fluid is important for fertilization to occur during natural conditions but contains some components that instead disfavor ART. It is thus important to separate spermatozoa from the seminal fluid and other factors such as leucocytes, epithelial cells, and dead spermatozoa, to generate a sample with a high number of morphologically normal and motile spermatozoa (World Health, 2010).

1.2.1 Density-gradient centrifugation

Density-gradient centrifugation (DGC) is one of the sperm preparation methods generally preferred that provides high-quality spermatozoa (Mortimer, 2000). The method separates the spermatozoa from leucocytes, other somatic cells, and degenerated spermatozoa based on the difference in cell density (Pinto et al., 2021, World Health, 2010). Motile and morphologically normal spermatozoa have a higher density of 1,10 g/ml compared to the immotile and immature spermatozoa with a density of 1,06-1,09 g/ml (Oshio et al., 1987), resulting in that after centrifugation, the cells end up in different gradient levels matching their density (Malvezzi et al., 2014). The upper gradient will contain abnormal and non-motile spermatozoa, with the seminal plasma and an interface of leukocytes and cell debris on top. The motile and mature spermatozoa will end up in the lower phase, primarily as a pellet at the bottom of the tube (Takeshima et al., 2017). A washing procedure is then usually performed to remove density-gradient media, adding a further centrifugation step to the method (Mortimer, 2000).

However, it has been questioned how centrifugation may negatively impact spermatozoa, and published results are somewhat conflicting. It has been shown that repetitive centrifugation greatly increases the production of reactive oxygen species (ROS) from spermatozoa (Aitken and Clarkson, 1988, Iwasaki and Gagnon, 1992), which might be a consequence of the mechanical stress causing damage to the plasma membrane (Aitken and Clarkson, 1988). Oxidative stress (OS), as a result of excessive ROS, is, in turn, a major contributor to sperm DNA fragmentation (SDF), affecting male infertility and reproductive outcomes (Agarwal et al., 2020, Dutta et al., 2021, Esteves et al., 2021, Sakkas and Alvarez, 2010). In some studies, however, when using gradient

centrifugation methods, a lower level of ROS has been observed in the fraction containing the motile spermatozoa, suggesting that these cells are not affected by the centrifugation procedure. Instead, the method separates the motile spermatozoa from toxic ROS (Aitken and Clarkson, 1988, Takeshima et al., 2017). Conversely, other studies have found that the lower fraction containing the motile spermatozoa have higher levels of ROS and SDF than the original semen sample (Iwasaki and Gagnon, 1992, Muratori et al., 2016, Muratori et al., 2019). Although, it is not clear that this increase is caused by the centrifugation step but could be a result of the removal of the seminal plasma, and also an indication that spermatozoa with normal morphology and density are producers of ROS (Iwasaki and Gagnon, 1992). Another study did instead show no difference in the percentage of SDF after centrifugation. This could either signify that DGC is not selective for spermatozoa with higher DNA integrity, or that DGC might increase DNA damage in spermatozoa (Zini et al., 2000).

1.3 Oxidative Stress & DNA Fragmentation

For the necessity to deliver the genetic information to the oocyte and to ensure that the DNA is physically protected, the DNA in spermatozoa is sixfold more highly condensed than in somatic cells (Ward and Coffey, 1991). During late spermatogenesis, the cells' DNA repair capacity is highly reduced, which is somewhat compensated for by the tightly compacted chromatin, increasing the cells' resistance to external impact. But even so, genetic damage may occur on testicular, epididymal, and post-ejaculatory levels (Lewis and Aitken, 2005).

SDF intends single- and double-strand DNA breaks (Esteves et al., 2021) which can be caused by defective maturation, abortive apoptosis, or OS, with some external risk factors being smoking, diet, drug abuse, and heat exposure (Agarwal et al., 2020). For the condensed packaging of the DNA to occur, most of the histones are replaced with a small protein called Protamine, with controlled DNA nicking appearing as a way to aid the chromatin arrangement (Okada, 2022). If the control for this process fails, abnormal chromatin structure could be formed, preventing access to the correct sequences to be used for embryo development (Agarwal et al., 2020, Sakkas and Alvarez, 2010). Apoptosis is induced in around half of all germ cells during spermatogenesis because of the germ cell screening mechanism performed by the Sertoli cell. This process however is not always so effective, resulting in defect cells still undergoing maturation and despite having a damaged genome still showing normal morphology (Sakkas and Alvarez, 2010). OS is said to be the most common factor causing SDF and a result from exceed ROS production (Agarwal et al., 2020, Dutta et al., 2021, Esteves et al., 2021, Sakkas and Alvarez, 2010). ROS are products of normal cellular metabolism and are in semen produced by leukocytes and immature spermatozoa (Ko et al., 2014, Robert et al., 2021, Tremellen, 2008). A physiologically normal level of ROS plays an important role in processes such as sperm maturation, zona pellucida binding, acrosome reaction, and sperm-oocyte fusion (Ko et al., 2014). Thus, on one hand, being essential for the spermatozoa's ability to fertilize the oocyte (Robert et al., 2021), an abundance of ROS can instead inflict damage to the spermatozoa.

Several studies measuring DNA damage caused by OS found a negative correlation with seminal parameters, such as the concentration (Guz et al., 2013, Kodama et al., 1997), motility (Kao et al., 2008), and total sperm number and morphology (Shen et al., 1999). It has been shown that spermatozoa with damaged DNA, both *in vivo* and using IVF/ICSI, have the ability to fertilize an oocyte (Ahmadi and Ng, 1999, Horta et al., 2020, Simon et al., 2010, Sivanarayana et al., 2014) and that the oocyte to some extent has the capacity to repair the fragmented DNA of spermatozoa (Horta et al., 2020). An explanation for this might be that the paternal genome activation occurs first at the 4-8 cell stage (Sivanarayana et al., 2014). The capability of repairment of the spermatozoa DNA could however be reduced due to defects in the oocyte repair mechanism or the amount of DNA damage in the spermatozoa, which instead can result in further development defects (Horta et al.,

2020). Several studies show that SDF significantly impacts embryo and blastocyst development, a process in which the paternal genome plays a major role (Horta et al., 2020, Seli et al., 2004, Simon et al., 2010). Some studies also indicate that high SDF correlates with low implantation and clinical pregnancy rates (Bungum et al., 2007, Simon et al., 2010, Simon et al., 2011, Malić Vončina et al., 2021).

1.4 Novel methods in ART

A potential revolutionization in the field of ART is the novel lab-on-a-chip concept with the idea that all the laboratory steps performed during IVF will be implemented in one single unit (Kushnir et al., 2022). As a step in this direction, there has been an increasing integration of microfluidics into the field of ART, particularly regarding sperm-sorting devices. Microfluidics is a system that controls a small amount of fluid, enabling the measurement and manipulation of biological processes at a single-cell level (Nosrati, 2022). Regarding the preparation of spermatozoa, an important aspect of microfluidics is that it eliminates the centrifugation step and has also been shown to generate samples consisting of highly motile spermatozoa with normal morphology and reduced DNA fragmentation compared to conventional preparation methods (Asghar et al., 2014, Kushnir et al., 2022, Parrella et al., 2019). The technology of microfluidics comes with several potential advantages such as the standardization of workflows, fewer sample transfers, and simpler protocols reducing human error, reduction in cost and contamination, as well as miniaturization and automation increasing the accessibility to use IVF. However, in terms of sperm-sorting devices, decreased sperm concentration and that the devices only can process small semen volumes are some limitations of microfluidics (Asghar et al., 2014, Kushnir et al., 2022, Nosrati, 2022, Parrella et al., 2019). One example of a microfluidic unit, and for the moment the only one available to order in Sweden, is the ZyMōt Multi Sperm Separation Device. This unit separates motile spermatozoa from less motile and morphologically abnormal spermatozoa by letting them swim through a microfilter containing 8 µm-sized pores (**Figure 1**).

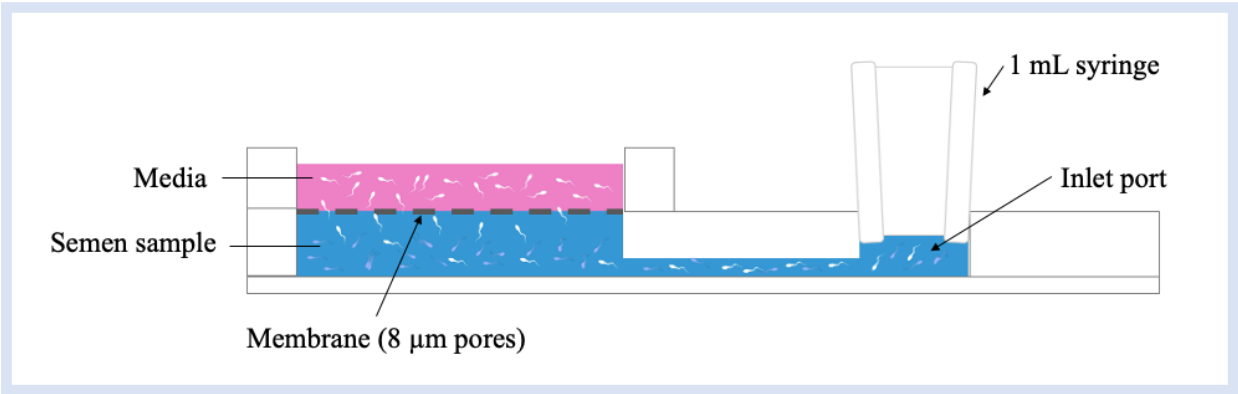

Figure 1. *Schematics of the mechanism of action for the ZyMōt Multi Sperm Separation Device. Fresh semen sample is injected in the inlet port. Motile spermatozoa swim thorough the membrane consisting of 8 µm-sized pores, leaving less motile and morphologically abnormal spermatozoa behind. The prepared sample is then collected from the upper chamber, containing high quality spermatozoa.*

1.5 Aim of the study

It has been shown that positive results within ART strongly depend on the quality of the prepared semen samples (Ahmadkhani et al., 2022), amplifying the significance of the ideal sperm preparation methods to be used. Thus, there is considerable interest in the assessment of the new microfluidic separation techniques that have appeared on the market within IVF for future clinical use.

The purpose of this study was to evaluate the ZyMōt Multi sperm separation device by comparing it to DGC, which is the method currently used by Sahlgrenska Reproductive Medicine Clinic. The methods were primarily assessed considering four specific parameters:

- The proportion of motile spermatozoa
- The degree of motility of the spermatozoa
- The yield
- The percentage of sperm DNA fragmentation.

Regarding the four parameters mentioned above, the following question was asked:

- How does the quality of the spermatozoa prepared with ZyMōt differ from those prepared with density-gradient centrifugation?

Also, with the aim to look for a relationship between oxidative stress and DNA fragmentation, the following question was asked:

- Is there any correlation between the level of oxidative stress in the origin semen sample and the percentage of sperm DNA fragmentation in the samples prepared with either ZyMōt or density-gradient centrifugation?

2. Material and Methods

This is a quantitative preclinical prospective comparative study performed at the Laboratory of Reproductive Medicine at Sahlgrenska University Hospital. In this study, the ZyMōt™ Multi (850 µL) Sperm Separation Device (ZyMōt Fertility Inc MD USA) has been evaluated and compared to the currently used method of DGC. The prepared sperm samples from both methods were compared considering the four main parameters mentioned above. Also, an evaluation of the level of OS in the native semen samples was performed.

2.1 Subjects & Samples

Patients included in the study were visiting the Sahlgrenska Reproductive Medicine Laboratory during Mars and April 2023 for routine sperm analysis as a part of preparations for fertility treatment. Participation in the study was completely voluntary. For the understanding of the verbal and written information about the project, an inclusion criterion for the patients was that they had to be Swedish speaking. The patients' approval was then confirmed with written consent. The samples used for analysis were leftover material that should have been discarded, no patient-specific data was collected, and all the material was discarded after analysis. An ethics application was submitted. But since no personal data was collected nor any intervention was performed on any subject, the study was not covered by the regulations in 3-4 §§ in the Ethics Review Act. On these premises, the Ethics Review Authority decided not to take the application into consideration, and they left an advisory opinion stating that they do not have any ethical objections to the project, see Appendix 1 (Dnr: 2022-05602-01-365143).

Semen samples were collected through masturbation and a total of 53 different patients approved participation. Criteria for the samples were that it had to be a normal semen sample according to the WHO parameters (World Health, 2010) and with a minimum volume of 1,9 mL. A certain number of dropouts occurred due to the failure to meet these criteria (n=24). Either one or two samples were analyzed simultaneously depending on available samples and capacity. If more acceptable samples were approved at that same time but not used, they were sorted as redundant (n=4). Thus, from a total of 53 samples, this project included 25 samples which are the base for the

results in this report. **Figure 2** shows a general description of the study design. After analysis, the initial semen samples were either excluded or tested for OS and then prepared with DGC and ZyMōt Multi, followed by an assessment of the parameters mentioned above and a blinded analysis for SDF.

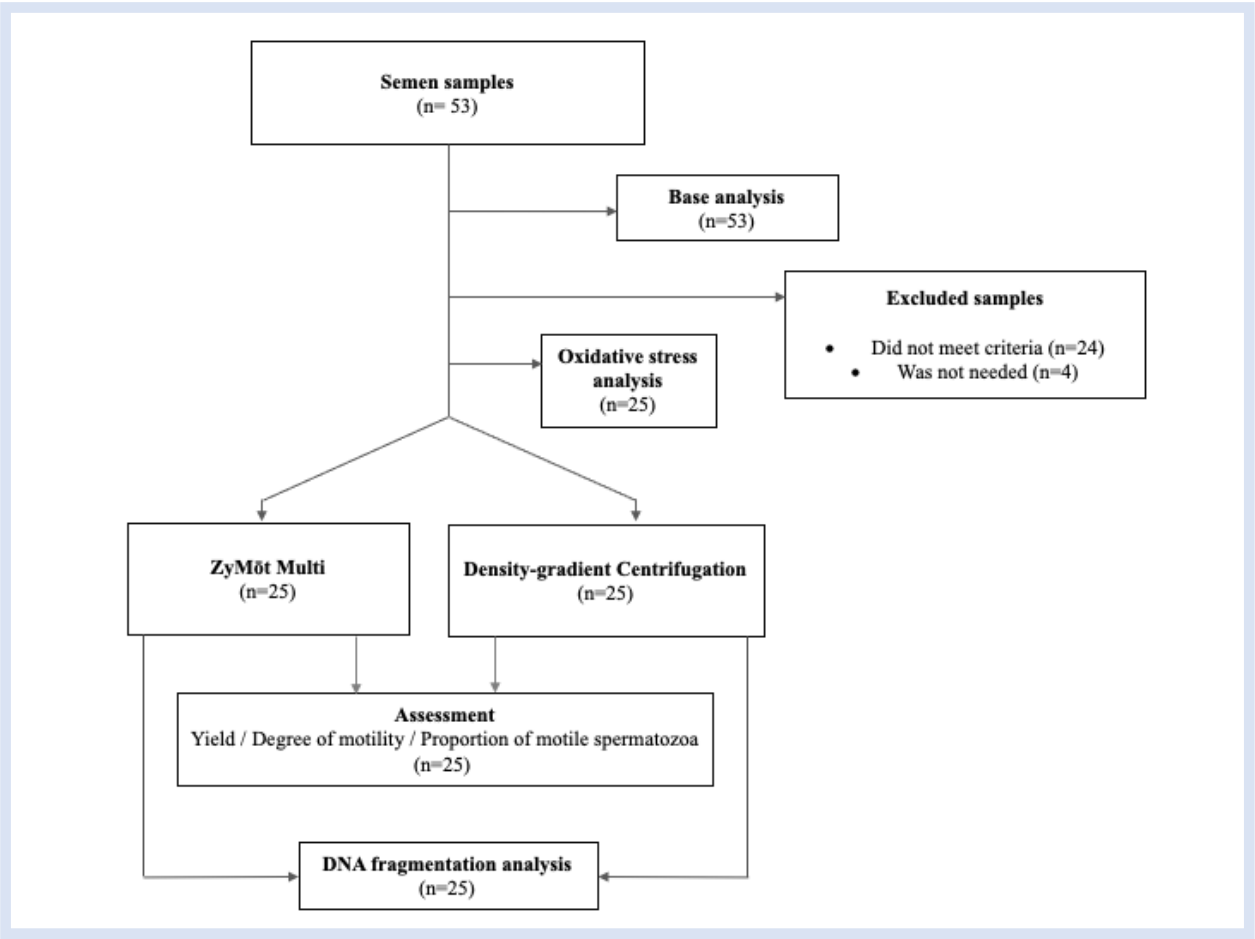

Figure 2. *Flowchart of the study design showing the number of samples included and excluded in the study. The native semen sample were split and prepared with two different sperm preparation methods followed by an assessment and DNA fragmentation analysis for comparison.*

2.2 Semen analysis

A semen analysis was performed both before and after the preparation of the semen sample with either one of the sperm preparation methods. All semen samples were set to liquify for a minimum of 15 minutes after collection before the analysis could start.

2.2.1 Before preparation

The volume of the unprocessed liquified semen sample was determined, and a small volume of the sample was placed on a microscope slide and covered with a cover glass for microscopic analysis. The proportion of motile spermatozoa, given as a percentage, was visually estimated and the presence of agglutination and/or aggregation was noted and taken into this estimation. The degree of motility was assessed depending on how actively the spermatozoa was moving and graded on a four-point scale, shown in **Table 2**. An average of the total degree of motility was estimated and grades of 2,25 to 3 were considered normal. The concentration of spermatozoa (10^6/mL) was determined using a Marienfeld Superior^{TM} Counting Chamber with a depth of 0,100 mm. 50 µL of the liquified semen sample was added to a test tube containing 4,95 mL NaCl 0,9% + 0,5% formaldehyde, for the killing and fixation of the spermatozoa. The sample was vortexed for at least

10 seconds so that aggregates would dissolve. 10 µL was then transferred to the counting chamber for microscopic analysis.

***Table 2. The degree of motility of spermatozoa**. A four-point scale was used to determine the degree of motility for spermatozoa by assessing how actively they were moving. E.g., if half of the spermatozoa was graded as a 2 and half a 2,5, a grade of 2,25 was given. Grades 2,25 – 3 were considered normal.*

Degree of motility
1 = Moving, but not actively
1,25–2 = Slow active motility
2,25-2,75 = Fast active motility
3 = Hyperactive motility

2.2.2. After preparation

After preparation with either sperm preparation method, a semen analysis was again performed and 10 µL of the prepared sample was added to a counting chamber for microscopic analysis. The degree of motility of the spermatozoa was analyzed and graded in the same way as before the preparation. The concentration of motile spermatozoa (10^6/mL) was estimated using the counting chamber. The proportion of motile spermatozoa was calculated by dividing the counted motile spermatozoa by the total amount of counted spermatozoa (motile + immotile), generating a percentage value. The yield was determined by calculating the total sperm count (TSPC) in the prepared sample. This was done by multiplying the concentration with the volume of the prepared sample, 0,52 mL for ZyMōt and 0,5 mL, 1 mL, or 3 mL for DGC.

2.3 Oxidative stress

For this method, the Oxisperm® (Halotech DNA SL Madrid Spain) Kit HT-OS20 was used to measure the level of OS in the native sample. The kit measures the possible excess of superoxide anions and is based on the nitro blue tetrazolium (NBT) assay in the form of a reactive gel (RG). The presence of superoxide anions in the semen sample converts tetrazolium salt along with other OS-associated molecules in the RG into blue crystals. This can be visualized as an increasing color intensity and then compared to a color scale.

The tube containing RG was placed in a 93 °C water bath for 5 minutes. The RG was then reduced to 37 °C by placing it in a 37 °C heat block for a few minutes. The volume of the semen sample and RG to be used (Proportion 1:1; semen-RG), was calculated by dividing 1000 by the concentration of spermatozoa in the sample. In an Eppendorf tube included in the kit, the semen sample and the RG were mixed and incubated at 4 °C for 5 minutes. The Eppendorf tube was then placed in a 37 °C heat block for 45 minutes. To decide the level of OS the color of the sample was compared to the color scheme included in the kit (**Figure 3**) where four levels of intensity have been preclassified: L1: Low; L2: Low-medium; L3: Medium; L4: High.

2.4 Sperm preparation methods

2.4.1 Density-gradient centrifugation

To a test tube, 2 mL of a 40% gradient (PureSperm® 40 Nidacon Gothenburg Sweden) and 2 mL of an 80% gradient (PureSperm® 80 Nidacon Gothenburg Sweden) were added, forming two layers with the 40% gradient at the top. A liquified semen sample of 850 µL was placed above the 40% gradient layer and the tube was centrifuged at 300 x g for 20 minutes, generating motile spermatozoa to end up in the pellet separated from other parts of the seminal fluid, as shown in **Figure 4**.

The upper layer, down to 0,5 mL, was then discarded and the pellet was transferred to a new test tube. The washing of the spermatozoa was performed by the addition of sperm wash (PureSperm Wash, Nidacon, Gothenburg, Sweden) to the test tube containing the pellet, up to a volume of 5 mL. The test tube was then centrifuged at 500 x g for 10 minutes. After the centrifugation, the upper layer was discarded down to a definite volume of 0,5 mL, 1 mL, or 3 mL dependent on the pellets size. The pellet was mixed with the solution by pipetting up and down and 10 µL was then used for further analysis on a counting chamber.

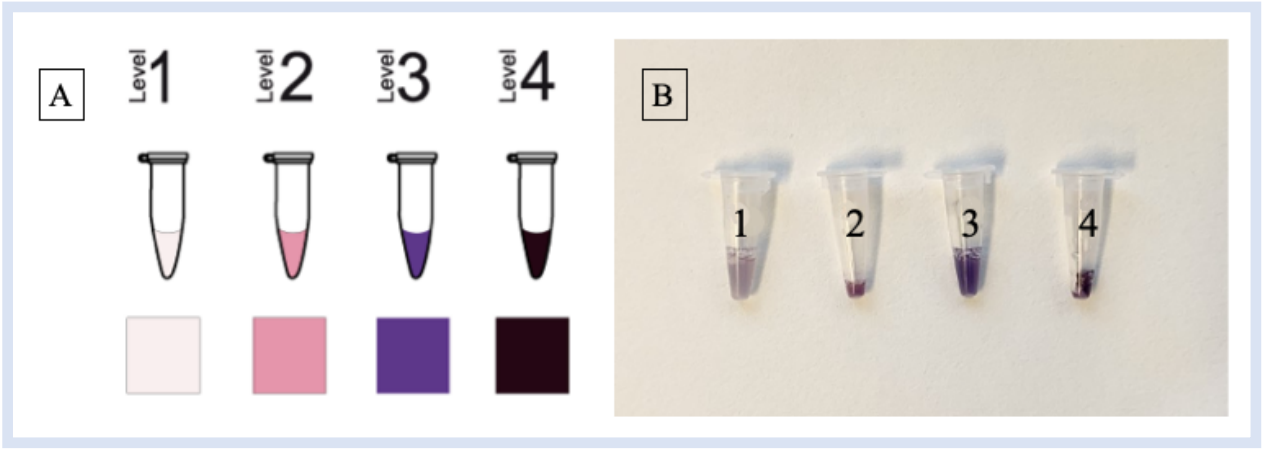

***Figure 3**. (A) The color scheme included in the Oxisperm Kit HT-OS20 used to decide the level of oxidative stress and (B) examples of tests corresponding these levels. Pre-classified levels of intensity: Level 1: Low; Level 2: Low-medium; Level 3: Medium; Level 4: High.*

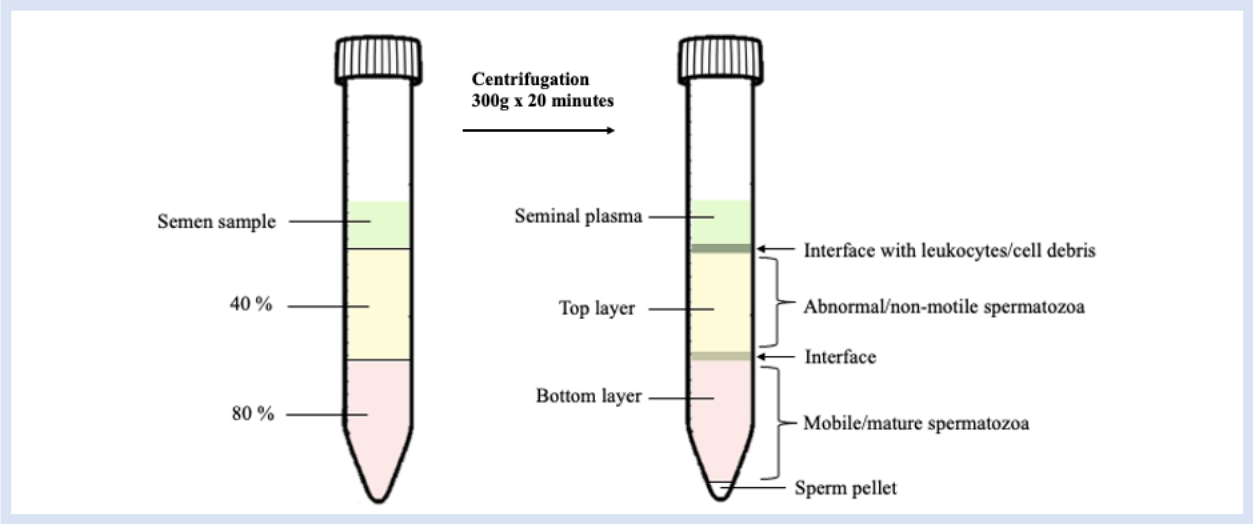

***Figure 4.** Schematics of density-gradient centrifugation (DGC). A fresh semen sample is placed upon two gradients followed by centrifugation, collecting motile and mature spermatozoa in a pellet at the bottom of the tube leaving abnormal/non-motile spermatozoa and cell debris in the phases above.*

2.4.2 ZyMōt Multi

To the ZyMōt™ Multi (850 µL) Sperm Separation Device (ZyMōt Fertility Inc MD USA) (**Figure 1**), 850 µL of liquified semen sample was injected into the inlet port using a 1 mL syringe. 800 µL of media (G-IVF™ PLUS, Vitrolife, Gothenburg, Sweden) was drawn into a new 1 mL syringe and 50 µL of the media was injected into the outlet port until the media reached the membrane. The remaining media was applied over the membrane by dropping so that the membrane was completely covered and connected with the droplet inserted in the outlet port. The device was put in a petri dish, covered, and incubated at 37 °C for 30 minutes. After incubation, 520 µL of the prepared

sample was drawn with a fresh 1 mL syringe and transferred to an Eppendorf tube. From the Eppendorf tube, 10 µL was used for further analysis in a counting chamber.

2.5 Sperm DNA-fragmentation analysis

For this method, 500 µL of the samples prepared with ZyMōt and DGC were placed in a 2 mL Eppendorf tube each, marked with either a "G" for Gradient or a "Z" for ZyMōt. The tubes were then blinded with the help of a colleague who covered up the markings with stickers of different colors. The samples were then connected to colors and after analysis the sticker was removed, and the method of origin was revealed. If necessary, the samples were diluted with sperm wash (Nidacon PureSperm Wash) to a maximum concentration of 20 million sperm/mL.

The analyzation was performed using the Halosperm® (Halotech DNA SL Madrid Spain) kit HT-HS10. The agarose-containing Eppendorf tube included in the kit was put in a water bath at 93 °C for melting and further kept at 37 °C to prevent gelification. 50 µL of prepared sperm sample was mixed with the agarose in the Eppendorf tube, then 10 µL from this mix was placed on the sample cell on the included microscope slide and covered with a coverslip. The microscope slide was then incubated for 5 minutes at 4 °C for solidification of the agarose. Next, the coverslip was removed, and the slide was put in an incubation tray containing a denaturation solution (120 µL denaturation acid in 15 mL distilled H_2O) for 7 minutes for the denaturation of DNA. The slide was then placed in a lysis solution (LS) for 25 minutes for the removal of nuclear proteins. The slide was transferred to a new tray containing distilled water to wash off the LS for 5 minutes. For dehydration, the slide was placed first in 70% ethanol and then in 100% ethanol for 2 minutes each. It was then left to dry on a paper towel at room temperature. The slide could be stored in the dark at room temperature or staining could be performed directly.

2.5.1 Mechanism of action

The kit used is based on the sperm chromatin dispersion technique where an acid treatment denatures the DNA, and a lysis solution removes most of the nuclear proteins. In sperms with non-fragmented DNA, loops of the DNA create halos that can be observed around the head of the sperm which does not occur in sperms with damaged DNA.

2.5.2 Staining

Staining of the slide was performed using Brightfield Staining Kit (Halotech DNA SL Madrid Spain) HT-BFS. A paraffin pen was used to draw a circle around the pre-marked circle on the microscope slide from the DNA fragmentation procedure. A few drops of Staining Solution A were placed inside the paraffine circle and incubated for 7 minutes. The stain was removed by carefully pipetting, then vertically turning the slide and gently knocking off the overflow solution. A few drops of Staining Solution B were then placed on the slide, incubated for 8 minutes, and removed in the same way as solution A. The slide was left to dry before analysis.

2.5.3 Visualization

For visualization and counting of spermatozoa to decide the degree of SDF, brightfield microscopy with a 40x objective was used. The sperms were divided into two groups, with or without fragmentation. This was done by grading the sperms on a scale of 1-5 using a scheme included in the kit (**Figure 5**). Spermatozoa with a big halo (grade 1) or a medium halo (grade 2) counted as non-fragmented, and spermatozoa with a small halo (grade 3) or without a halo (grade 4), as well as degraded (grade 5), counted as spermatozoa with DNA fragmentation. The percentage of spermatozoa with fragmented DNA was calculated by dividing the number of spermatozoa graded 3-5 by the total amount of counted spermatozoa and then multiplying it by 100. A total of 200 spermatozoa

with distinct tails and reasonably normal morphology, regardless of grade, were counted. **Figure 6** shows the visualization of spermatozoa in the brightfield microscopy, examples of cells excluded, and the cells counted with their designated grade.

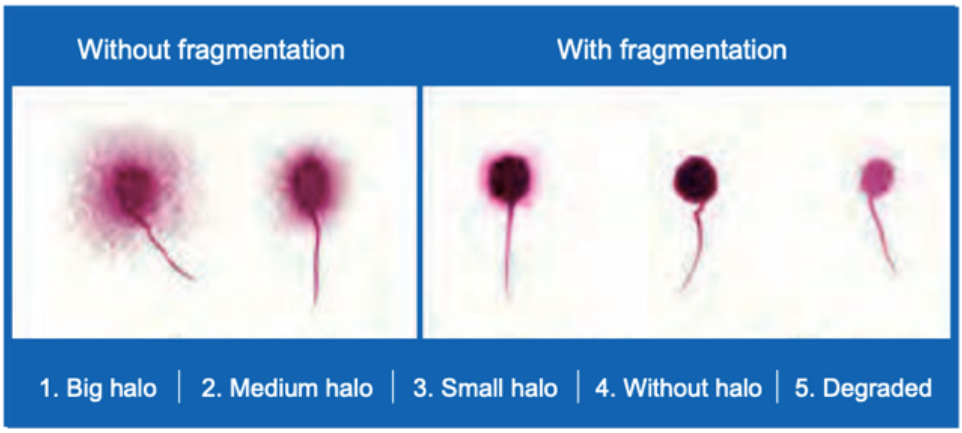

Figure 5. *Visualization and grading scheme included in the Halosperm ® kit HT-HS10 of spermatozoa after denaturation treatment and coloring. Spermatozoa graded 1-2 were classified as spermatozoa without DNA fragmentation. Spermatozoa graded 3-5 were classified as spermatozoa with DNA fragmentation.*

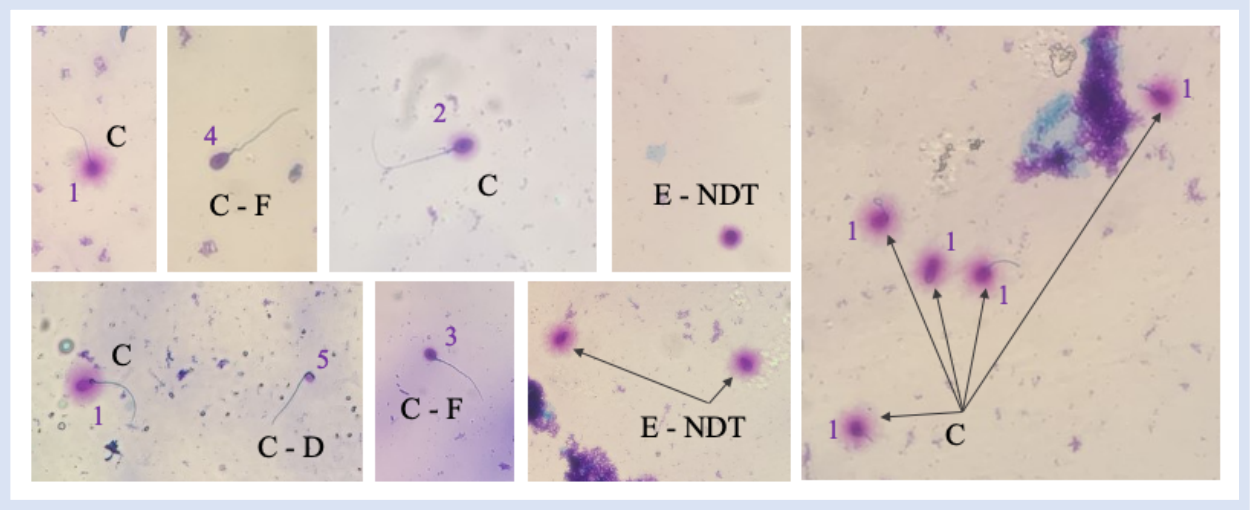

Figure 6. *Brightfield microscopy image of spermatozoa after denaturation treatment and coloring. A total of 200 sperms were counted for the assessment of DNA fragmentation. The abbreviations show which cells were excluded and why, cells counted and their designated grading. C = counted, E = Excluded, F = Fragmented, D = Degraded, NDT = No distinct tail. Grading: 1: Big halo; 2: Medium halo; 3: Small halo; 4: Without halo; 5: Degraded. Grade 1-2 equals spermatozoa without DNA fragmentation. Grade 3-5 equals spermatozoa with DNA fragmentation.*

2.6 Statistical analysis

RStudio v. 4.3.0. was used to perform all statistical analyses and a value of $p < 0,05$ was considered significant. Shapiro-Wilks test was used to test for normal distribution which was excluded for all parameters. For paired comparisons between the methods, Wilcoxon matched-pairs test was used, and the descriptive statistics were displayed with median values and interquartile ranges (IQR). Median-based linear models were used to test for a relationship between the level of oxidation in the unprocessed semen sample and SDF for each method.

3. Results

3.1 Origin semen samples

A total of 25 samples that met the criteria for a normal semen sample were analyzed regarding certain parameters, including OS, before preparation. These analyzed values are presented in **Table 3** as medians IQR. The median level of oxidation was 2 (IQR: 1-3), corresponding to a low-medium OS level.

Table 3. Analysis parameters of the fresh semen samples*. Shown as medians with interquartile ranges (IQR).*

Parameter	Origin semen sample
Volume (mL)	2,6 (2,1–3,7)
Grade of motility	2,5 (2,5–2,5)
Concentration (10^6/mL)	68,5 (28,8–109)
Proportion of motile spermatozoa (%)	60 (40–70)
Level of Oxidation	2 (1–3)

3.2 Analysis parameters by method of process

Semen analysis parameters obtained after preparation by method of process are shown in **Table 4**. No difference in the grade of motility of spermatozoa was observed, and the data showed only moderate evidence that the TSPC was lower after being processed with ZyMōt compared to DGC ($p = 0,04647$), indicating that preparation with DGC provides a higher yield. The proportion of motile spermatozoa, displayed as a percentage, proved to be significantly higher in the samples prepared with ZyMōt compared to DGC ($p < 0,0001$). This is shown in **Figure 7**, displaying a median of 98% (IQR: 96-98) for ZyMōt and a median of 87% (IQR: 75-93) for DGC. The distribution of SDF by method of process is presented in **Figure 8**. The SDF was significantly lower in the samples being processed by ZyMōt than compared to DGC ($p < 0,0001$), with a median of 2% (IQR: 1-9) for DGC and a median of 0% (IQR: 0-1) for ZyMōt.

Table 4. Semen analysis parameters obtained after preparation by method of process*. Values are shown as medians with interquartile ranges (IQR). Wilcoxon matched-pairs test for comparison between methods, *p < 0,05 versus ZyMōt. N=25.*

Parameter	Density-gradient centrifugation	ZyMōt
Grade of motility	2,75 (2,5–2,75)	2,75 (2,5–2,75)
Total sperm count (10^6)	3,2 (1,2–13,2) *	2,7 (1–4,9)

3.3 Impact of origin semen sample on the outcome parameters

Non-parametric regression models were used to look for an association between the level of oxidation in the origin semen sample and the SDF rate of the prepared semen sample. No significant relationship was observed for either DGC ($p = 0,7949$) or ZyMōt ($p = 0,373$).

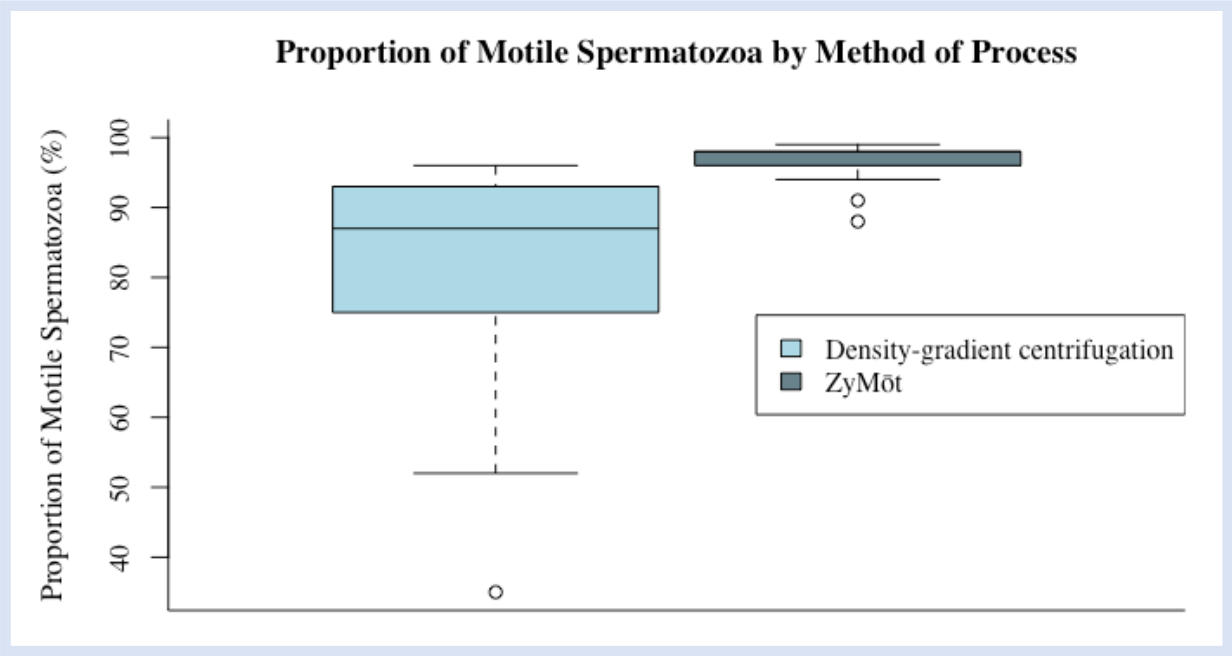

Figure 7. *The proportion of motile spermatozoa in samples prepared by either ZyMōt or density-gradient centrifugation (DGC) displayed as a percentage value. Wilcoxon matched-pairs test for comparison between the methods showed a significant difference ($p < 0,0001$). Box plots: Median is represented as a horizontal line within the box, the top and bottom of the box refers to the first and third quartile, and whiskers showing the highest and lowest data point excluding outliers. ° = outliers. N=25.*

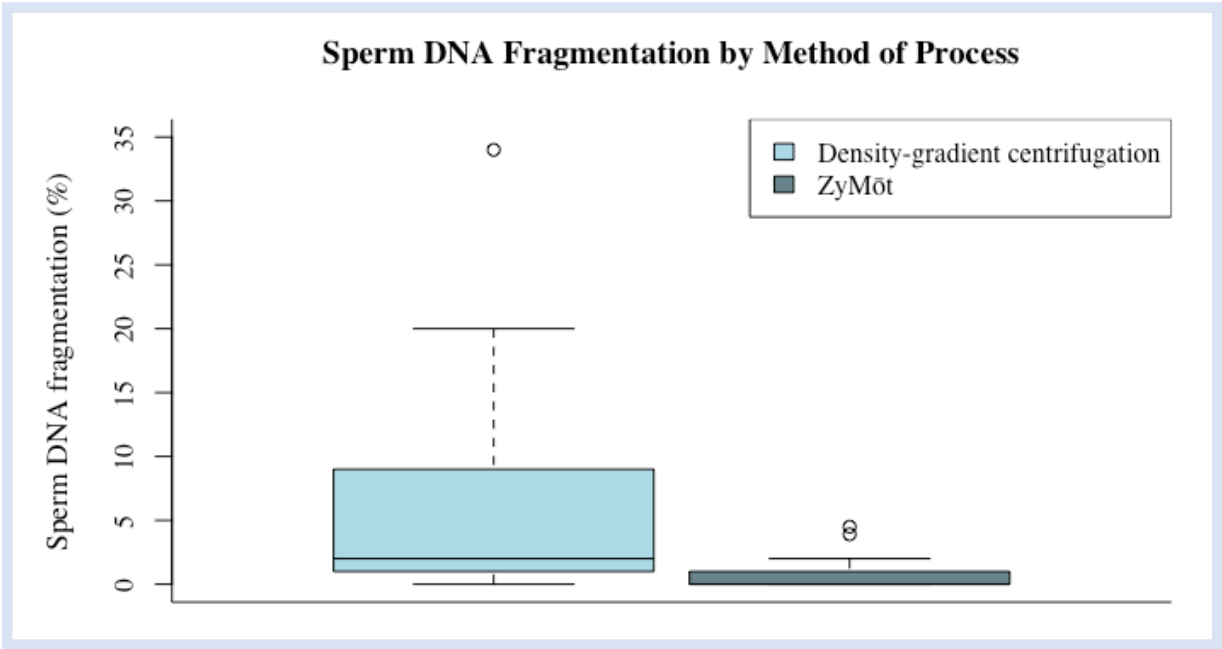

Figure 8. *The percentage of Sperm DNA fragmentation (SDF) of spermatozoa in samples prepared by either ZyMōt or density-gradient centrifugation (DGC). Wilcoxon matched-pairs test for comparison the between methods showed a significant difference ($p < 0,0001$). Box plots: Median is represented as a horizontal line within the box, the top and bottom of the box refers to the first and third quartile, and whiskers showing the highest and lowest data point excluding outliers. ° = outliers. N=25.*

4. Discussion

The quality of the prepared semen sample has a great impact on the outcome within ART (Ahmadkhani et al., 2022), thus, this pilot study was performed with the aim to evaluate one of the new microfluidic methods appearing on the market of sperm-selecting techniques. The results from this study indicate that the microfluidic ZyMōt Multi sperm separation device generates a sample

containing a high number of motile spermatozoa with an almost complete absence of DNA fragmentation.

4.1 Main parameters

The spermatozoa retrieved from the samples prepared with ZyMōt showed significantly lower SDF than those prepared with DGC. Other studies have reported similar results when using microfluidic devices and comparing the outcome to conventional sperm preparation methods (Asghar et al., 2014, Mirsanei et al., 2022, Parrella et al., 2019, Quinn et al., 2018, Cabello et al., 2023), supporting the observations made in this study. An important part of this evaluation was the blinding of the tests, excluding potential bias and verifying the results obtained even further. The removal of the centrifugation step might be one reason why the SDF was lower after the microfluidic process since centrifugation is proven to generate more ROS (Aitken and Clarkson, 1988, Iwasaki and Gagnon, 1992) which can lead to DNA fragmentation (Agarwal et al., 2020, Dutta et al., 2021, Esteves et al., 2021, Sakkas and Alvarez, 2010). However, it cannot be said that this is the cause of the difference observed in this study since no measurements of SDF were made on the origin sample. It might instead be so that the DGC has an inferior capability to sort out spermatozoa with a damaged genome (Zini et al., 2000). SDF has been shown to have a great impact on embryo development and in some cases even affects implantation and pregnancy rates (Horta et al. 2020; Seli et al. 2004; Simon et al. 2010, Bungum et al. 2007; Malić Vončina et al. 2021; Simon et al. 2011). The result for this parameter is thus of great interest since the grade of DNA fragmentation cannot be estimated by only evaluating the quality of the spermatozoa using the conventional semen parameters. And since the evaluation of SDF is not part of the base semen analysis when using IVF or ICSI, a method for sperm preparation minimizing DNA-damaged spermatozoa might be an important step towards better outcomes within IVF.

The proportion of motile spermatozoa showed to be significantly higher in the samples prepared with ZyMōt than with DGC, a result also obtained in similar studies (Mirsanei et al., 2022, Parrella et al., 2019, Quinn et al., 2018, Cabello et al., 2023).

The total amount of spermatozoa obtained after preparation was somewhat lower when ZyMōt was used compared to DGC. This has also been shown in other studies comparing conventional semen preparation methods to microfluidic methods and might indicate the devices' high selectivity (Mirsanei et al., 2022, Parrella et al., 2019, Quinn et al., 2018, Cabello et al., 2023). Since a low sperm count might be one parameter influencing the choice of ART treatment to be used (Hreinsson et al., 2005), the lower yield obtained with ZyMōt might result in increased use of ICSI treatments.

For the degree of motility, both methods generated equally good quality spermatozoa. However, the measurement of this parameter was somewhat difficult since the evaluation was performed as a visual estimation and was to be fitted on a four-point scale. In most cases, the spermatozoa in the samples from both methods were observed to move just as actively. Perhaps in the future, differences could be observed more clearly if other methods involving automized technology were to be used. One example being a study where they investigate a microfluidic system developed to electrically measure the motility of the spermatozoa (de Wagenaar et al., 2016).

Several other studies have demonstrated that OS is the most common factor causing SDF production (Agarwal et al., 2020, Dutta et al., 2021, Esteves et al., 2021, Sakkas and Alvarez, 2010) leading to a presumption that the level of OS in the native semen sample would reflect the outcome for SDF. However, in this study, no correlation was observed between the level of OS in the origin semen sample and the DNA fragmentation rate for the samples obtained from either of the methods. A factor that might have had an impact on these results was that DNA fragmentation analysis was performed only after preparation. Thus, it could have been of interest also to measure SDF on the

origin semen sample, ruling out the effect either one of the sperm preparation methods might have had on these parameters.

In this study, no measurements were performed to compare the effectiveness between ZyMōt and DGC. However, an individual estimate agrees with other studies (Asghar et al., 2014, Kushnir et al., 2022, Parrella et al., 2019, Quinn et al., 2018) that ZyMōt is experienced to improve laboratory efficiency with a less complex protocol and with minimized sample transfers. The exclusion of the centrifugation step may not only rule out the production of ROS but also eliminates the use of large equipment. The advantages of this new microfluidic method have the potential to lead to a more standardized and automized process for sperm preparation within ART, which could also minimize human error as well as reduce hands-on time.

4.3 Limitations and Future Work

The parameters in this study were analyzed as visualized estimations or counting, which quite easily could be affected by bias. This was somewhat excluded by blinding for some tests and others were restrained by counting chambers. As a way of minimizing this potential subjectivity for further studies, a suggestion might be that the analysis is performed by two or more persons.

The limited sample size may be challenging and diminish the results' dependability. However, this has merely been a pilot study with the aim to gather sufficient data to give an implication on the capacity of this new microfluidic method, which I would say have been accomplished. However, in this study, nothing can be said about which one of these methods will generate a better final ART outcome. Some studies have examined this, observing the outcomes of ICSI treatments with spermatozoa prepared with either microfluidics or conventional techniques. One showed no difference in fertilization rate between the methods but did observe a moderately significant difference in clinical pregnancy rates (Zaha et al., 2023). Contradictory, another study observed no significant difference in clinical pregnancy and ongoing pregnancy rates (Quinn et al., 2022). Further, a study where a group of couples with a history of low fertilization rate underwent ICSI and was compared to a control group with first-time ICSI couples, testing for different outcomes if either microfluidics or a conventional sperm preparation method were used (Mirsanei et al., 2022). They showed that for the fertilization rate, a significant difference was observed between the methods for the study group but not for the control group. They also showed that embryo quality was improved when microfluidics was used in both groups. However, further studies need to be performed to gather sufficient data on how the use of microfluidics affects the final clinical outcomes of ART. Primarily ICSI treatments have been performed after processing with microfluidics and if possible, it would be interesting to see how the method affects the outcome within normal IVF treatments, which might be somewhat challenging regarding the limited volume that the device can hold. Another limitation that comes with the evaluation of sperm preparation methods for final outcomes within ART, is that other factors than the sperm preparation technique used can affect and influence pregnancy rates (Zaha et al., 2023). It might also be important to notice that a result implicating no difference between the methods might still be in favor of the new microfluidics since it can improve laboratory efficiency, as long as it does not affect the clinical outcome.

4.4 Conclusion

The use of assisted reproduction technology is increasing every year and will presumably continue to do so, carrying out the very important mission to help build families all over the world. With knowledge of the global decrease in sperm quality, the preparation of semen samples in this process might come to play a larger role than ever before. Samples prepared with ZyMōt generated spermatozoa with a higher proportion of motility and a lower grade of DNA fragmentation compared to those prepared with DGC. No difference observed for the results regarding the motility grade

between the methods still indicates ZyMōt being a method generating a good quality sample, and the lesser yield might prove the selectiveness of this method regarding the very best spermatozoa. Regardless of the parameter outcome, ZyMōt proved to be easier to perform minimizing hands-on time, and thus might be a preferred alternative to conventional preparation methods.

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Appendix

Appendix 1

Dnr 2022-05602-01

Lund avdelning 1 medicin

BESLUT OCH YTTRANDE

2023-01-24

Sökande forskningshuvudman
Västra Götalandsregionen

Forskare som genomför projektet
Charles Christian Hanson

Projekttitel
Mikroflödespreparation av spermier för assisterad befruktning - Utvärdering av Zymot - multisperm separation device

Uppgifter om ansökan
Ansökan inkom till Etikprövningsmyndigheten 2022-10-20 och blev valid 2022-10-27. Ansökan är tidigare behandlad vid sammanträde 2022-11-15. Av myndigheten begärd komplettering enligt beslut inkom 2022-12-12.

Etikprövningsmyndigheten beslutar enligt nedan. Etikprövningsmyndigheten lämnar samtidigt ett rådgivande yttrande enligt 4 a § förordningen (2003:615) om etikprövning av forskning som avser människor.

BESLUT

Etikprövningsmyndigheten avvisar ansökan, det vill säga tar inte upp ansökan till prövning.

Skäl för beslutet
I det aktuella projektet kommer det inte att göras något ingrepp på en forskningsperson eller annan intervention på sätt som anges i 4 § etikprövningslagen. Det kommer inte att ske någon behandling av personuppgifter på så sätt som anges i 3 § etikprövningslagen. Mot bakgrund härav omfattas inte studien av bestämmelserna i 3-4 §§ etikprövningslagen och ska därför inte etikprövas.

RÅDGIVANDE YTTRANDE

Etikprövningsmyndigheten har inte några etiska invändningar mot forskningsprojektet.
