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Efficiency of sperm separation by using microfluidic chips compared to the swim up method

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Abstract

The effectiveness of assisted reproduction methods is still not as high as was expected recently. For effective therapy, it is necessary to use only the highest quality sperm for oocyte fertilization, as the fertilization process most often takes place using the ICSI technique, i.e. direct injection of sperm into the oocyte. For these reasons, separation techniques are constantly being developed, and in a relatively short period of time the view of these procedures has changed. In recent years, microfluidic chip techniques have been increasingly applied. In this study, we compared the swim up technique with the two most used chips (ca0 and ZYMOT). We tested the efficiency of these separation methods against the swim-up method. In this study, we evaluated sperm concentration, motility, morphology, acrosome status and DNA integrity before and after separation methods. Each patient's ejaculate was separated simultaneously by the swim up method and both chips. It turned out that the separation methods are very similar in motility, concentration and acrosome status and sperm morphology. However, in the case of DNA fragmentation, a significant difference in the reduction of the proportion of fragmented spermatozoa was found only with the chips and not with the swim-up method. Moreover, when comparing the relative effectiveness of sperm reduction with fragmented DNA, the ZYMOT method was more effective than Ca0. However, the differences between the chips were not statistically significant with an average of 18%.

Running title: Microfluidic chips separation and swim-up

Keywords: spermatozoa, microfluidic, DNA integrity, swim-up, sperm preparation

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Introduction

Ejaculated spermatozoa are selected very intensely in *in vivo* conditionts. Sperm separation in vivo is, therefore, very intense and is a multistep process that starts immediately after ejaculation at the level of cervix uteri. Is it massive selection from the several hundreds of millions, only a few tens or hundreds sperm cells reach the ovulated oocyte [1]. Before in vitro fertilization, sperm cells suitable for utilization must be carefully selected. Choice of a suitable sperm separation method for ICSI is an important step and there are several methods available currently [2]. There are many ways of separating human sperm (review). The most widely used are the traditional methods (swim-up, density gradient centrifugation – DGC), which were developed at a time when the primary method of fertilization was metocouple conventional IVF. With the development of the ICSI method, there was an increased requirement for careful sperm selection. At this time, other methods were developed based on various parameters such as their zeta potential (zeta quotation) sperm morphology (IMSI) or binding to hyaluronic acid (PICSI). Some of these methods are almost no longer used today (IMSI). However, in recent years there has been a massive development of MFSS methods. These methods are simple elegant, no need for centrifugation and are often presented as very gentle and effective. However, it is always important to verify the efficacy of these methods. Standard parameters such as concentration, motility or sperm morphology are not sufficient to objectively assess the efficiency of separation. In a number of cases of male infertility, the cause cannot be determined by these basic parameters [2,3]. The state of the acrosome is indicative of the overall level of sperm damage and is a relatively sensitive parameter in their evaluation. DNA fragmentation is another key parameter of sperm condition critical to the final outcome of fertilization. Sperm with intact DNA is essential for successful IVF and embryo development. Tested microfluidic separation methods have been presented as a very efficient and gentle sperm separation system [4-6]. Microfluidic separation is often referred to as a technique that removes sperm with fragmented DNA and yields better IVF results compared to standard sperm selection techniques [4,5,7].

The aim of our study was to compare the swim-up sperm separation method with two microfluidic chip methods.

Material and methods

Patients

In this study was included six non-smokers patients from Center of Reproductive Medicine in age (24-39) with an ejaculate volume higher than 2.5 mL. The study was approved by the ethics committee of University Hospital Brno. All the patients enrolled in this study consented to participate and signed an informed consent form. To exclude the negative effect of high ROS levels, the level of ROS/RNS in seminal plasma was measured before the start of the analyses. The level of ROS/RNS was measured using a commercial kit OxiSelect™ (In Vitro ROS/RNS Assay Kit).

Spermiogram analyses

The semen specimens were obtained following an abstinence period of 2–5 days and collected in sterile containers via masturbation. Following liquefaction for 60 min, a semen drop of 10 µl was loaded to a Makler counting chamber for evaluation of motility and concentration. Morphology was detected using fixed spermatozoa coloured with a specific dye Diff-Quik (MICROPTIC SL Co., Barcelona, Spain) and evaluated under 100x magnification. For all of these evaluations, an inverted phase contrast microscope (Nikon ECLIPSE Ts2, Germany) was used. The analysis of sperm concentration and motility was performed in raw semen samples according WHO manual [8].

Experimental design

For each patient, the ejaculate was separated into four parts after liquefaction (60 min after ejaculation): the 1st part of the ejaculate (0.2 mL) was not separated and only analysed on ROS/RNS concentration, the 2nd part (900 µL of ejaculate) was separated using a Ca0 microfluidic chip (LensHOOKE Bonraybio, Taichung, Taiwan), 3rd part (850 µL of ejaculate) was processed by using ZYMOT microfluidic chip (Cooper, USA) and the 4th part (the rest of the ejaculate; ≥500 µL) was processed using the swim-up method (Figure 2). After separation all samples were collected and evaluated on sperm concentration, morphology, motility, DNA fragmentation and acrosomal status (**Fig. 1**).

Preparation of spermatozoa

Following the liquefaction of the semen sample, the total volume in each individual subject was divided into four subgroups:

1) The unprocessed ejaculate for basic examination of concentration, motility, morphology, DNA fragmentation and quantification of ROS/RNS.

2) Microfluidic chip Zymote: We used the microfluidic sperm sorting chip ZYMOTE® (Cooper, USA). It is a device with an inlet and outlet port. This chip does not require any pre-treatment of the semen sample. At first, 900 µl of liquefied semen are loaded to the inlet port by a syringe. After adding the sample to the chip, both ports are overlaid by 2 µl of mineral oil. The samples are incubated for 30 min at 37 °C at humidified incubator. After that, the spermatozoa are carefully removed in 500 ul suspension from outlet port by using a pipette.

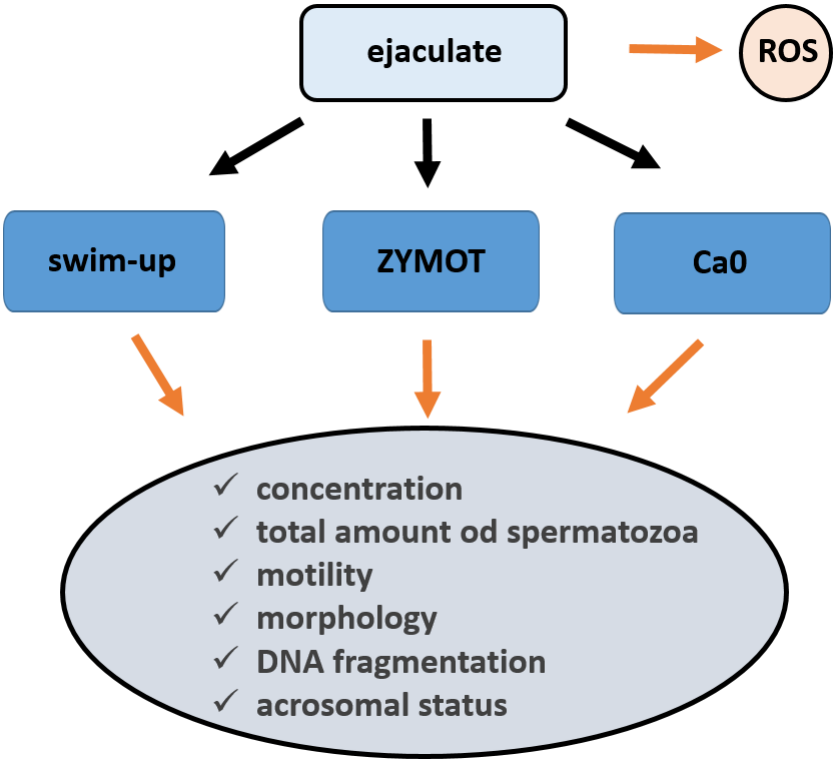


FIGURE 1 Flow chart of analyses

3) Microfluidic chip Ca0: Second microfluidic sperm sorting chip was also a commercial chip – Ca0 device (LensHOOKE, Bonraybio, Taichung, Taiwan) it was used in accordance with the manufacturer's instructions. In brief, 1000 µL of neat semen was filled into the lower part of chamber. Subsequently 900 µL of Sperm Preparation Medium (Origio, Denmark) was added into the upper chamber and covered by cover piece of chip. The device was then placed into a humidified incubator at 37°C for a duration of 30 minutes. Following the incubation period, 500 µL of sperm suspension was extracted from the upper chamber for subsequent analysis

4) Swim-up method

Semen samples were pipetted into 15 ml conical centrifuge tubes and washed twice in 2 ml Sperm Preparation Medium (Origio, Denmark) in accordance with the manufacturer's instructions. Following the second wash, 1000 µL Universal IVF medium (Origio, Denmark) was gently overlaid onto the spermatozoa. The tube was then inclined at an angle of approximately 45° to increase the surface area of the semen-culture medium interface and subsequently incubated for 60 min at 37 °C. Following this incubation period, only 400 µL from the surface were collected for analysis. After the second washing, the spermatozoa were gently overlaid by 1000 µL Universal IVF medium (Origio, Denmark) was gently overlaid onto the spermatozoa.The tube was put an angle 45°, to increase the surface area of the medium interface, and incubated for 30 min at 37 °C. After this time, only 500 µl from the surface were collected for the sperm analyses.

Evaluation of DNA integrity

Sperm DNA fragmentation was assessed by the Halosperm G2 kit (Halosperm G2 kit, Halotech, Spain in accordance with manufacturer's instructionts. As provided in the manufacturer's instructions, the spermatozoa with big and medium halo were considered free from DNA fragmentation [9]. The proportion of spermatozoa with fragmented DNA was evaluated. The DNA fragmentation index (DFI) was calculated by this formula: DFI (%) = (fragmented spermatozoa + degenerated spermatozoa / total spermatozoa counted) × 100. Minimum of 500 spermatozoa were evaluated in each sample under the x40 objective of the microscope. To mitigate potential bias, at least 250 spermatozoa were analyzed by two independent technicians. The DFI value of the unprocessed samples was used as the starting point for calculating the relative separation efficiency.

Evaluation of acrosomal status

For acrosomal visualisation was used lectin peanut agglutinine labeled fluorescein isothiocyanate (FITC-PNA). Sperm samples (5 µl) were firtly airdried on glass slides and afterthat were fixed in ice-cold ethanol, again dried, and coated with solution containing FITC-PSA (Sigma Adrich, Germany) for 30min in dark. Samples were mounted under antifade Vectashield medium with DAPI (Vector Laboratories, USA) and covered by coverslipes. A fluorescence micro-scope (Nikon ECLIPSE Ts2, Nikon, Japan) was used to examine the fluorescence patterns of 200 sperm cells from random areas, at

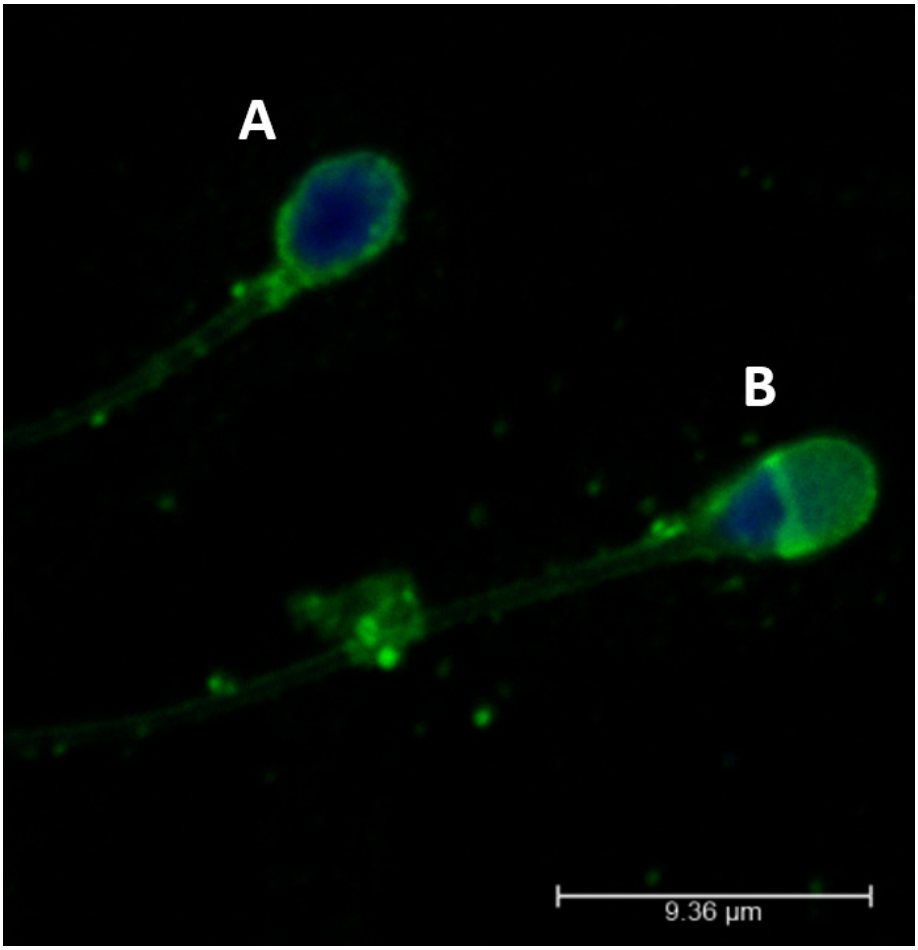


FIGURE 2 Visualisation of acrosome by FITC-PNA: A – non detected acrosome, B – intact acrosome, green – acrosome, blue – DNA

x1000 magnification. Acrosomal status was categorized using distinct lectin staining patterns: 1) complete acrosome staining, indicating an intact acrosome; 2) partial acrosome staining, signifying sperm with disturbed acrosome; and 3) no staining of the entire sperm head, indicating a sperm with detached acrosome (**Fig. 2**).

ROS/RNS assessment

The concentration of reactive oxygen/nitrogen species (ROS/RNS; including hydrogen peroxide as one of the primary reactive oxygen species present in semen) in semen samples was quantified using a commercially available assay kit. The total quantity of ROS/RNS was determined using the OxiSelect™ In Vitro ROS/RNS Assay Kit (Cell Bi-olabs, San Diego, CA, USA), in accordance with the manufacturers protocol. The fluorescence signal was measured using a Fluostar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) at an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. Sample was freeze in liquid nitrogen immediately after end of 60 min (time for liquefaction) after ejaculation.

Statistical analysis

The STATISTICA CZ software, version 10 (StatSoft, Inc., Prague, Czech Republic) was used to perform the statistical analysis. Data were expressed as mean ± standard error of the mean. Comparison of numeric variables between the groups was performed using one-way analysis of variance. Differences were considered statistically significant when $P< 0.05$.

Results

In this study, 6 patients with an average age of 32.5 years and an average ejaculate volume of 5.4 ml were included. Five patients were normozoospermic and one was teratozoospermic. Due to the large volume of ejaculate, three types of sperm separation were performed after liquefaction: swim-iup, Ca0 and ZYMOT.

After separation, there was a significant decrease in sperm concentration compared to the untreated ejaculate, but no significant difference in either method compared to the other separations. In terms of motility, there was a significant increase in the proportion of motile sperm after separation but no difference was found between separations.

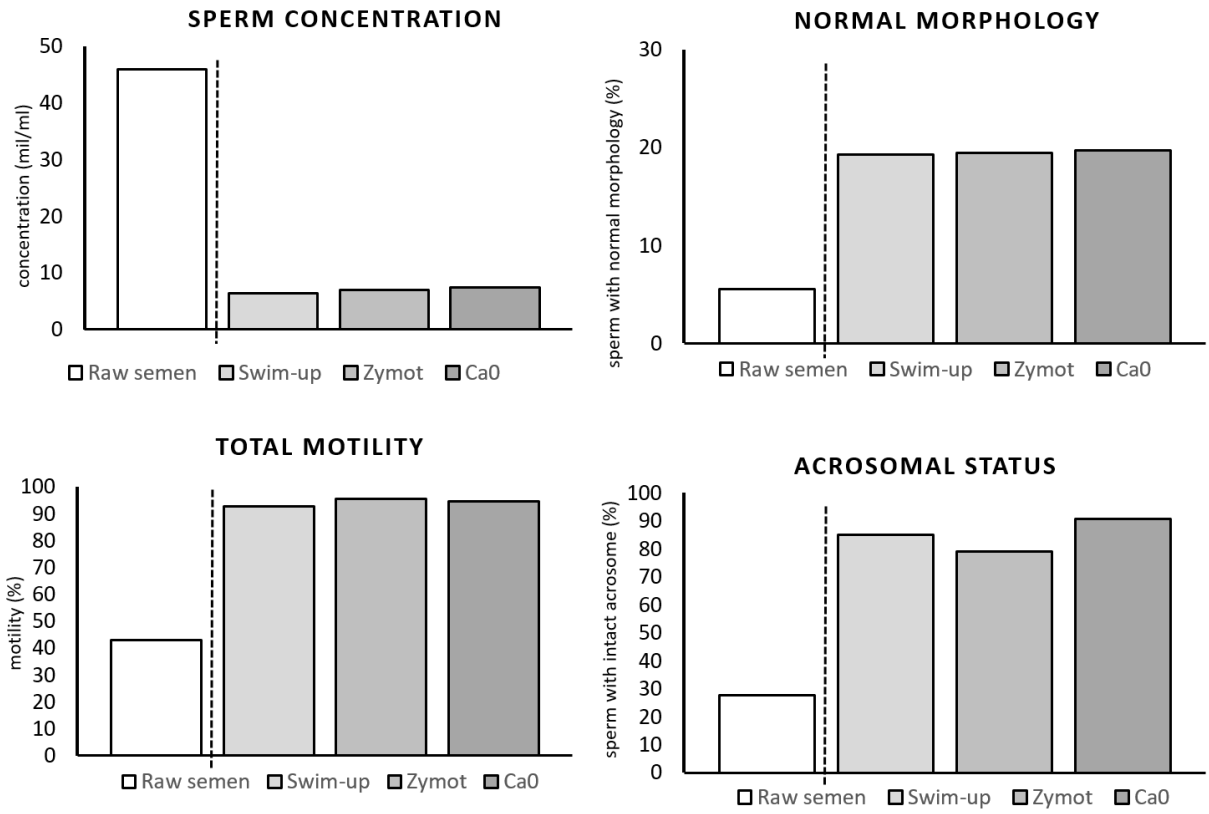


FIGURE 3 Graphical presentation of separation efficiency in context proportion of spermatozoa with fragmented DNA (DFI-DNA fragmentation index) by the swim-up, Zymot and Ca0 methods. A – Comparison of absolute values. Statistically significant differences are indicated by asterics (P< 0.1); B – Visualisation of relative effectivity separation in context DFI values. The numbers express the relative efficiency of separation in terms of the proportion of sperm with fragmented DNA

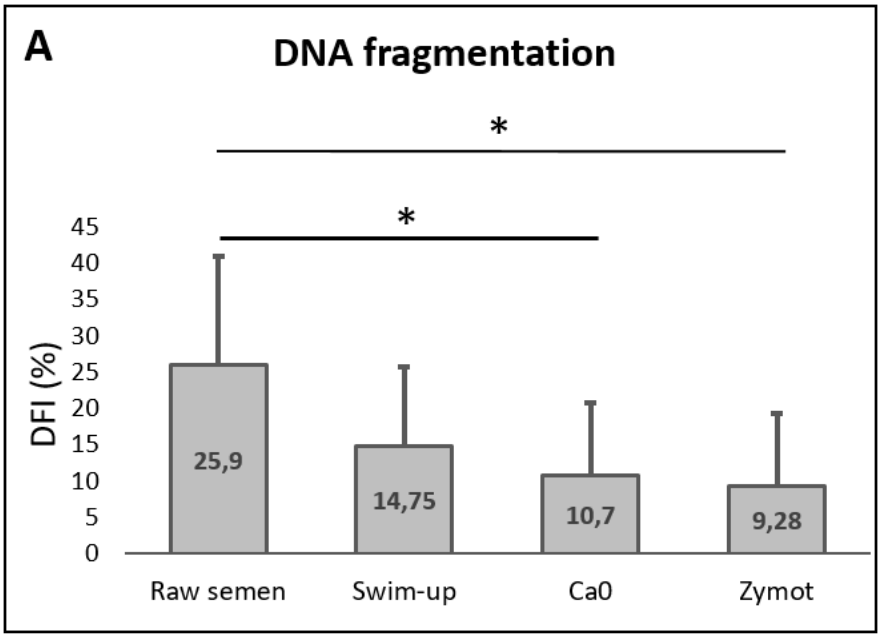
In the evaluation of morphology, a significantly higher proportion of sperm with normal morphology was found compared to the control but there was no difference in this parameter between the three separation methods studied. When the proportion of sperm with intact acrosome was observed, again a difference was found compared to the untreated sample, but no significant difference was found between the methods in terms of separation techniques (**Fig. 3**).

In the case of sperm DNA fragmentation detection analysis, differences between separations were found. The initial DNA fragmentation values before separation were relatively low (on average 25.3%) and only one patient had a really high proportion of sperm with fragmented DNA (53.13%). After separation with the swim-up method, there was a non-significant decrease in the DFI value to 14.75%. In the case of the Ca0 method, there was a statistically significant difference between the proportion of DFI before and after separation. There was a reduction to 10.7% DFI. In the case of the ZYMOT method, an even more statistically significant reduction from the control was found to 9.28% DFI. When comparing all three methods, there was no statistically significant difference in the proportion of DFI between the methods.

A comparison of absolute values expressing how significant the reduction in the proportion of sperm with fragmented DNA was is shown in Figure 2B. Here it can be seen that the swim-up technique itself reduced the proportion of sperm with DNA fragmentation to about 50%. The Ca0 method was more effective, reducing to 38.8% of the original DFI values and the most effective in this effect was the ZYMOT method, which reduced the DFI values to about a quarter of the original values, i.e. down to 27.7% of the original DFI (**Fig. 4**).

ROS/RNS concentration

It was previously described that H_2O_2 (one of the most important member of ROS family) demages sperm sperm, but it was observed only at concentrations above 200 μM. Concentrations of H_2O_2 below 200 μM do not have a significant negative effect on sperm [10]. In our cohort, we detected a total H_2O_2 levels and found in averaged at 36.78 μM with a maximum value of 76.09 μM (**Fig. 5**). These values are significantly below the threshold level of 200 μM.



B

Raw semen	Swim-up	Ca0	Zymot
100	48,11	15,66	13,72
100	47,53	15,99	9,5
100	52,79	30,45	23,1
100	62,13	33,03	35,5
100	22,7	59,31	11,36
100	68,53	78,66	71,33
Ø	50,30	38,85	27,42

FIGURE 4 Proportion of spermatozoa with fragmented DNA (DFI): A – values before and after separation by swim-up, Ca0 and FERTILE method. B – processing efficiency expressed in relative numbers, the initial DFI value of the unprocessed ejaculate is considered 100%

Discussion

In this study, we compared three different methods of human sperm separation. We observed the effectiveness of the swim-up method and two microfluidic chips, which are very common in the Czech Republic. These were the Lenshooke Ca0 and ZYMOT separation chips. We selected only patients who had a sufficient volume of ejeculate (to make it realistic to perform all 3 methods simultaneously) and who had a low content of oxygen radicals in seminal plasma (to exclude the negative effect of high concentrations of oxygen radicals). We evalue ated the spermatozoa in terms of their motility concentration, acrosome morphology and the degree of DNA fragmentation.

The method of sperm separation using the microfluidic chips is often used and is often presented as a very gentle method which significantly decreases the proportion of spermatozoa with fragmented DNA [1,11,12]. MFSS techniques are often considered to have a higher sperm selection efficiency for ART techniques than conventional swim-up or DGC methods [13-15]. In our study, the comparison of separation technics in case of concentration, morphology, motility or acrosomal status we did not find differences. In case of DNA fragmentation we presented that the DFI values of unprocessed samples were significantly higher than after the MFSS separations. However, no significant differences were detected in the DFI values between swim-up and unprocessed samples. In comparison the density gradient centrifugation (DGC) and the microfluidic method (FERTILE) and their effect on separation of spermatozoa with non-fragmented DNA was found a significant difference in efficiency of the separations, with a significant decrease of the spermatozoa with fragmented DNA after MFSS when compared to the DGC method. This phenom-

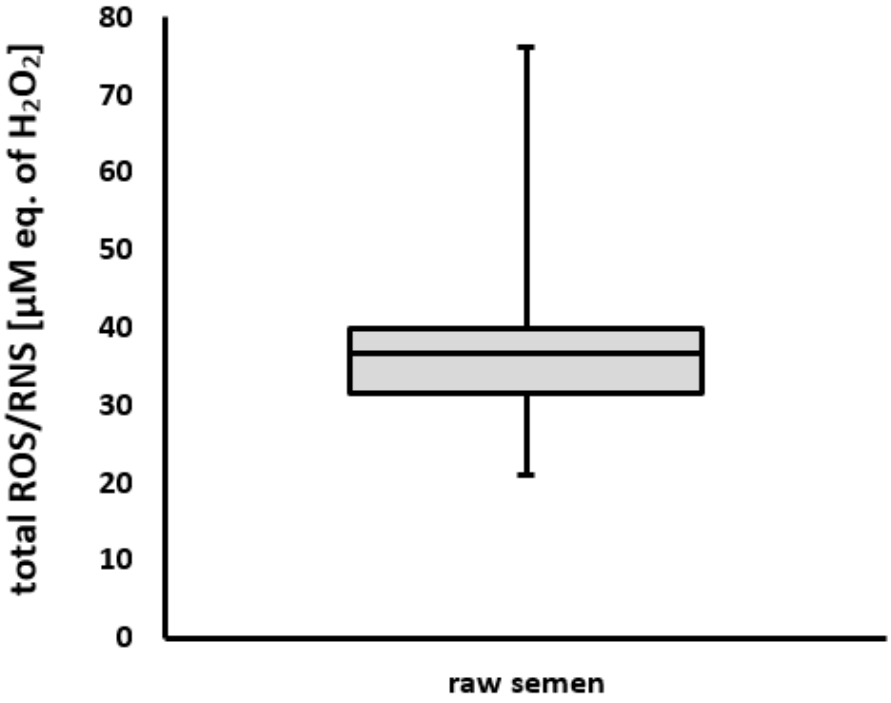


FIGURE 5 H_2O_2 values in seminal plasma. Graphical presentation of H_2O_2 values in analyses samples. The 25th and 75th percentiles are represented by boxes, with the median value, while the 10th and 90th percentiles are represented by whiskers

enon may be attributed to the utilization of the DGC method, which is considered less gentle than the swim-up method employed in the present study. The observed difference could potentially be caused by the adverse effect of the DCG method on sperm DNA integrity. Our laboratory primarily employs the swim-up method, as it has been reported to be more gentle and significantly decrease the proportion of fragmented spermatozoa when compared to the DGC [16].

While MFSS methods work well, the disadvantage of using them is the limited amount of sperm obtained compared to swim-up method. For this reason, the use of the MFSS separation method may not be always advantageous for patients, as it will significantly limit the amount of usable sperm. For microinjection methods this is not a problem, but when is used conventional IVF or even using the IUI method, it may give similar or even worse results than the swim-up method [17].

A comparison between the traditional swim-up method and the FERTILE microfluidic chip, focusing on embryological factors, revealed only one significant difference: the FERTILE technique produced a higher percentage of high-quality blastocysts. No other parameters showed notable variations between the two methods [5].

The appropriate selection of high-quality spermatozoa with intact DNA is crucial. It has been well established that sperm DNA fragmentation negatively impacts the overall efficacy of IVF methodologies [18].

Conclusions

The MFSS separation methods are simple, easy and efficient. They do not require any expensive instrumentation and can be implemented with only basic laboratory equipment without the use of additional chemicals.

In our study, sperm concentration, motility, morphology, acrosome status and DNA fragmentation were examined in spermatozoa after separation. There was no significant difference between the parameters studied. Only in the case of DNA fragmentation, MFSS methods were more efficient than swim-up, and in terms of absolute values in individual patients, Zymot was found to be more efficient than Ca0.

Ethical approval

This research has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by University Hospital Brno Ethical committee with reference number 11-110123/EK project 11/23.

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Conflict of interest

We certify that no actual or potential conflict of interest in relation to this article exists.

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