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Comparison of microfluidic and swim-up sperm separation methods for IVF

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Abstract

Sperm separation for ICSI is an essential step in realization of the IVF procedures. The method of microfluidic separation of sperm cells using chips has been applied more and more frequently in recent years. This method is often presented as extremely gentle to spermatozoa and decreasing significantly concentration of sperm cells with fragmented DNA when compared to conventional methods. The aim of our study was to verify a microfluidic chip system from the perspective of its potential to select spermatozoa with non-fragmented DNA. We tested the efficiency of this separation method against the swim-up method. In this study we evaluated sperm DNA integrity before and after the separation methods in ten patients. Ejaculate of each patient was separated by both the swim up method and the microfluidic chip method at the same time. It was shown that both the methods are very similar in reduction of spermatozoa with fragmented DNA. Interestingly, the concentration of spermatozoa with fragmented DNA was lower after the microfluidic separation than after the swim-up method in all the patients. Nevertheless, the differences were not statistically significant with only 2.1% on average, which is negligible in terms of practical use.

Running title: Microfluidic chip and DNA fragmentation

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

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Introduction

During fertilization in vivo, the ejaculated spermatozoa is being selected very intensely. From the initial number of hundreds of millions, only a few tens or hundreds sperm cells reach the ovulated oocyte. During fertilization in vitro, sperm cells suitable for utilization must be selected carefully. Choice of a suitable sperm separation method for ICSI is an important step and there are several methods available currently [1]. Some of these methods are commonly used (swim-up, density gradient centrifugation - DGC), some are already obsolete (IMSI) and some are applied in specific cases (MACS). In present time, the methods of microfluidic chips are more and more popular. Nevertheless, before implementation of a new method to operation of a laboratory, it is appropriate to verify its efficiency. Standard parameters, such as concentration, motility or morphology of spermatozoa, are not sufficient for an objective evaluation of separation efficiency. In about 30% of male infertility cases, the cause cannot be detected by these parameters [2,3]. DNA fragmentation represents such morphologically undetectable damage of spermatozoa most often. Correct selection of a sperm cell with non-damaged DNA is one of the prerequisites for achieving successful fertilization and embryo development in assisted reproductive technologies. Data from the literature suggest that the frequency of spermatozoa with massive DNA fragmentation is a marker of sperm quality and also possible predictor of fertility [4]. The tested microfluidic separation method was presented as a very effective and gentle sperm separation system [5,6,7]. Many reports indicate that microfluidic separation is a beneficial technique to remove spermatozoa with fragmented DNA and provides higher IVF outcomes compared to standard sperm selection techniques [5,6,8]. The possible beneficial effects of this technique in clinical application are still debatable. Recent analyses reported that microfluidic chip had a positive effect on total number of grade 1 embryos after ICSI, but in other IVF parameters, this method did not improve the reproductive outcomes [5].

The aim of our study was to compare the conventional swim-up sperm separation method with the new microfluidic chip method with emphasis on reducing the concentration of sperm cells with fragmented DNA.

Material and methods

Patients

The analysis of sperm concentration and motility was performed in raw semen samples according WHO manual (2010) [9]. 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 and evaluated using immersion objective with 100x magnification. For all of these evaluations, a phase contrast microscope (Nikon, Germany) with 20x or 100x magnification was used.

A total of ten patients of our center were included in the study. The ejaculate was obtained by masturbation. After liquefaction, the ejaculate of each patient was divided into three parts. Spermatozoa from each part was examined using the Halosperm G2 kit (Halotech, Spain) and the proportion of spermatozoa with fragmented DNA was determined (expressed as DFI – DNA fragmentation index). The DFI of the unprocessed samples was taken as the control value for efficiency of the separation.

Experimental design

A total of 10 patients of Center of Assisted Reproduction of University Hospital Brno aged 27 – 40 years were included in the study. All the patients gave their consent to realization of this test. Each sample was further subgrouped as follows: 1) part of the ejaculate (0.4 ml) was left without further processing 2) 0.1 ml of the ejaculate was separated using the microfluidic chip FERTILE® (KOEK Biotechnology), 3) the remaining part of the ejaculate was processed using the swim-up separation method.

Preparation of spermatozoa

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

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

2) Microfluidic chip: We work with the microfluidic chip according to the manufacturer's instructions. For this technique, we used the microfluidic sperm sorting chip FERTILE® (KOEK Biotechnology). It is a device with an inlet sample chamber connected to an outlet collection chamber by a narrow microfluidic channel (50 µl deep, 400 µl broad and 1.5 cm long). It has been designed as a flow, chemical-free and single use device with 5 parallel channels for each sample. This chip does not require any pre-treatment of the semen sample. At first, 2 µl of liquefied semen are loaded to the inlet port by a micro-pipette. After adding the sample to the sorting chip, inlet and outlet ports are carefully overlaid with 2 µl of mineral oil. The samples are incubated for 30 min at 37 °C. After that, the spermatozoa are carefully removed from outlet port with a micro-pipette.

3) Swim-up: Pipetted semen samples in 15 ml conical centrifuge tubes were washed twice in 2 ml Sperm Preparation Medium (Origio, Denmark) according to the manufacturer's instructions. After the second washing, the spermatozoa were gently

overlaid with 1 ml Sperm Medium (COOK, Ireland). The tube was inclined at an angle of about 45°, to increase the surface area of the semen-culture medium interface, and then incubated for 40 min at 37 °C. After this time, only 100 µl from the surface were collected for the DNA integrity analyses.

After these separation procedures, the spermatozoa were analysed by the Halosperm G2 method. This test is focused on nuclear DNA fragmentation.

Halosperm

Sperm DNA fragmentation was assessed by the Halosperm G2 kit. The semen sample was diluted to 20 mil/ml in an appropriate sperm extender. Eppendorf tubes with agarose were placed in a water bath at 92 °C for 5 min. They were subsequently tempered to 37 °C. After that, the diluted semen sample was transferred to the melted agarose tube and gently mixed. A drop of 8 µl of the mix was placed onto the sample well (provided in the kit) immediately and covered with a coverslip. The slides were then placed on a glass plate in 4 °C for a minimum of 5 min. After that, the coverslips were gently removed. Finally, the samples were denatured for 7 min and lysed for 20 min afterwards. After washing, the slides were dehydrated (using 70% and 96% ethanol) and air-dried. Strong staining is preferred to visualise the periphery of the dispersed DNA loops halos. As provided in the manufacturer's instructions, the spermatozoa with big and medium halo were considered free from DNA fragmentation [10].

The DNA fragmentation index (DFI) was calculated:

DFI (%) = Fragmented + Degenerated/Total cells counted.

For this present study, a minimum of 600 spermatozoa per sample were scored under the x40 objective of the microscope. To reduce the bias, two different technicians counted at least 300 sperm cells each.

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 $P< 0.05$.

Ethical approval

The research related to human use 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 review board. Ethical committee of University Hospital with reference number 14-240620/EK project 94/20 has approved the present study.

Informed consent statement

Informed consent has been obtained from all individuals included in this study.

Results

Based on a standard examination of spermiogram, normozoospermia was found in 8 patients, while results of 2 patients were classified as asthenozoospermia (**Tab. 1**). Patients with very low sperm concentration (oligozoospermia) were not included in this study intentionally. In oligozoospermic patients, it is often very difficult to obtain sufficient amount of spermatozoa to perform the separation techniques and final results of the Halosperm test can be affected negatively by the very low number of sperm cells analysed. The risk of error caused by a very low number of sperm cells analysed is significantly increased.

In nine patients, the number of spermatozoa with fragmented DNA was very low already before processing (DFI below 30%), only one patient showed

TABLE 1 List of all patients

PATIENT	AGE	CONCENTRATION (MIL/ML)	MOTILITY (%)	NORMAL MORPHOLOGY (%)	DIAGNOSIS
1	27	42	62	11	Normozoospermia
2	30	27	43	6	Normozoospermia
3	38	37	54	12	Normozoospermia
4	29	45	64	15	Normozoospermia
5	32	49	45	13	Normozoospermia
6	33	104	61	12	Normozoospermia
7	37	34	28	7	Asthenozoospermia
8	40	78	67	11	Normozoospermia
9	35	56	39	5	Asthenozoospermia
10	27	38	48	9	Normozoospermia

a higher value (patient No. 9, DFI 43%) (**Tab. 2**). After separation by the swim-up method, this parameter decreased in all the patients, to 48% of the initial DFI value on average (15.4% - 69%). If the same samples were processed using the FERTILE system, the DFI values were always lower in comparison with the swim-up method in all the patients. Using the FERTILE method, the values of DFI were reduced to 38% of the initial values in the ejaculate on average (6.3% - 66%). The separation with microchip was more efficient than the swim-up separation method by 10.5 % on average (2% - 27.7%) (**Tab. 3**). Nevertheless, no statistically significant difference was found in the number of fragmented spermatozoa between the swim-up separation method and the FERTILE method (**Fig. 1**). When expressed in absolute numbers, the mean DFI in the separated samples decreased from 18.2% in the unprocessed samples to 8.7% in the samples separated by the swim-up method and to 6.6% in the samples separated by the FERTILE method (**Tab. 2**). Efficiency of the FERTILE method was then better than efficiency of the swim-up method by 2.1%.

In the patient No. 9 (asthenozoospermia; 56 mil/ml, progressive motility 29%, non-progressive motility 10%, normal morphology 5%), the initially high DFI value (43%) was decreased to 14% by the swim-up method and to 11% by the FERTILE method (**Tab. 2**).

Discussion

The present study evaluated the extent to which using of the microfluidic chip for spermatozoa separation discarded the spermatozoa with fragmented DNA in contrast to the classical swim-up method. The method of sperm separation using the micro-

TABLE 2 DFI values of all patients before and after separation by swim-up and FERTILE method

PATIENT	DFI (%; ABSOLUTE VALUE)			DIFFERENCES BETWEEN SWIM-UP AND FERTILE
	SEMEN	SWIM-UP	FERTILE	
1	13.0	7.2	6.6	0.6
2	9.4	6.4	6.2	0.2
3	21.0	9.0	5.5	3.5
4	23.0	16.0	9.5	6.5
5	16.0	13.0	10.0	3.0
6	11.0	1.4	0.7	0.7
7	22.0	12.3	10.3	2.0
8	16.0	4.5	3.4	1.1
9	43.0	14.0	11.0	3.0
10	8.0	3.0	2.7	0.3
Ø	**18.2**	**8.7**	**6.6**	**2.1**

TABLE 3 Demonstration of efficiency of the separation methods. Ability of the separation methods to reduce the number of spermatozoa with fragmented DNA was evaluated, the initial DFI value of the unprocessed ejaculate is considered 100%

PATIENT	DFI (%; RELATIVE VALUE)			DIFFERENCES BETWEEN SWIM-UP AND FERTILE
	SEMEN	SWIM-UP	FERTILE	
1	100	55.0	50.7	4.3
2	100	68.0	66.0	2.0
3	100	42.8	26.2	16.0
4	100	69.0	41.3	27.7
5	100	81.0	62.5	18.5
6	100	15.4	6.3	9.1
7	100	55.9	46.8	9.1
8	100	28.1	21.3	6.8
9	100	32.6	25.5	7.1
10	100	37.5	33.7	3.8
Ø	**100**	**48.5**	**38.0**	**10.5**

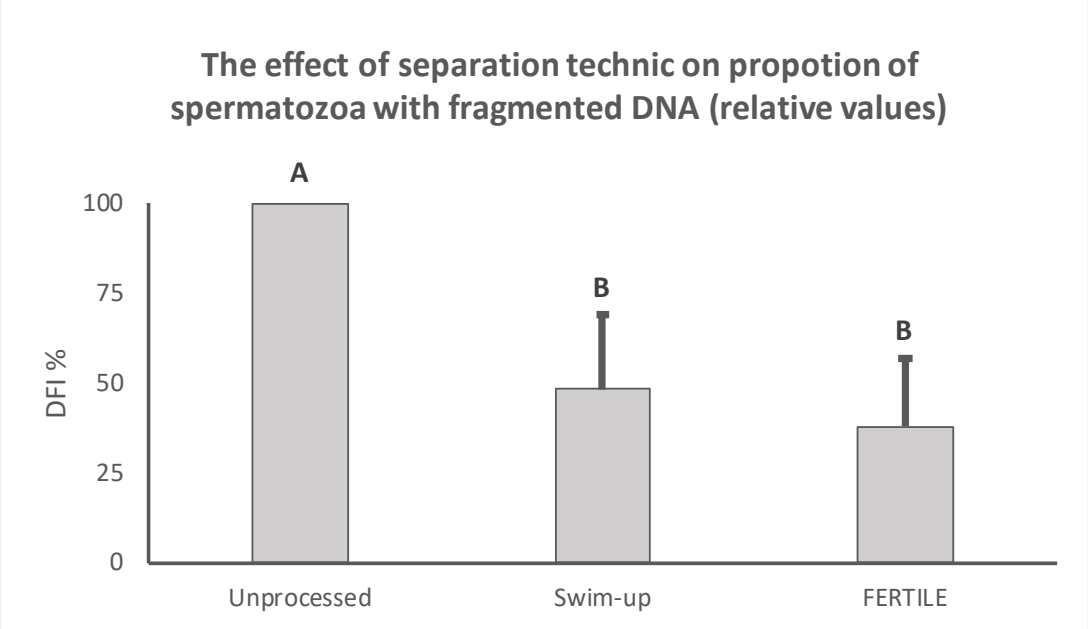

FIGURE 1 Graphical presentation of separation efficiency by the swim-up and FERTILE methods. Statistically significant differences are indicated by different symbols (A, B) (P< 0.05)

fluidic chips is increasingly utilized and is often presented as a very gentle method which significantly decreases the proportion of spermatozoa with fragmented DNA [1,11]. In this study, we presented that the DFI values of unprocessed samples were significantly higher than after the swim-up or FERTILE separations. However, no significant differences were detected in the DFI values between the separations by swim-up and FERTILE method (Figure 1). A study comparing the density gradient centrifugation (DGC) and the microfluidic method (FERTILE) and their effect on separation of spermatozoa with non-fragmented DNA has been published recently [6]. Unlike our experiment, this work revealed a significant difference in efficiency of the separation methods, with a significant decrease of the spermatozoa with fragmented DNA after utilization of the microfluidic chip when compared to the DGC method. This can be due to utilization of the DGC method, which is a method less gentle than the swim-up method used in the present study, and the observed difference can be caused by the negative effect of the DCG method on sperm DNA integrity. We work primarily with the swim-up method at it has been reported to be more gentle and significantly decreased proportion of fragmented spermatozoa when compared to the DGC [12].

A study on DNA integrity and comparison of the swim-up method and a sperm separation method using the microfluidic chips has already been published. It has reported a significantly lower proportion of spermatozoa with fragmented DNA after the separation using the microfluidic chips than after the swim-up separation method [11]. However, the study was realized with the Sperm Sorter Qualis system which uses laminar flow for separation of spermatozoa. Therefore it is a different principle of separation and in this respect maybe more efficient than the FERTILE system tested in the present study.

It is remarkable that separation by the FERTILE method did not decrease the DFI below 10%, not even in the patient with a high DFI value (see Results table 2, patient No. 9). This finding suggests that despite being able to select spermatozoa better due to its strict selection criteria, this separation system is not specific for spermatozoa with fragmented DNA.

It has been reported previously, that using of the microfluidic chip method significantly reduced total sperm number suitable for utilization in comparison to other methods like swim-up, DGC or MACS [1]. It is not considered a problem for ICSI cycles, but it should be taken into account for the classical IVF or IUI in the context of the limited input volume (max 10 µl). When the conventional swim-up method and the FERTILE were compared with focus on embryological parameters, only a significantly higher proportion of good blastocysts was observed after using the microfluidic chip FERTILE. No differences were found in neither of the other parameters [5]. Correct selection of good spermatozoa with intact DNA is very important. It is well known that sperm DNA fragmentation affects negatively total efficiency of the IVF methods [13].

Conclusions

The FERTILE separation method is a simple, undemanding and efficient. It requires no additional instrumentation and it can be realized only with basic laboratory equipment without utilization of other

chemicals. In our study, spermatozoa after separation were examined for DNA fragmentation, which was lower after utilization of the microfluidic chip method than after the swim-up method in all the patients. Nevertheless, absolute difference of the DFI value was better for the FERTILE method than for the swim-up by just 2.1%. The difference between the FERTILE and the swim-up methods was not statistically significant in the observed parameter.

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Conflict of interest statement
The authors declare they have no conflict of interest.

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