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IMPROVING SPERM DNA INTEGRITY THROUGH CUMULUS CELL-BASED SELECTION: A DISH EVALUATION

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ABSTRACT

Sperm selection is a fundamental process in assisted reproductive techniques. Selecting the "best spermatozoa" can improve embryo development and increase pregnancy rates. Recent studies have shown that cumulus cells (CCs) surrounding the oocyte improve the selection of sperm with the best morphology and motility and with lower DNA fragmentation, improving the results of other methods, such as conventional density gradient centrifugation (CDG), which can modify different physiological characteristics of the sperm. In this study, we used a dish inspired by microfluidic devices to select spermatozoa that could cross the physical barrier formed by CCs (Proto-CCD). For this purpose, six CCs clusters from six oocyte donors were used along with semen samples from six different sperm donors. Sperm concentration, motility, and DNA fragmentation results from the control group (freshly ejaculated samples) were compared with those from the study group (fresh semen samples filtered with CCs using Proto-CCs D). The sperm concentration was reduced by 91% in the filtered sample with respect to the sperm concentration observed in the fresh samples. In all six cases, 100 % of motile sperm was obtained after selection with CCs filter cells. With respect to DNA fragmentation, a mean decrease of 81% was observed after filtering sperm with the CCs. Proto-CC D allows for the effective selection of higher-quality spermatozoa for ICSI by increasing motility and reducing DNA fragmentation levels. Moreover, the prototype was designed to allow the ICSI to be performed on the same device. This facilitates the full protocol by integrating sperm selection and fertilization using the same device.

Keywords
Cumulus cells, DNA fragmentation, spermatozoa, ICSI.

INTRODUCTION

Sperm selection is an essential aspect of assisted reproductive technologies (ART). After ejaculation, gametes are placed in a non-physiological environment, and their selection is performed in the laboratory by qualified personnel. However, this selection process differs significantly from natural selection. In general, spermatozoa are selected based on motility and morphological characteristics using routine techniques such as density gradient centrifugation (DGC) and the swim-up (SU) method. However, these methodologies require an aggressive step of sperm centrifugation that can increase the levels of sperm DNA damage owing to the higher generation of reactive oxygen species (ROS) that can be generated during the process (Punjabi *et al.,* 2018). Furthermore, this process requires intensive manipulation of the sample, expanding the opportunity for an increase in the level of iatrogenic damage, which is highly time dependent. This scenario has promoted the development of alternative techniques that allow for more physiological selection of sperm.

During the last decade, more specialized sperm selection techniques have been developed to target sperm with enhanced fertilization potential. These techniques are based on different physiological aspects of the sperm in the ejaculate, such as motility (microfluidic devices; Olatunji and More, 2022), physiological characteristics such as the presence of hyaluronic binding capacity (PICSI: Physiological ICSI; Kirkman-Brown *et al.,* 2019), and factors such as the presence of apoptotic cells presenting DNA fragmentation (MACS; Sánchez-Martín *et al.,*

2017) or membrane integrity (Baldini *et al.,* 2021). In particular, sperm DNA fragmentation (SDF) has attracted the attention of researchers in the human biology of reproduction, as high levels of SDF are associated with an increased risk of miscarriage and have a negative impact on embryonic development (Borges *et al.,* 2019). Therefore, selecting the most competent sperm for microinjection is a key step for improving reproductive success (Rienzi *et al.* 2019).

With this scenario, we investigated the effects of sperm interaction with cumulus cells (CCs). CCs are granulosa cells surrounding the oocyte that play a crucial role in supporting the development and maturation of human oocytes. These specialized cells surround the oocyte and form the CCs, which is a cluster of cells attached to the oocyte during maturation of the ovarian follicle. There is bidirectional communication between CCs and oocytes through gap junctions, which facilitate the exchange of nutrients and regulatory molecules that support and promote cellular growth (Martínez *et al.,* 2023). In this microenvironment, hormone levels such as FSH and LH, along with various nutrients and other factors, promote oocyte maturation and ovulation (Turathum *et al.* 2021).

These factors also modulate gene expression, protein synthesis, and antioxidant production, thereby enabling oocytes to counteract oxidative stress (Martínez *et al.,* 2023). Moreover, CCs secrete progesterone, a key hormone that triggers the acrosome reaction, which is essential for oocyte fertilization and supports embryo development and implantation. Granulosa cells naturally participate in sperm selection before fertilization, making them vital for this process. Their network of hyaluronic acid, glycosaminoglycans, and other proteins helps select the best sperm and activate them to enhance fertilization potential (Soto-Heras *et al.,* 2023). Recently, studies that have been published using CCs for sperm selection have shown that sperm capable of passing through CCs exhibit better morphology, motility, and DNA integrity, resulting in higher fertilization rates and improved embryo quality (Wang *et al.,* 2018). Sperm exposed to the CCs secretome have shown improved mitochondrial function and motility, and reduced DNA damage (Luongo *et al.,* 2023).

The aim of this pilot study was to test a device sperm selection dish (Proto-CCs D) that was envisioned to standardize and facilitate sperm selection through the insertion of CCs as natural and operational natural filters using a microfluidic platform. The device was tested for sperm selection that fulfilled the expectations of the recruited samples for intracytoplasmic sperm injection (ICSI). The primary objective of this study was to assess the benefit of this device in providing sperm subsamples with improved sperm motility, sperm concentration and lower SDF compared to the native ejaculate.

MATERIALS AND METHODS

This study was conducted at the Instituto Bernabéu, Alicante, from January to May 2023. Gametes were obtained from six sperm and six oocyte donors. Samples were used fresh on the day of donation. The study was approved by the Institutional Review Board on November 2022 (reference number BR34/2022).

GENERAL STRUCTURE OF PROTO-CCS D: The dish contains two parallel lanes running from right to left (Figure 1a). The lanes were divided into three wells labeled A, B, and C (Figure 1a). Well A is located at the right end of each lane and was used to place the ejaculated raw semen sample (PRE sample; Figure 1b). Well B is located at the center of each lane, and CCs freshly collected after oocyte denudation must be placed in this well. Well C is located at the left end of each lane; it collects sperm that has passed through the CCs (upper lane, study group; POST-samples; Figure 1c). This well gathers the sperm sample that will be used for fertilization. The bottom lane, which does not contain CCs (Figure 1c), is used as a control to compare the behavior of sperm in the absence of these cells. Above these two lanes, there is a physical space allowing ICSI once the sperm samples have been selected. This allows selected sperm from well C to be directly injected into the oocyte in the same dish.

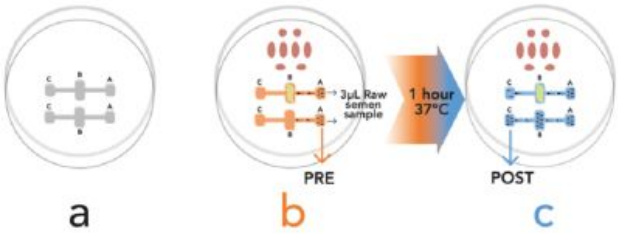

Figure 1. *a: Proto-CCs D dish with the 3 wells (A, B and C). b: upper lane with CCs in the B well and botton lane without CCs. Raw semen samples (PRE samples) are deposited in well A. c: after 1 hour incubation at 37 ºC, sperm are collected from well C (POST-samples).*

PREPARATION OF CCS: Oocytes were collected following puncture, and cumulus cells were isolated before decumulation. These cells were cultured in 60 mm round dishes (Falcon®, USA) with Global Total LP medium with HEPES (Cooper Surgical®, Denmark) and manipulated using insulin needles (Nipro®, Japan). CCs were trimmed and transferred to dishes containing the same medium at 37 °C.

Both lanes of the dish were prepared by filling well A with 5 µL of Sperm Washing medium (Fujifilm Irvine Scientific®, USA) and well C with the same volume. Central well B was filled with 2.5 µL of HEPES (Cooper Surgical®, Denmark) and 2.5 µL of Sperm Washing medium in the control lane. Once the CCs were placed in well B, the dish was covered with

LiteOil (Cooper Surgical®, Denmark), and 3 µL of raw semen was added to well A in each lane (Figure 1b. The Proto-CC D was incubated for 1 h at 37 °C. Finally, 3 µL of selected sperm were collected from well C. Sperm concentration, sperm motility (only linear motility) and sperm DNA fragmentation in this well were considered as the variables to be measured.

Due to the anticipated low sperm concentration at well C, the SCD (Sperm Chromatin Dispersion) method was employed using the Halosperm G2 kit (HalotechDNA, Madrid, Spain). This methodology allows the investigation of sperm DNA quality even after selecting only a single sperm (Gosálvez *et al.,* 2013). Sperm were centrifuged to increase concentration in Sperm Washing medium (Fujifilm Irvine Scientific® USA) and rapidly processed for DNA damage visualization using the standard methodology as described by the manufacturer. Given the expected low sperm concentration and to increase the capacity to visualize sperm using low-magnification lenses (10x), fluorescence microscopy was used. Finally, to determine the levels of DNA fragmentation, 300 spermatozoa were counted in the PRE (well A) and POST (well C) samples in five ejaculates. In only one sample, where the concentration obtained was low, 300 spermatozoa were counted in the PRE sample and only 100 in the POST sample.

Slides were stained with Fluorogreen (Halotech DNA, Madrid, Spain). Images were visualized and captured using a Nikon Eclipse microscope equipped with a high-resolution Nikon 12 bits CCD (Nikon DS-Q) and using 40x fluorite objective. The general images representing SDF are shown in Figure 2. Basically, three different sperm morphologies are produced. Those spermatozoa presenting a large halo of dispersed chromatin represent a normal and non-fragmented DNA molecule (N in Figure 2), where those presenting no-halo or a small halo around a compact core (F in Figure 2) represents spermatozoa presenting a fragmented DNA molecule. In this case we pay attention to a particular class of sperm morphology defined as degraded sperm (D in Figure 2). These spermatozoa are interpreted as presenting a highly affected DNA molecule where all DNA fragments have been removed after the action of the lysing solution used in the Halosperm protocol (Gosálvez *et al.,* 2014). This specific class has been taking into account because it was found to be efficiently selected using alternative selection sperm techniques such as MACS (González-Martínez *et al.*, 2018).

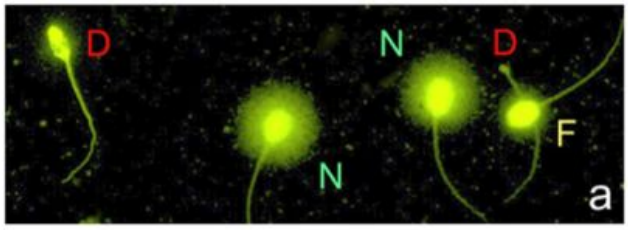

Figure 2. *A general view of sperm DNA fragmentation associated to different sperm morphologies after using Halosperm. Visualization using fluorescence microscopy. N: normal sperm F: Fragmented sperm D: Degraded sperm.*

STATISTICAL ANALYSIS. Descriptive and analytical statistics were performed using the original dataset in Microsoft Excel files exported to SPSS (IBM SPSS v25 Statistics Package, NY, USA). Given the low number of case studies in the present pilot study, non-parametric statistics for paired groups were used. The Wilcoxon signed-rank test (confidence interval α = 0.05) was applied using a two-tailed test. Spearman Rho was used to assess for the correlation analysis.

RESULTS

SPERM CONCENTRATION

Figure 3a shows a comparative graphical representation of the sperm concentration results (M/ml). A decrease in sperm concentration was observed between the sperm present in well A (raw sperm samples) and well C (selected sperm samples). Considering the mean of both values, the sperm concentration decreased by approximately 92 %. Accordingly, significant differences were observed between the values obtained for PRE and POST selection (Wilcoxon = 2.201; p = 0.028). No significant correlation was observed between initial and final sperm concentrations (Spearman's rho = 0.609; p = 0.200). This indicates that the proportion of decrease in sperm concentration was different for each individual.

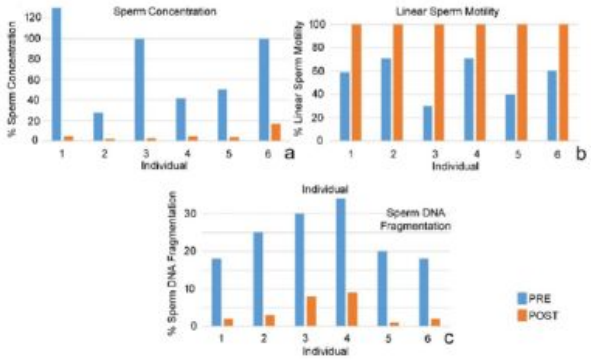

Figure 3. *Graphic representation of the values obtained in preselected sperm samples (blue columns) and post-sperm selection using cumulus cells as a physical filter (orange columns). a) Summary of sperm concentration data; b) summary of sperm linear motility data; c) summary of sperm DNA fragmentation data.*

LINEAR SPERM MOTILITY

Figure 3b shows the linear sperm motility results of the evaluated samples. A clear increase in the proportion of linear motility was observed between sperm present in well A (raw sperm samples) and those collected in well C (selected sperm samples). Considering the mean of both values, linear sperm motility increased by approximately 81 %. Accordingly, significant differences were observed between the values obtained for PRE and POST selection (Wilcoxon = 2.207; p = 0.027). Correlation analysis was not performed because one of the variables (sperm linear motility in well C) was constant.

SPERM DNA FRAGMENTATION

When comparing SDF values between raw semen samples and those processed with Proto-CCs D (Figure 3c), significant differences were found between PRE and POST samples (Wilcoxon -2.201; p = 0.028). On average, an 83 % decrease in the final SDF values was observed. This percentage varied when each sperm sample was analyzed separately, and the efficiency range varied from 73 % to 92.8 %.

However, it is interesting to note that a limited positive correlation existed between the SDF values in the PRE and POST samples (Spearman Rho = 0.182; P = 0.050). The elimination of degraded sperm was practically complete, and only one spermatozoon with degraded DNA was found in well C (POST) in one of the samples.

DISCUSSION

The findings from this pilot study underscore the significant benefits associated with the use of CCs as biological filters in microfluidic environments to improve some characteristics of semen samples to be used for fertilization. The most pronounced effect was observed on sperm concentration, which experienced a notable reduction of 92 %. Despite this marked decrease, the sperm concentration in the POST sample (collected in well C) ranged between 2.3 and 17 million sperm/ml. This concentration is adequate for performing intracytoplasmic sperm injection (ICSI) without any issue.

Additionally, pooling sperm collected from multiple lanes would yield a sufficient concentration and volume to facilitate procedures such as in vitro fertilization (IVF) or intrauterine insemination (IUI), ensuring the feasibility of these techniques. After selection, the sperm exhibited extremely high linear motility, with 100 % motility observed across all samples.

Furthermore, there was a substantial reduction in SDF, with an 83 % decrease when comparing the PRE and POST samples. According to the relatively positive correlation found between the SDF values of the PRE and POST samples, this reduction seems to be associated with the initial presence of SDF in the PRE sample.

This reduction brings SDF levels below the thresholds typically reported in fertile men and even sperm donors, where SDF levels generally remain under 20 %, using Halosperm as a reference technique (Esteves *et al.,* 2020).

The improvements in sperm quality observed in this experiment may be attributed to the role of CCs in Proto-CC D, which appears to function synergistically when interacting with sperm. These CCs likely confer final capacitation properties to the sperm, a process that is bypassed when the sperm is directly selected for ICSI. In addition to capacitation, CCs appear to act as selective barriers, retaining sperm with deficiencies that prevent them from traversing this natural obstacle. This selective filtration and capacitation mechanism could explain the enhanced quality of sperm selected using the Proto-CC D device. Regarding the effects of CCs as physiological barriers on sperm motility, it is expected that only motile sperm are capable of crossing this barrier (Handzhiyska *et al.,* 2024; Hong *et al.,* 2009), and this would be effective in both natural and experimental models. The enhanced proportion of linear motility in the recovered samples is robust and intriguing.

Thus, this methodology, in addition to the selection of more active motile sperm in the original sample, also enhances some sperm characteristics prior to fertilization. Some published studies have supported this statement. For example, it has been reported how progesterone, secreted by CCs, activates CatSper calcium channels in sperm, enhancing capacitation by increasing calcium influx, which is crucial for hyperactivation and the acrosome reaction (Strünker *et al.,* 2011). In preparation for fertilization, it has been reported that cumulus cells and factors such as progesterone help to "prime" the sperm for the acrosome reaction, a critical event that allows sperm to penetrate the zona pellucida (Jin and Yang, 2017).

It is likely that these findings are directly related to the fact that CCs can provide metabolites, such as pyruvate and lactate, to sperm, thereby enhancing their energy metabolism and supporting capacitation (Dacheux and Dacheux, 2014). It can be speculated that sperm that are able to cross the physical barrier of CCs are not only the most prepared to do so, but they also improve their capacity to penetrate the oocyte more efficiently. This may explain, in part, the massive linear motility observed after sperm selection.

The most dramatic change observed after using Proto-CCs D was a significant decrease in the sperm concentration. This response is consistent with the general behaviour of semen samples processed using microfluidic devices. In these cases, a significant reduction in sperm concentration has been repeatedly reported. The device geometry, flow rates, and channel sizes influence the number of sperms that can successfully navigate through the microfluidic system. Some devices may inadvertently trap or exclude viable sperm, leading to loss of sperm concentration in the final sample.

Thus, the efficiency of sperm recovery varies depending on the microfluidic system design (Nagata *et al.,* 2018; Iqbal *et al.,* 2020). The system we used was quite simple, and the microchannels were wide enough to integrate a relatively large number of sperms. We believe that the observed benefits are related more to the physiological and mechanical benefits of the sperm interacting with the CCs than to the physical fact of the sperm moving through these microchannels.

With respect to the decrease in the levels of SDF, the first information published using microfluidics reported that these devices improve the proportion of sperm with good-quality DNA and motility (Shirota *et al.,* 2016; Nosrati *et al.,* 2017; Naknam *et al.,* 2019), an effect that was also observed in our experiments. However, it is noteworthy that a minor proportion of sperm with fragmented DNA persisted in the final sample. This indicates that motile sperm containing fragmented DNA molecules can trans pass this natural filter. On average, microfluidic devices can result in a 20–50 % improvement in sperm DNA fragmentation levels, depending on the device, sample characteristics, and baseline DNA fragmentation rates (Quinn *et al.,* 2018; Samuel *et al.,* 2018;. Parrella *et al.,* 2019; Xu *et al.,* 2021).

The current study demonstrated a significant reduction in SDF, with sperm selection using Proto-CCs D, achieving an average efficiency of 83 %. We did not perform PRE and POST comparisons in the group without CCs, as our primary aim was to evaluate the specific impact of CCs intercalation compared to standard sperm selection techniques. Previous attempts using processed sperm samples (density gradient centrifugation) were ineffective; therefore, we opted to use raw semen samples with CCs to better approximate the natural biological conditions.

The fact that the degraded sperm present in the ejaculated neat semen sample were practically abolished is also noteworthy. This effect was also observed when MACS for apoptotic sperm selection was used (González-Martínez *et al.,* 2018). In terms of other semen characteristics, previous studies, including those by Franken (Franken and Bastiaan, 2009) and Carrel (Carrel *et al.,* 1993), have consistently shown that sperm selected via CCs contact display better morphology, whether in the head, midpiece, or tail regions. There has also been reported that sperm selected by CCs displayed improvements in morphology, motility, capacitation, and acrosome reaction and even a greater capacity to bind to the zona pellucida (Handzhiyska *et al.,* 2024). These studies are directly connected with those performed by Hong *et al.* (2004) and Yazdanpanah *et al.* (2021), indicating that sperm that traverse CCs exhibit a higher acrosome reaction, demonstrating their maturity and capacitation.

The results obtained in this experiment clearly show that sperm are capable of crossing the CCs barrier placed in this device and produce a series of benefits in the sperm sample that will be used for fertilization. Additionally, the possibility of selecting sperm and performing the microinjection using the same device diminishes the negative impact of iatrogenic damage to oocytes and sperm during the sperm injection protocol.

A further advantage of this protocol is the possibility of using fresh unprocessed semen, and it seems assumable that cryopreserved samples will benefit from these advantages. This option reduces extra sperm handling after ejaculation because the sperm can be placed in the insertion well once the sperm sample is liquefied.

As a proof-of-concept study, additional studies are required to verify these results. In any case, even when using a reduced number of samples, the benefits are evident, even improving those reported using microfluidic devices without the intercalation of CCs, which results in high stimulation. One potential drawback of this selection method is CCs saturation.

This saturation could occur if the sperm concentration is too high, which may reduce or even negate the filtering effect of CCs, or if the cells trigger mechanisms to produce reactive oxygen species (ROS) that may result in sperm damage. This potential issue can be addressed by adjusting the sperm concentration or by reducing the incubation time on the plate. Conversely, if the concentration is too low, the sperm may not be able to cross the barrier, rendering the technique unusable without further sample manipulation to increase its concentration.

In conclusion, CCs in a microfluidic device using Proto-CC D may offer the advantages of selecting motile sperm with an increased proportion of linear motile sperm, reduced DNA fragmentation and improved laboratory workflow.

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