Sperm selection by rheotaxis improves sperm quality and early embryo development

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Abstract

The objective of this work was to elucidate whether a sperm selection method that combines rheotaxis and microfluidics can improve the selection of spermatozoa over density gradient and swim-up. For this purpose human sperm selected by rheotaxis were compared against density gradient, swim-up and a control group of non-selected spermatozoa in split frozen-thawed (FT) and fresh (F) semen samples. Sperm quality was assessed in terms of motility, morphology, DNA fragmentation index (DFI), viability, acrosome integrity and membrane fluidity. Using a mouse model, we compared fertilisation and embryo development rates after performing ICSI with spermatozoa, sorted using rheotaxis or swim-up. Selection by rheotaxis yielded a sperm population with reduced DFI than the control ($P < 0.05$), improved normal morphology ($P < 0.001$) and higher total motility (TM; $P < 0.001$) than the other techniques studied in F and FT samples. Swim-up increased TM compared to density gradient and control in FT or F samples ($P < 0.001$), and yielded lower DFI than the control with F samples ($P < 0.05$). In FT samples, selection by rheotaxis yielded sperm with higher viability than control, density gradient and swim-up ($P < 0.01$) while acrosomal integrity and membrane fluidity were maintained. When mouse spermatozoa were selected for ICSI using rheotaxis compared to swim-up, there was an increase in fertilisation ($P < 0.01$), implantation ($P < 0.001$) and foetal development rates ($P < 0.05$). These results suggest that, in the absence of non-destructive DNA testing, the positive rheotaxis can be used to select a population of low DNA fragmentation spermatozoa with high motility, morphology and viability, leading to improved embryo developmental rates.

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Introduction

Intracytoplasmic sperm injection (ICSI) was initially developed for patients with severe male factor infertility, but its application has widened for cases with low oocyte yields, cryopreserved oocytes, insemination for patients at risk for HIV or even to comply with laws that limit the number of eggs to be inseminated (Palermo *et al.* 2017). Recent reports on the use of assisted reproductive technology (ART) show a dominance of ICSI over conventional *in vitro* fertilisation (IVF) and now makes up approximately 70% of all fertilisations *in vitro* (Adamson *et al.* 2018, De Geyter *et al.* 2020) despite major country to country variations.

The existing technologies used to select better quality spermatozoa for use in both IVF and ICSI involve sperm cells either being forced to swim through a density

gradient using centrifugation or swim upwards in a tube of sperm preparation medium. These techniques have been used for decades in the preparation of spermatozoa for traditional IVF and by default are being used for sperm selection for ICSI as well, but seem to be suboptimal for this purpose (Oseguera-López *et al.* 2019). Embryologists must then select through visual observation the best spermatozoon based on their morphology, a step that can be extremely time-consuming (Auger *et al.* 2016, Sikka & Hellstrom 2016). In addition, they have no information on the DNA integrity of the selected sperm as there is no non-destructive test for DNA fragmentation.

The capacity of human spermatozoa to produce a good-quality embryo with a high implantation and development potential depends on many factors, including its DNA integrity (Miller *et al.* 2010). High levels of DNA fragmentation in spermatozoa can slow

down embryo morphokinetic parameters after ICSI (Wdowiak *et al.* 2015), and there is a positive relationship between levels of DNA damage in spermatozoa and spontaneous pregnancy loss (Robinson *et al.* 2012). Up to 30% of patients seeking ART had high rates of sperm DNA breaks (Bungum *et al.* 2004), and therefore ICSI is the preferred method when DNA fragmentation index (DFI) exceeds 30% (Bungum *et al.* 2007, Chi *et al.* 2017).

The need for a better sperm selection method has led to the development of new technologies to increase the quality of sperm selection for ICSI. Among others, sodium hyaluronate has been used to select spermatozoa based upon their maturity state in the form of droplets (Parmegiani *et al.* 2010) or coating culture dishes (Worilow *et al.* 2013); Magnetic activated cell sorting (MACS) combined Annexin V with magnetic beads to remove apoptotic sperm cells (Ziarati *et al.* 2019), and a new group of devices have emerged to select spermatozoa based on microfluidics (Kishi *et al.* 2015, Shiota *et al.* 2016, Chinnasamy *et al.* 2018, Nakao *et al.* 2020).

Enroute to the site of fertilisation in the ampulla, mammalian spermatozoa have the ability to be actively guided towards the oocyte by different mechanisms (Pérez-Cereales *et al.* 2015). Among them, rheotaxis – or swimming against a flow – has been revealed to be a long-distance biophysical guiding mechanism for human spermatozoa (Kantsler *et al.* 2014). In a recent preliminary study by De Martin *et al.* (2017), the use of a rheotaxis related sperm selection method resulted in an improvement in sperm morphology and chromatin maturity. However the study did not assess the impact of the selection method on DNA fragmentation or fertility potential of the selected sperm.

Here we report a new sperm selection method which combines, in a single-use microfluidic device, the ability of spermatozoa to orientate and swim against a flow of media using microfluidic technology. We compared sperm selection by rheotaxis using a proprietary designed microfluidic system with the current standard procedures for sperm selection, namely density gradient and swim-up. Sperm quality was assessed in terms of motility, morphology, viability, acrosome integrity, membrane fluidity and DNA fragmentation using frozen-thawed and fresh spermatozoa. Using a mouse model, we compared fertilisation and embryo development rates after performing ICSI with spermatozoa sorted using rheotaxis or swim-up.

Materials and methods

Unless otherwise indicated, all chemicals were of analytical grade and obtained from Sigma–Aldrich Ireland Ltd.

Ethical approval

All donors gave written informed consent, and the use of human sperm samples for research was approved by Faculty

of Science and Engineering Research Ethics Committee at the University of Limerick.

Experiments in mice were carried out in strict accordance with recommendations of the guidelines of European Community Council Directive 86/609/EEC. Every effort was made to minimise suffering. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the INIA (Madrid, Spain; permit number CEEA 2014/025).

Experimental design

Comparison of the selection performed by different sperm preparation techniques in split semen samples using frozen-thawed and fresh human spermatozoa

In this experiment, we aimed to compare the selection performed by the current standard procedures for sperm selection, namely density gradient and swim-up, with the selection performed by rheotaxis using a proprietary designed microfluidic system. A control group of non-selected spermatozoa was also included, and sperm quality was assessed in terms of motility, morphology, and DNA fragmentation. Ten replicates/donors were assessed with frozen-thawed spermatozoa, and ten replicates/donors were assessed with fresh sperm cells. A replicate for frozen-thawed semen was a pool of three straws (one from each of three ejaculates) from each donor while for fresh semen a replicate was one ejaculate per donor.

Next, to confirm the improvement in sperm morphology, we conducted a morphology evaluation at high magnification in frozen-thawed human sperm samples. For this, samples from three different ejaculates of each donor were thawed and pooled, the experiment was replicated four times with different donors, and at least 200 spermatozoa were assessed per sample. Additionally, to further characterise the selection performed by the aforementioned sperm preparation methods, viability, membrane fluidity and acrosome integrity of frozen-thawed human spermatozoa were analysed by flow cytometry. This experiment had 12 replicates/donors and at least 10,000 events were analysed per sample.

ICSI, in vitro culture and embryo transfers using a mouse model

After we demonstrated that human spermatozoa selected by rheotaxis displayed better motility, morphology and lower DNA fragmentation than spermatozoa selected using current techniques, we used the mouse (*Mus musculus*) as a model to assess fertilisation and embryo development rates after ICSI (Yanagimachi 1998, Lyu *et al.* 2010, Ma *et al.* 2020).

We compared spermatozoa sorted using rheotaxis with the technique routinely used for the preparation of mouse sperm cells for ICSI, namely swim-up ($n = 10$ males), and we completed ICSI of mouse oocytes ($n = 944$ oocytes), *in vitro* culture and embryo transfers ($n = 25$ over 10 days). The technician selecting spermatozoa for ICSI was blinded to the selection method.

Semen samples

Semen samples were donated by healthy donors after presenting for semen assessment at a fertility clinic. Samples were

obtained by masturbation after 3–5 days of sexual abstinence and were processed and assessed in accordance with the WHO Guidelines (World Health Organization 2010). Samples were allowed to liquefy at room temperature (RT; approximately 22°C) for 30–60 min prior to processing. The transport to the laboratory was performed at RT, and the same temperature was used to hold the samples while the sperm aliquots were distributed to the experimental groups. The inclusion criteria for semen samples were as follows: semen volume >1.5 mL, sperm concentration >15 × 10⁶ spermatozoa/mL, progressive motility >32% and normal forms >4%. Frozen human sperm samples were donated by Cryos International – Denmark ApS (Aarhus, Denmark). Briefly, following liquefaction of the semen samples obtained from selected healthy donors, one part of SpermCryo™ All-round (Gynotec, Malden, the Netherlands) was added to three parts of semen while gently swirling before leaving the sample for 10 min to equilibrate. The semen was transferred to CBS™ High Security straws (Cryo Bio Systems, L'Aigle, France), frozen in liquid nitrogen vapour and stored at -196°C. For each replicate one straw from each of three different ejaculates from the same donor were thawed (30°C for 30 s), pooled to avoid intra-individual differences and diluted 1:1 in sperm washing medium (SWM; PureSperm®Wash, Nidacon, Mölndal, Sweden). All experiments were completed blinded to the evaluator within 1 h after sperm collection/thawing.

Sperm preparation techniques

Density gradient centrifugation

A density gradient was prepared in a 15-mL conical tube by layering 2 mL of 40% (v/v) density gradient medium (PureSperm®40, Nidacon) over 2 mL of 80% (v/v) density gradient medium (PureSperm®80, Nidacon). An aliquot of 500 µL of semen was placed above the density gradient and centrifuged at 300 *g* for 20 min. Following removal of the supernatant, the sperm pellet was resuspended in 5 mL SWM after which the pellet was disturbed by gentle pipetting and centrifuged at 500 *g* for 10 min. Following centrifugation, the final pellet was resuspended in 500 µL SWM.

Swim-up

To select spermatozoa by the swim-up method, a semen aliquot of 500 µL was carefully pipetted under 1.5 mL SWM in a sterile 15 mL conical tube. The falcon tube was inclined at an angle of 45° to increase the surface area of the semen-culture medium interface and was incubated for 1 h at 37°C in a water bath. Afterwards, the tube was returned to the upright position, and the uppermost 1 mL of medium was collected, transferred to a new tube and centrifuged at 500 *g* for 10 min. Finally, the supernatant was discarded, and the sperm pellet was resuspended in 500 µL SWM.

Microfluidic chip

A disposable device with a microfluidic channel was manufactured by CNC machining from polymethyl methacrylate (PMMA). The microfluidic channel measured

Figure 1 Microfluidic chip. A disposable chip with a microfluidic channel was manufactured from polymethyl methacrylate (PMMA). (A) The microfluidic channel measured 300 µm wide, 100 µm deep and 10 mm in length. (B) It was connected to a syringe pump (Standard Infuse/Withdraw PHD ULTRA™ Syringe Pump, Harvard Apparatus, Holliston, USA) by silicone tubing and a connector (Male Mini Luer fluid connector, Microfluidic ChipShop, Jena, Germany). The microfluidic channel was primed with sperm washing medium (PureSperm®Wash, Nidacon, Mölndal, Sweden) to remove air bubbles, following this, the flow velocity was stabilised at 30 µm/s. (C) A 20 µL sperm sample was loaded into the starting inlet of the microfluidic channel allowing spermatozoa to orient against the oncoming flow and swim against it (positive rheotaxis) for 30 min. Sperm progression was assessed under 200× magnification on an inverted microscope (CK40; Olympus). (D) At the end of this period, spermatozoa that reached the collection wells were collected and submitted to further analysis. (E) Waste well.

300 µm wide, 100 µm deep and 10 mm in length (Fig. 1). It was connected to a syringe pump (Standard Infuse/Withdraw PHD ULTRA™ Syringe Pump, Harvard Apparatus, Holliston, USA) by silicone tubing and a connector (Male Mini Luer fluid connector, Microfluidic ChipShop, Jena, Germany). The microfluidic channel was primed with SWM to remove air bubbles, following this, the flow velocity was stabilised at 30 µm/s. A 20 µL sperm sample was loaded into the starting inlet of the microfluidic channel allowing spermatozoa to orient against the oncoming flow and swim against it (positive rheotaxis) for 30 min. Sperm progression was assessed under 200× magnification on an inverted microscope (CK40; Olympus). At the end of this period, spermatozoa that reached the collection wells (20 µL per well) were collected and submitted to further analysis. For a more comprehensive understanding of the microfluidic device see Videos 1, 2, 3, 4 and 5.

Video 1

Start of the microfluidic channel demonstrating how human spermatozoa gain entry into the channel. Arrow indicates the direction of the flow. An asterisk indicates the starting well. Sperm progression was recorded under 100X magnification on an inverted microscope (CK40; Olympus, Center Valley, USA). This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-20-0364/video-1>) is available from the online version of the article at <https://doi.org/10.1530/REP-20-0364>.

Video 2

Middle of the microfluidic channel showing how human spermatozoa progress upstream. Arrow indicates the direction of the flow. Sperm progression was recorded under 200X magnification on an inverted microscope (CK40; Olympus, Center Valley, USA). This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-20-0364/video-2>) is available from the online version of the article at <https://doi.org/10.1530/REP-20-0364>.

Video 3

The lateral channel of the microfluidic device showing how human spermatozoa progress through it. Sperm progression was recorded under 200X magnification on an inverted microscope (CK40; Olympus, Center Valley, USA). This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-20-0364/video-3>) is available from the online version of the article at <https://doi.org/10.1530/REP-20-0364>.

Video 4

End of the lateral channel of the microfluidic device showing how human spermatozoa advance into the collection zone/well. An asterisk indicates the collection well. Sperm progression was recorded under 200X magnification on an inverted microscope (CK40; Olympus, Center Valley, USA). This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-20-0364/video-4>) is available from the online version of the article at <https://doi.org/10.1530/REP-20-0364>.

Video 5

Selected human sperm population at the collection well of the microfluidic device. Sperm progression was recorded under 100X magnification on an inverted microscope (CK40; Olympus, Center Valley, USA). This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-20-0364/video-5>) is available from the online version of the article at <https://doi.org/10.1530/REP-20-0364>.

Motility evaluation

As part of the quality control, initial sperm motility was assessed within 30 min after liquefaction of the sample or thawing and pooling the straws. It was also evaluated after each sperm preparation technique. To this end, a volume of 10 µL of semen was delivered onto a clean glass slide and covered with a 22 mm × 22 mm coverslip. The procedure was performed at 37°C with a heated microscope stage using a phase-contrast microscope (BX60; Olympus) equipped with an eyepiece reticle with grid at 200X magnification. Motility was assessed as follows: (i) progressive motility (PR): spermatozoa moving actively, either linearly or in a large circle, regardless of speed, (ii) non-progressive motility (NP): all other patterns of motility with an absence of progression, and (iii) immotility (IM): no movement. At least 200 spermatozoa were scored per replicate for the percentage of different motile categories.

Morphology evaluation

To determine sperm morphology, a 10 µL aliquot of the sperm preparation was smeared onto a clean slide, allowed to dry in air and stained using the Diff-Quik kit (RAL Diagnostics, Martillac, France). The slide was examined immediately after drying, with brightfield optics at 1000X magnification using oil immersion. A normal/abnormal classification, along with a detailed recording of the location of sperm abnormalities, was performed following the criteria proposed in the 5th edition of the WHO guidelines (World Health Organization 2010). The following categories of defects were noted: (i) head defects, (ii) neck and midpiece defects, (iii) principal piece defects, and (iv) excess residual cytoplasm. A minimum of 200 spermatozoa were evaluated in each replicate.

Morphology evaluation at high magnification

Spermatozoa (frozen-thawed only) were visualised using a 60X objective on a Nomarski differential interference contrast (DIC) inverted microscope (Nikon Eclipse Ti, Nikon Instruments Europe BV) equipped with a specific package for intracytoplasmic morphologically selected sperm injection (IMSI; RI IMSI, Research Instruments Ltd, Cornwall, UK) and further magnified using RI Viewer software up to 6000X. Spermatozoa were graded and classified into four groups according to the presence or size of vacuoles following the criteria described by Vanderzwalmen *et al.* (2008): (i) Grade I, absence of vacuoles; (ii) Grade II, maximum of two small vacuoles; (iii) Grade III, more than two small vacuoles or at least one large vacuole and (iv) Grade IV, large vacuoles in conjunction with abnormal head shapes or other abnormalities.

DNA fragmentation assay

Sperm DNA integrity was evaluated by the acridine orange (AO) test as described by Ajina *et al.* (2017). To avoid rapidly fading fluorescence, all the slides were observed within 5 min under a fluorescence microscope (BX60; Olympus) at 1000X magnification, with excitation at 450–490 nm. Spermatozoa with normal (double-stranded) DNA showed green fluorescence, and those with denatured or ssDNA were red, orange, or yellow. The DFI (Jiang *et al.* 2011) was used to represent the ratio of spermatozoa with red, orange, or yellow fluorescence relative to all the spermatozoa analysed per sample. At least 200 spermatozoa were assessed for each replicate.

Flow cytometry

The aim of the flow cytometry analysis was to detect viability, membrane fluidity and acrosome integrity in spermatozoa (frozen-thawed only) selected by the different sperm preparation techniques. In all samples, the sperm concentration was set at 1×10^6 spermatozoa/mL, and sperm suspension was incubated with a three stain combination of fluorochromes Yo-Pro1 (15 nM, ThermoFisher Scientific), Merocyanine M540 (2.6 µM, Sigma–Aldrich) and Lectin PNA from *Arachis hypogaea* conjugated with Alexa Fluor™ 647 (2.5 µg/mL, ThermoFisher

Scientific) for 15 min at 37°C. Subsequently, samples were subjected to analysis by CytoFlex flow cytometry (Beckman Coulter Inc., Brea, USA) equipped with violet (405 nm), blue (488 nm) and red (635 nm) lasers. Sperm population was discriminated from debris with the forward scatter (FSC) and side scatter (SSC) signals. Yo-Pro1 and M540 were excited with the 488 nm laser, while PNA-A647 was excited with the 635 nm laser and emission was detected by the 525/40BP, 585/42BP and 690/50BP filters, respectively. The analysis of the flow cytometry data was carried out using CytExpert, version 2.3.0.84 (Beckman Coulter Inc.). All variables were assessed using logarithmic amplification, 10,000 gated events were collected for each sample and flow cytometry data are expressed as percentage from the total analysed population.

Animals, gamete collection, and sperm freezing

Both male and female mice were fed with a standard diet *ad libitum* and maintained in a temperature- and light-controlled room (23°C, 14h light : 10 h darkness). When required, animals were euthanised by cervical dislocation.

Spermatozoa were collected from the caudal epididymis of 3-month-old B6D2 males (F1 hybrid of C57BL/6J females and DBA/2J males) and suspended in a non-capacitating modified human tubal fluid medium (ncHTF) (Pérez-Cerezales *et al.* 2018). The pH of the ncHTF medium was adjusted to 7.4, and spermatozoa were incubated for 1 h at 37°C without CO₂ to allow motile spermatozoa to swim to the surface of the droplet. Following sperm collection and transfer to a sterile eppendorf tube, the sample was either subjected to sperm selection using the microfluidic channel described earlier (rheotaxis) or not (swim-up). Once selected, sperm samples were frozen by immersion in liquid nitrogen (Lacham-Kaplan *et al.* 2003) and stored for periods ranging from 1 day to 4 weeks at -80°C. Frozen-thawed spermatozoa were mixed with 40–50 µL of a 10% polyvinyl-pyrrolidone (PVP-360) in M2 medium before being placed in the culture dish for microinjection.

Mouse oocyte collection

Metaphase II oocytes were collected from the oviducts of 6- to 8-week-old B6D2 female mice superovulated with 7.5 IU of eCG, and with an equivalent dose of hCG 48 h later, as described by Fernández-González *et al.* (2008).

Intracytoplasmic sperm injection in mice

ICSI with frozen-thawed mouse spermatozoa was performed in M2 medium at RT, as previously described (Moreira *et al.* 2007). Individual sperm heads decapitated by the snap-freezing procedure were injected into oocytes. Oocytes were injected in groups of ten per treatment with spermatozoa from one male.

In vitro embryo culture

After 15 min of recovery at 37°C in M2 medium, surviving oocytes were returned to mineral oil-covered KSOM and cultured in groups of 20–40 embryos in 25 µL drops at 37°C in a 5% CO₂ atmosphere. Fertilisation was evaluated as the

percentage of embryos reaching the 2-cell stage (Cleavage rate) after 24 h in culture.

Embryo transfer

Embryo transfer was performed as described previously (Fernández-González *et al.* 2008). Briefly, recipient pseudopregnant females (CD1 females mated with vasectomized males) 0.5 days post coitum were anaesthetised with inhaled isoflurane and 2-cell embryos were transferred into the oviducts. Each female received 10–12 embryos from both treatments into either the left or right oviducts. On day 15 days after ICSI, surrogate females were euthanized, and the number of foetuses and reabsorptions were scored as described by Pérez-Cerezales *et al.* (2018). Subsequently, the percentage of transferred embryos implanted (Implantation rate) and the percentage of implanted embryos developed until the foetus stage (foetus development rate) were calculated.

Statistical analysis

Data were analysed using Statistical Package for the Social Sciences (SPSS) (version 25, IBM). Data were first examined for normality of distribution using the Shapiro–Wilk test and later for homogeneity of variance using Levene's test. Unpaired *t*-tests for independent samples were performed for simple two-group comparisons. For group-wise comparisons a univariate ANOVA (ANOVA) was used. *Post hoc* tests were conducted using the Bonferroni test. Results were reported as the mean $\pm$ S.E.M. *P* < 0.05 was considered statistically significant.

Results

Comparison of the selection performed by different sperm preparation techniques in split semen samples using frozen-thawed and fresh human spermatozoa

First, the motility of the samples was assessed, and total motility (TM, %) was calculated as follows: TM = PR + NP. Spermatozoa selected by rheotaxis had higher TM than the other selection techniques studied, both with frozen and fresh samples (*P* < 0.001; Fig. 2A and B, respectively). The swim-up selection also increased TM when compared to density gradient and control groups, irrespective of whether they were frozen-thawed or fresh (*P* < 0.001). However, surprisingly, no significant improvement in TM was achieved through the density gradient in both frozen-thawed and fresh spermatozoa.

Subsequently, we analysed the levels of DNA fragmentation of the sperm samples and calculated the DFI. In frozen-thawed samples (Fig. 2C), the level of DNA fragmentation of the initial samples (18.0 ± 4.9) was only reduced by rheotaxis selection (13.2 ± 4.6 lower; *P* < 0.05). This trend was also evident with fresh samples (Fig. 2D) where the initial DFI of the samples (10.3 ± 2.3) was reduced by rheotaxis selection (8.6 ± 1.4 lower; *P* < 0.05), but also through the selection achieved with the swim-up (6.9 ± 1.2 lower; *P* < 0.05).

Figure 2 Motility, DNA fragmentation and morphology of frozen-thawed and fresh human spermatozoa by the method of processing. Total motility (A and B), DNA fragmentation index (DFI; C and D) and normal morphology (E and F) of frozen-thawed (A, C and E) and fresh (B, D and F) human spermatozoa selected by density gradient (gradient), swim-up or rheotaxis. Non selected (control) treatment is also represented. Each bar is a mean $\pm$ S.E.M. of the ten replicates/donors. ^{abc}Differing superscripts differ significantly.

Samples were also stained to assess sperm morphology. In frozen-thawed samples (Fig. 2E), we found that selection by rheotaxis improved the percentage of spermatozoa with normal morphology (NM; 40.5 ± 5.8 higher; $P < 0.001$), while no improvement was achieved by density gradient or swim-up selection. In addition, when sperm

morphology of fresh samples was assessed after performing the selection procedures (Fig. 2F), selection by rheotaxis not only increased NM from the starting population (49.2 ± 2.9 higher; $P < 0.001$), but also yielded a superior NM than density gradient and swim-up (41.5 ± 3.8 and 39.2 ± 3.6 higher, respectively; $P < 0.05$).

The type of sperm morphological abnormalities was also characterised (Table 1). In frozen-thawed and fresh samples, rheotaxis selected spermatozoa had fewer head abnormalities, in comparison to control, density gradient and swim-up ($P < 0.05$). A similar trend was observed in midpiece defects, but no further reduction was obtained when comparing gradient vs rheotaxis in frozen-thawed samples. None of the selection techniques affected abnormalities relating to the principal piece and excess residual cytoplasm in frozen-thawed samples. However, in fresh samples, sperm rheotaxis and swim-up selection reduced the presence of abnormalities in the principal piece ($P < 0.001$), while the presence of excess residual cytoplasm decreased with the three selection techniques studied ($P < 0.001$).

When sperm morphology was evaluated at high magnification, spermatozoa were graded into four groups according to the presence or size of the vacuoles, however, as Grade I and Grade II (absence of vacuoles and maximum of two small vacuoles, respectively) are deemed acceptable for ICSI, in Fig. 3 we present the percentage of Grade I+Grade II together. Sperm selection by rheotaxis increased the percentage of Grade I+II spermatozoa in comparison to the starting unselected sperm population, density gradient and swim-up ($P < 0.001$).

When sperm subjected to flow cytometry were classified based on the fluorescence of Yo-Pro-1, the percentage of viable spermatozoa after density gradient centrifugation was similar to the neat sample. However, spermatozoa processed either by swim-up or rheotaxis had increased sperm viability (19.5 ± 3.5 and $35.5 \pm 4.2\%$ higher, respectively), with the latter having the highest increase (Fig. 4A; $P < 0.01$). The population of sperm selected by rheotaxis had similar acrosome integrity to the other treatments ($P > 0.05$) (Fig. 4B). Rheotactically selected sperm exhibited a similar

Table 1 Distribution of sperm morphologic abnormalities by method of processing. Main abnormalities found in the sperm morphology assessment in unprocessed (control) semen or following density gradient (gradient), swim-up or rheotaxis: head, midpiece (MP), principal piece (PP) and excess residual cytoplasm (ERC). Results expressed as mean $\pm$ S.E.M. (%).

Groups	Head	MP	PP	ERC
Fresh samples				
Control	73.2 $\pm$ 3.3 ^a	30.4 $\pm$ 3.6 ^a	12.3 $\pm$ 1.9 ^a	2.8 $\pm$ 0.7 ^a
Gradient	68.1 $\pm$ 2.4 ^a	20.1 $\pm$ 3.4 ^a	9.9 $\pm$ 2.3 ^{a,b}	0.7 $\pm$ 0.2 ^b
Swim-up	68.1 $\pm$ 2.8 ^a	19.7 $\pm$ 4.0 ^a	4.3 $\pm$ 1.1 ^{b,c}	0.5 $\pm$ 0.1 ^b
Rheotaxis	32.6 $\pm$ 3.3 ^b	6.8 $\pm$ 1.6 ^b	1.9 $\pm$ 0.5 ^c	0.3 $\pm$ 0.3 ^b
Frozen-thawed samples				
Control	64.6 $\pm$ 4.4 ^a	31.4 $\pm$ 2.7 ^a	14.8 $\pm$ 2.1 ^a	2.0 $\pm$ 1.1 ^a
Gradient	59.4 $\pm$ 6.0 ^a	26.2 $\pm$ 2.7 ^{a,b}	14.2 $\pm$ 1.5 ^a	1.1 $\pm$ 0.3 ^a
Swim-up	53.0 $\pm$ 4.1 ^a	29.0 $\pm$ 3.3 ^a	12.3 $\pm$ 2.2 ^a	1.3 $\pm$ 0.4 ^a
Rheotaxis	31.8 $\pm$ 4.3 ^b	18.5 $\pm$ 3.8 ^b	12.8 $\pm$ 3.4 ^a	2.1 $\pm$ 0.8 ^a

^aValues with different superscripts differ significantly within column within fresh or frozen-thawed semen. $n = 10$ men in each of the fresh and frozen-thawed groups.

Figure 3 Frozen-thawed human sperm morphology evaluated at high magnification (6000×). Spermatozoa selected by density gradient (gradient), swim-up, rheotaxis, or non-selected (control) spermatozoa were graded and classified into four groups according to the presence or size of vacuoles following the criteria described by Vanderzwalmen *et al.* (2008). As only grade I and II are suitable for use in ICSI, the percentage of Grade I+Grade II are shown together. Samples from three different ejaculates of each donor were thawed and pooled, the experiment was replicated four times with different donors, and at least 200 spermatozoa were assessed per donor/sample. Each bar is a mean $\pm$ S.E.M of the four replicates. ^{abc}Differing superscripts differ significantly ($P < 0.001$).

membrane fluidity profile to swim-up selected sperm, but was lower than that of the neat sample as well as density gradient selected sperm (Fig. 4C; $P < 0.001$).

ICSI, *in vitro* culture and embryo transfer in a mouse model

Using a mouse model, we have demonstrated that when mouse spermatozoa were selected using rheotaxis or swim-up, there was a significant increase in fertilisation rates following ICSI, evaluated as the percentage of embryos reaching the 2-cell stage ($P < 0.01$; Fig. 5A). The resultant embryos were then transferred to recipient females, and those fertilised using rheotactically selected spermatozoa had a 12% higher implantation rate

($P < 0.001$; Fig. 5B) and a 20% higher foetal development rate ($P < 0.05$; Fig. 5C).

Discussion

Using both fresh and frozen-thawed spermatozoa, we have applied a novel simple method which can provide the embryologist with a population of sperm cells with superior DNA integrity as well as improved morphology and motility than the currently used methods of density gradient and swim-up. When mouse spermatozoa were selected using rheotaxis, it yielded higher fertilisation and implantation rates than when spermatozoa were selected using swim-up, and the offspring had normal embryonic development. Critically this biomimicry process does not involve centrifugation of spermatozoa or the use of exogenous binding substances.

Since the successful application of ICSI in 1992 to treat male factor infertility (Palermo *et al.* 1992), there has been a lack of an efficient physiological method for selecting the most suitable spermatozoon to be injected into a mature oocyte. The predominance of ICSI over IVF (De Geyter *et al.* 2020), is increasing the need to replace the traditional and rudimentary sperm preparation techniques (density gradient and swim-up). Mimicking the journey spermatozoa would undergo on their way to the fallopian tubes *in vivo* and based on rheotaxis, a natural functional test of swimming against a flow, we have developed a technology to help the embryologists to select the best sperm cells for ICSI.

Similar to Yamanaka *et al.* (2016), the percentage of motile spermatozoa in samples processed by density gradient in this study was similar to that in the original semen but increased with swim-up. Moreover, our device increased the total motility of the sample, both with frozen and fresh semen compared to the other selection techniques studied. A similar improvement was achieved by other authors who compared microfluidic sperm sorters with the sperm preparation procedure of density gradient centrifugation combined with swim-up; 100% vs 91% progressive motility, respectively, in Quinn *et al.* (2018), or 95.4% vs 60.3% total motility, respectively, in Shirota *et al.* (2016).

Figure 4 Viability, acrosome integrity and membrane fluidity of frozen-thawed human spermatozoa evaluated by flow cytometry. Viability (denoted A), acrosome integrity (denoted B) and membrane fluidity (denoted C) of frozen-thawed human spermatozoa selected by density gradient (gradient), swim-up or rheotaxis. Non selected (control) treatment is also represented. Data are expressed as percentage from the total analysed population. Each bar is a mean $\pm$ S.E.M of the 12 replicates/donors. ^{abc}Differing superscripts differ significantly ($P < 0.05$).

Figure 5 Cleavage, implantation, and foetal development rates following intracytoplasmic sperm injection of mouse spermatozoa preselected by swim-up or rheotaxis. Fertilization was evaluated as the percentage of embryos reaching the 2-cell stage (cleavage rate; denoted A). Subsequently, after transferring the embryos to recipient females, the percentage of embryos implanted (implantation rate; denoted B) and the percentage of embryos developed until the foetus stage (foetus development rate; denoted C) were calculated. Twenty replicates were completed using a total of 944 oocytes and spermatozoa from ten males. Embryos reaching the 2-cell stage were transferred to 25 females. Each female received 10–12 embryos from both treatments into either the left of right oviducts. Females were sacrificed at D14 of gestation. Vertical bars represent mean $\pm$ S.E.M. **Differing superscripts differ significantly.

Our rheotactically driven selection not only has increased the percentage of selected spermatozoa with normal morphology, both in fresh and frozen-thawed samples, but also, yielded a higher percentage of normal morphology than density gradient and swim-up (three and two to three-fold, respectively). We have also shown that rheotaxis selected spermatozoa had fewer head abnormalities, in comparison to control, density gradient and swim-up. Moreover, when sperm morphology was evaluated at high magnification, sperm selection by rheotaxis increased the percentage of spermatozoa with normal head morphology and no or small vacuoles (Grades I+II) in comparison to the starting unselected sperm population, density gradient and swim-up. The selection of spermatozoa for ICSI with normal head morphology and size at high magnification has been reported to be associated with improved fertilisation (Cassuto *et al.* 2009), blastocyst development (Vanderzwalmen *et al.* 2008), implantation and pregnancy rates (Hazout *et al.* 2006), as well as lower miscarriage rates (Antinori *et al.* 2008).

Nowadays, all the techniques to evaluate sperm DNA integrity render the sperm cells non-useful for human application. In cases where high levels of DNA fragmentation are diagnosed, the chance of spontaneous conception declines at sperm DFI values above 20% and approaches zero for values over 30–40% (Cissen *et al.* 2016). Our device selected a sperm population with decreased levels of DNA fragmentation for both fresh and frozen samples, as assessed by DFI. This is in agreement with other authors that have reported less DNA fragmentation in spermatozoa sorted using microfluidic

devices (Kishi *et al.* 2015, Shiota *et al.* 2016). Shiota *et al.* (2016) reported a significantly lower sperm DFI with a microfluidic chip compared with a centrifugation plus swim-up procedure (0.8% vs 10.1%, respectively). Regarding the traditional sperm preparation techniques, we found that only the swim-up method could reduce DFI, at least with fresh sperm samples. A similar reduction was recently reported by Oguz *et al.* (2018) who showed that swim-up but not density gradient method yielded a significant reduction in the DFI compared to basal rates, in semen samples of both unexplained and mild male factor subgroups. These results partially agree with other authors who reported DFI was reduced with density gradient, but unsurprisingly swim-up alone or density gradient followed by swim-up yielded greater reductions in DFI (Volpes *et al.* 2016, Yamanaka *et al.* 2016).

Unfortunately, the literature regarding a deep sperm characterisation after different sperm preparation techniques in human species is scarce, and it is infrequent to have other parameters analysed apart from motility, morphology and DNA fragmentation. In this regard, we report here that, among the sperm selection techniques, rheotaxis selected a population of spermatozoa with the highest viability, being even higher than the improvement shown by the swim-up technique and which had been previously reported (Force *et al.* 2001, Ricci *et al.* 2009, Highland *et al.* 2016). Moreover, when sperm plasma membrane fluidity was analysed to detect early signals of sperm capacitation (reviewed by Leahy & Gadella 2015), we report the same degree of membrane fluidity for the traditional sperm preparation techniques and the neat sample, but not for rheotaxis, which showed a slightly higher percentage of spermatozoa with low membrane fluidity. The results of the current study are in line with previous reports in which under non-capacitating conditions, similar to ours, there was no increase in membrane fluidity between the neat sample and the sperm population selected by density gradient centrifugation (Buffone *et al.* 2009) or swim-up (Force *et al.* 2001). Zhang *et al.* (2016) reported that there is no active signal transduction during human sperm rheotaxis, as it is a passive process, resulting from hydrodynamic interactions between the sperm flagellum and the surrounding fluid flow, where without extra energy consumption, sperm can reserve energy for executing later important events prior to fertilisation.

Using mouse oocytes, we demonstrated that when mouse spermatozoa were selected for ICSI using rheotaxis compared to the swim-up method, there was a significant increase in fertilisation rate. This improvement was also reflected by increased implantation and development until the foetal stage after transferring the embryos to recipient females. This may be due to spermatozoa with lower levels of DNA fragmentation being used in the rheotaxis treatment. A similar experimental approach was recently used to demonstrate an improved ICSI efficiency, and a rise in the embryo quality with mouse spermatozoa selected by

thermotaxis (Pérez-Cerezales *et al.* 2018), which lead us to validate not only the use of the mouse model for obtaining data on fertilisation, implantation and foetal development, but also the safety of this method of sperm selection.

Conclusion

Here we report a single-use novel microfluidic device that stimulates spermatozoa to swim against a flow of media driven by an electronic syringe pump. Over a 30 min period spermatozoa swim into a collection well from where a highly selected sperm fraction with low DFI can be removed and used for ICSI. This method selects spermatozoa not only with lower DNA fragmentation but also with higher motility, morphology and viability than currently used sperm selection methods in clinical practice. In addition, it has the potential to significantly reduce the time embryologists spend preparing the sample and selecting sperm for treatment. Using a mouse ICSI model, we have reported significantly improved fertilisation and embryo development rates. Further studies should be performed in fertility clinics to provide clinical data on the benefit of this sperm preparation method.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J R contributed to the design of the study, performed the experiments, analysed the data, and drafted the manuscript. R L and R F carried out the ICSI and the embryo transfers in mice. MS performed and analysed the flow cytometry experiments. F W contributed to the design of the experiments with human sperm samples and conducted the morphology evaluation at high magnification. J C, D M and P B L provided human semen samples and contributed to the design of the experiments with the human sperm samples. A G A designed and analysed the mouse ICSI and embryo transfer experiments while A M G contributed to the interpretation of the data. D N secured funding for the work, contributed to the design of the microfluidics system and the experiments. S F secured funding for the work, conceived and designed the study, analysed the data, and edited the manuscript. All authors read and revised the article and approved the final manuscript.

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