1 **TITLE:** Sperm selection by rheotaxis improves sperm quality and early embryo
2 development.
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23 **Short title:** Improving sperm selection using rheotaxis.

24 **Keywords:** rheotaxis, microfluidics, sperm, DNA fragmentation, morphology, motility.

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26

27 **ABSTRACT**

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

49 **INTRODUCTION**

50 Intracytoplasmic sperm injection (ICSI) was initially developed for patients with severe
51 male factor infertility, but its application has widened for cases with low oocyte yields,
52 cryopreserved oocytes, insemination for patients at risk for HIV or even to comply with
53 laws that limit the number of eggs to be inseminated (Palermo *et al.*, 2017). Recent
54 reports on the use of assisted reproductive technology (ART) show a dominance of ICSI
55 over conventional *in vitro* fertilization (IVF) and now makes up approximately 70% of
56 all fertilizations *in vitro* (Adamson *et al.*, 2018; De Geyter *et al.*, 2020) despite major
57 country to country variations.
58
59 The existing technologies used to select better quality spermatozoa for use in both IVF
60 and ICSI involve sperm cells either being forced to swim through a density gradient
61 using centrifugation or swim upwards in a tube of sperm preparation medium. These
62 techniques have been used for decades in the preparation of spermatozoa for traditional
63 IVF and by default are being used for sperm selection for ICSI as well, but seem to be
64 suboptimal for this purpose (Oseguera-López *et al.*, 2019). Embryologists must then
65 select through visual observation the best spermatozoon based on their morphology, a
66 step that can be extremely time-consuming (Auger *et al.*, 2016; Sikka and Hellstrom,
67 2016). In addition, they have no information on the DNA integrity of the selected sperm
68 as there is no non-destructive test for DNA fragmentation.
69
70 The capacity of human spermatozoa to produce a good-quality embryo with a high
71 implantation and development potential depends on many factors, including its DNA
72 integrity (Miller *et al.*, 2010). High levels of DNA fragmentation in spermatozoa can
73 slow down embryo morphokinetic parameters after ICSI (Wdowiak *et al.*, 2015) and

74 there is a positive relationship between levels of DNA damage in spermatozoa and
75 spontaneous pregnancy loss (Robinson *et al.*, 2012). Up to 30% of patients seeking
76 ART had high rates of sperm DNA breaks (Bungum *et al.*, 2004), and ICSI is the
77 preferred method when DNA fragmentation index (DFI) exceeds 30% (Bungum *et al.*,
78 2007; Chi *et al.*, 2017).
79
80 The need for a better sperm selection method has led to the development of new
81 technologies to increase the quality of sperm selection for ICSI. Among others, sodium
82 hyaluronate has been used to select spermatozoa based upon their maturity state in the
83 form of droplets (Parmegiani *et al.*, 2010) or coating culture dishes (Worrilow *et al.*,
84 2013); Magnetic activated cell sorting (MACS) combined Annexin V with magnetic
85 beads to remove apoptotic sperm cells (Ziarati *et al.*, 2019), and a new group of devices
86 have emerged to select spermatozoa based on microfluidics (Kishi *et al.*, 2015; Shirota
87 *et al.*, 2016; Chinnasamy *et al.*, 2018; Nakao *et al.*, 2020).
88
89 Enroute to the site of fertilization in the ampulla, mammalian spermatozoa have the
90 ability to be actively guided towards the oocyte by different mechanisms (Pérez-
91 Cerezales *et al.*, 2015). Among them, rheotaxis - or swimming against a flow - has been
92 revealed to be a long-distance biophysical guiding mechanism for human spermatozoa
93 (Kantsler *et al.*, 2014). In a recent preliminary study by De Martin *et al*., (2017), the use
94 of a rheotaxis related sperm selection method resulted in an improvement in sperm
95 morphology and chromatin maturity. However the study did not assess the impact of the
96 selection method on DNA fragmentation or fertility potential of the selected sperm.
97

98 Here we report a new sperm selection method which combines, in a single-use
99 microfluidic device, the ability of spermatozoa to orientate and swim against a flow of
100 media using microfluidic technology. We compared sperm selection by rheotaxis using
101 a proprietary designed microfluidic system with the current standard procedures for
102 sperm selection, namely density gradient and swim-up. Sperm quality was assessed in
103 terms of motility, morphology, viability, acrosome integrity, membrane fluidity and
104 DNA fragmentation using frozen-thawed and fresh spermatozoa. Using a mouse model,
105 we compared fertilization and embryo development rates after performing ICSI with
106 spermatozoa sorted using rheotaxis or swim-up.
107

108 **MATERIALS AND METHODS**

109 Unless otherwise indicated, all chemicals were of analytical grade and obtained from
110 Sigma Aldrich Ireland Ltd (Arklow, Ireland).
111

112 **Ethical approval**

113 All donors gave written informed consent and the use of human sperm samples for
114 research was approved by Faculty of Science and Engineering Research Ethics
115 Committee at the University of Limerick.
116

117 Experiments in mice were carried out in strict accordance with recommendations of the
118 guidelines of European Community Council Directive 86/609/EEC. Every effort was
119 made to minimize suffering. The study protocol was approved by the Committee on the
120 Ethics of Animal Experiments of the INIA (Madrid, Spain; permit number CEEA
121 2014/025).
122

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 3 straws (1 from each of 3 ejaculates) from each donor while for fresh semen a replicate was 1 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 4 times with different donors, and at least 200 spermatozoa were assessed per sample. Additionally, to further characterise the selection performed by the above-mentioned 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 fertilization 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 x10^6 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

173 and stored at -196 ºC. For each replicate one straw from each of three different
174 ejaculates from the same donor were thawed (30°C for 30 s), pooled to avoid intra-
175 individual differences and diluted 1:1 in sperm washing medium (SWM;
176 PureSperm®Wash, Nidacon, Mölndal, Sweden). All experiments were completed
177 blinded to the evaluator within 1 h after sperm collection/thawing.

178

179 **Sperm preparation techniques**

180 ***Density gradient centrifugation***

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

188

189 ***Swim-up***

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

197

198 ***Microfluidic chip***

199 A disposable device with a microfluidic channel was manufactured by CNC machining
200 from polymethyl methacrylate (PMMA). The microfluidic channel measured 300 µm
201 wide, 100 µm deep and 10 mm in length (Fig. 1). It was connected to a syringe pump
202 (Standard Infuse/Withdraw PHD ULTRA™ Syringe Pump, Harvard Apparatus,
203 Holliston, USA) by silicone tubing and a connector (Male Mini Luer fluid connector,
204 Microfluidic ChipShop, Jena, Germany). The microfluidic channel was primed with
205 SWM to remove air bubbles, following this, the flow velocity was stabilised at 30 µm/s.
206 A 20 µL sperm sample was loaded into the starting inlet of the microfluidic channel
207 allowing spermatozoa to orient against the oncoming flow and swim against it (positive
208 rheotaxis) for 30 min. Sperm progression was assessed under 200X magnification on an
209 inverted microscope (CK40; Olympus, Center Valley, USA). At the end of this period,
210 spermatozoa that reached the collection wells (20 µL per well) were collected and
211 submitted to further analysis. For a more comprehensive understanding of the
212 microfluidic device see Videos 1-5.
213

214 **Motility evaluation**

215 As part of the quality control, initial sperm motility was assessed within 30 min after
216 liquefaction of the sample or thawing and pooling the straws. It was also evaluated after
217 each sperm preparation technique. To this end, a volume of 10 µL of semen was
218 delivered onto a clean glass slide and covered with a 22 mm × 22 mm coverslip. The
219 procedure was performed at 37°C with a heated microscope stage using a phase-contrast
220 microscope (BX60; Olympus) equipped with an eyepiece reticle with grid at 200X
221 magnification. Motility was assessed as follows; (i) progressive motility (PR):
222 spermatozoa moving actively, either linearly or in a large circle, regardless of speed, (ii)

223 non-progressive motility (NP): all other patterns of motility with an absence of
224 progression, and (iii) immotility (IM): no movement. At least 200 spermatozoa were
225 scored per replicate for the percentage of different motile categories.

226

227 **Morphology evaluation**

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

237

238 **Morphology evaluation at high magnification**

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

248 vacuole; and (iv) Grade IV, large vacuoles in conjunction with abnormal head shapes or
249 other abnormalities.
250
251 **DNA fragmentation assay**
252 Sperm DNA integrity was evaluated by the acridine orange (AO) test as described by
253 Ajina et al., (2017). To avoid rapidly fading fluorescence, all the slides were observed
254 within 5 min under a fluorescence microscope (BX60; Olympus) at 1000X
255 magnification, with excitation at 450-490 nm. Spermatozoa with normal (double-
256 stranded) DNA showed green fluorescence, and those with denatured or single-stranded
257 DNA were red, orange, or yellow. The DFI (Jiang *et al.*, 2011) was used to represent the
258 ratio of spermatozoa with red, orange, or yellow fluorescence relative to all the
259 spermatozoa analysed per sample. At least 200 spermatozoa were assessed for each
260 replicate.
261
262 **Flow cytometry**
263 The aim of the flow cytometry analysis was to detect viability, membrane fluidity and
264 acrosome integrity in spermatozoa (frozen-thawed only) selected by the different sperm
265 preparation techniques. In all samples, the sperm concentration was set at $1x10^6$
266 spermatozoa per mL and sperm suspension was incubated with a three stain
267 combination of fluorochromes Yo-Pro1 (15nM, ThermoFisher Scientific, Waltham,
268 USA), Merocyanine M540 (2.6 µM, Sigma-Aldrich) and Lectin PNA from Arachis
269 hypogaea conjugated with Alexa Fluor™ 647 (2.5 µg/mL, ThermoFisher Scientific) for
270 15 min at 37°C. Subsequently, samples were subjected to analysis by CytoFlex flow
271 cytometry (Beckman Coulter Inc., Brea, USA) equipped with violet (405 nm), blue (488
272 nm) and red (635 nm) lasers. Sperm population was discriminated from debris with the

273 forward scatter (FSC) and side scatter (SSC) signals. Yo-Pro1 and M540 were excited
274 with the 488nm laser, while PNA-A647 was excited with the 635nm laser and emission
275 was detected by the 525/40BP, 585/42BP and 690/50BP filters, respectively. The
276 analysis of the flow cytometry data was carried out using CytExpert, version 2.3.0.84
277 (Beckman Coulter Inc.). All variables were assessed using logarithmic amplification,
278 10,000 gated events were collected for each sample and flow cytometry data are
279 expressed as percentage from the total analysed population.

280

281 **Animals, Gamete Collection, and Sperm Freezing**

282 Both male and female mice were fed ad libitum with a standard diet and maintained in a
283 temperature-and light-controlled room (23 ºC, 14L:10D). When required, animals were
284 euthanised by cervical dislocation.

285

286 Spermatozoa were collected from the caudal epididymis of 3-month-old B6D2 males
287 (F1 hybrid of C57BL/6J females and DBA/2J males) and suspended in a non-
288 capacitating modified human tubal fluid medium (ncHTF)(Pérez-Cerezales *et al.*, 2018).
289 The pH of the ncHTF medium was adjusted to 7.4 and spermatozoa were incubated for
290 1 h at 37 °C without CO_2 to allow motile spermatozoa to swim to the surface of the
291 droplet. Following sperm collection and transfer to a sterile Eppendorf tube, the sample
292 was either subjected to sperm selection using the microfluidic channel described earlier
293 (rheotaxis) or not (swim-up). Once selected, sperm samples were frozen by immersion
294 in liquid nitrogen (Lacham-Kaplan *et al.*, 2003) and stored for periods ranging from 1
295 day to 4 weeks at -80 ºC. Frozen-thawed spermatozoa were mixed with 40-50 µL of a
296 10% polyvinyl-pyrrolidone (PVP-360) in M2 medium before being placed in the culture
297 dish for microinjection.

298

299 *Mouse oocyte collection.* Metaphase II oocytes were collected from the oviducts of 6- to
300 8-wk-old B6D2 female mice superovulated with 7.5 IU of eCG, and with an equivalent
301 dose of hCG 48 h later, as described by Fernández-González et al., (2008).

302

303 **Intracytoplasmic sperm injection in mice**

304 ICSI with frozen-thawed mouse spermatozoa was performed in M2 medium at RT, as
305 previously described (Moreira *et al.*, 2007). Individual sperm heads decapitated by the
306 snap-freezing procedure were injected into oocytes. Oocytes were injected in groups of
307 10 per treatment with spermatozoa from one male.

308

309 **In vitro embryo culture**

310 After 15 min of recovery at 37 ºC in M2 medium, surviving oocytes were returned to
311 mineral oil-covered KSOM and cultured in groups of 20-40 embryos in 25 µl drops at
312 37 ºC in a 5% CO_2 atmosphere. Fertilization was evaluated as the percentage of
313 embryos reaching the 2-cell stage (Cleavage rate) after 24 h in culture.

314

315 **Embryo transfer**

316 Embryo transfer was performed as described previously (Fernández-Gonzalez *et al.*,
317 2008). Briefly, recipient pseudopregnant females (CD1 females mated with
318 vasectomized males) 0.5 days post coitum were anesthetised with inhaled isoflurane and
319 2-cell embryos were transferred into the oviducts. Each female received 10-12 embryos
320 from both treatments into either the left or right oviducts. On day 15 days after ICSI,
321 surrogate females were euthanised and the number of foetuses and reabsorptions were
322 scored as described by Perez-Cerezales et al, (2018). Subsequently, the percentage of

323 transferred embryos implanted (Implantation rate) and the percentage of implanted
324 embryos developed until the foetus stage (Foetus development rate) were calculated.
325
326 **Statistical analysis**
327 Data were analysed using Statistical Package for the Social Sciences (SPSS) (version
328 25, IBM, Chicago, USA). Data were first examined for normality of distribution using
329 the Shapiro-Wilk test and later for homogeneity of variance using Levene's test.
330 Unpaired t-tests for independent samples were performed for simple two-group
331 comparisons. For group-wise comparisons a univariate analysis of variance (ANOVA)
332 was used. Post hoc tests were conducted using the Bonferroni test. Results were
333 reported as the mean ± standard error of the mean (s.e.m). $P < 0.05$ was considered
334 statistically significant.
335
336 **RESULTS**
337 **Comparison of the selection performed by different sperm preparation techniques**
338 **in split semen samples using frozen-thawed and fresh human spermatozoa.**
339 Firstly, the motility of the samples was assessed, and total motility (TM, %) was
340 calculated as follows: TM=PR+NP. Spermatozoa selected by rheotaxis had higher TM
341 than the other selection techniques studied, both with frozen and fresh samples ($P <$
342 0.001; Fig. 2A, Fig. 2B, respectively). The swim-up selection also increased TM when
343 compared to density gradient and control groups, irrespective of whether they were
344 frozen-thawed or fresh ($P < 0.001$). However, surprisingly, no significant improvement
345 in TM was achieved through the density gradient in both frozen-thawed and fresh
346 spermatozoa.
347

348 Subsequently, we analysed the levels of DNA fragmentation of the sperm samples and
349 calculated the DFI. In frozen-thawed samples (Fig. 2C), the level of DNA fragmentation
350 of the initial samples (18.0 ± 4.9) was only reduced by rheotaxis selection (13.2 ± 4.6
351 lower; $P < 0.05$). This trend was also evident with fresh samples (Fig. 2D) where the
352 initial DFI of the samples (10.3 ± 2.3) was reduced by rheotaxis selection (8.6 ± 1.4
353 lower; $P < 0.05$), but also through the selection achieved with the swim-up (6.9 ± 1.2
354 lower; $P < 0.05$).
355
356 Samples were also stained to assess sperm morphology. In frozen-thawed samples (Fig.
357 2E), we found that selection by rheotaxis improved the percentage of spermatozoa with
358 normal morphology (NM; 40.5 ± 5.8 higher; $P < 0.001$), while no improvement was
359 achieved by density gradient or swim-up selection. In addition, when sperm
360 morphology of fresh samples was assessed after performing the selection procedures
361 (Fig. 2F), selection by rheotaxis not only increased NM from the starting population
362 (49.2 ± 2.9 higher; $P < 0.001$), but also yielded a superior NM than density gradient and
363 swim-up (41.5 ± 3.8 and 39.2 ± 3.6 higher, respectively; $P < 0.05$).
364
365 The type of sperm morphological abnormalities was also characterised (Table 1). In
366 frozen-thawed and fresh samples, rheotaxis selected spermatozoa had fewer head
367 abnormalities, in comparison to control, density gradient and swim-up ($P < 0.05$). A
368 similar trend was observed in midpiece defects, but no further reduction was obtained
369 when comparing gradient vs. rheotaxis in frozen-thawed samples. None of the selection
370 techniques affected abnormalities relating to the principal piece and excess residual
371 cytoplasm in frozen-thawed samples. However, in fresh samples, sperm rheotaxis and
372 swim-up selection reduced the presence of abnormalities in the principal piece (P <

373 0.001), while the presence of excess residual cytoplasm decreased with the three
374 selection techniques studied ($P < 0.001$).
375
376 When sperm morphology was evaluated at high magnification, spermatozoa were
377 graded into four groups according to the presence or size of the vacuoles, however, as
378 Grade I and Grade II (absence of vacuoles and maximum of two small vacuoles,
379 respectively) are deemed acceptable for ICSI, in Fig. 3 we present the percentage of
380 Grade I + Grade II together. Sperm selection by rheotaxis increased the percentage of
381 Grade I+II spermatozoa in comparison to the starting unselected sperm population,
382 density gradient and swim-up ($P < 0.001$).
383
384 When sperm subjected to flow cytometry were classified based on the fluorescence of
385 Yo-Pro-1, the percentage of viable spermatozoa after density gradient centrifugation
386 was similar to the neat sample. However, spermatozoa processed either by swim-up or
387 rheotaxis had increased sperm viability (19.5 ± 3.5 and 35.5 ± 4.2 % higher,
388 respectively), with the latter having the highest increase (Fig. 4A; $P < 0.01$). The
389 population of sperm selected by rheotaxis had similar acrosome integrity to the other
390 treatments ($P > 0.05$) (Fig. 4B). Rheotactically selected sperm exhibited a similar
391 membrane fluidity profile to swim-up selected sperm, but was lower than that of the
392 neat sample as well as density gradient selected sperm (Fig. 4C; $P < 0.001$).
393

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

395 Using a mouse model, we have demonstrated that when mouse spermatozoa were
396 selected using rheotaxis or swim-up, there was a significant increase in fertilization
397 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 fertilized 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.

423 Similar to Yamanaka *et al.,* (2016), the percentage of motile spermatozoa in samples
424 processed by density gradient in this study was similar to that in the original semen but
425 increased with swim-up. Moreover, our device increased the total motility of the
426 sample, both with frozen and fresh semen compared to the other selection techniques
427 studied. A similar improvement was achieved by other authors who compared
428 microfluidic sperm sorters with the sperm preparation procedure of density gradient
429 centrifugation combined with swim-up; 100 versus 91% progressive motility,
430 respectively, in Quinn *et al.,* (2018), or 95.4 versus 60.3% total motility, respectively, in
431 Shirota *et al.,* (2016).
432

433 Our rheotactically driven selection not only has increased the percentage of selected
434 spermatozoa with normal morphology, both in fresh and frozen-thawed samples, but
435 also, yielded a higher percentage of normal morphology than density gradient and
436 swim-up (3 and 2 to 3-fold, respectively). We have also shown that rheotaxis selected
437 spermatozoa had fewer head abnormalities, in comparison to control, density gradient
438 and swim-up. Moreover, when sperm morphology was evaluated at high magnification,
439 sperm selection by rheotaxis increased the percentage of spermatozoa with normal head
440 morphology and no or small vacuoles (Grades I+II) in comparison to the starting
441 unselected sperm population, density gradient and swim-up. The selection of
442 spermatozoa for ICSI with normal head morphology and size at high magnification has
443 been reported to be associated with improved fertilization (Cassuto *et al.*, 2009),
444 blastocyst development (Vanderzwalmen *et al.*, 2008), implantation and pregnancy rates
445 (Hazout *et al.*, 2006), as well as lower miscarriage rates (Antinori *et al.*, 2008).
446

447 Nowadays, all the techniques to evaluate sperm DNA integrity render the sperm cells
448 non-usable for human application. In cases where high levels of DNA fragmentation are
449 diagnosed, the chance of spontaneous conception declines at sperm DFI values above
450 20% and approaches zero for values over 30-40% (Cissen *et al.*, 2016). Our device
451 selected a sperm population with decreased levels of DNA fragmentation for both fresh
452 and frozen samples, as assessed by DFI. This is in agreement with other authors that
453 have reported less DNA fragmentation in spermatozoa sorted using microfluidic devices
454 (Kishi *et al.*, 2015; Shirota *et al.*, 2016). Shirota *et al.*, (2016) reported a significantly
455 lower sperm DFI with a microfluidic chip compared with a centrifugation plus swim-up
456 procedure (0.8 vs 10.1%, respectively). Regarding the traditional sperm preparation
457 techniques, we found that only the swim-up method could reduce DFI, at least with
458 fresh sperm samples. A similar reduction was recently reported by Oguz *et al*., (2018)
459 who showed that swim-up but not density gradient method yielded a significant
460 reduction in the DFI compared to basal rates, in semen samples of both unexplained and
461 mild male factor subgroups. These results partially agree with other authors who
462 reported DFI was reduced with density gradient, but unsurprisingly swim-up alone or
463 density gradient followed by swim-up yielded greater reductions in DFI (Volpes *et al.*,
464 2016; Yamanaka *et al.*, 2016).

465

466 Unfortunately, the literature regarding a deep sperm characterisation after different
467 sperm preparation techniques in human species is scarce and it is infrequent to have
468 other parameters analysed apart from motility, morphology and DNA fragmentation. In
469 this regard, we report here that, among the sperm selection techniques, rheotaxis
470 selected a population of spermatozoa with the highest viability, being even higher than
471 the improvement shown by the swim-up technique and which had been previously

472 reported (Force *et al.*, 2001; Ricci *et al.*, 2009; Highland *et al.*, 2016). Moreover, when
473 sperm plasma membrane fluidity was analysed to detect early signals of sperm
474 capacitation (reviewed by Leahy and Gadella, 2015), we report the same degree of
475 membrane fluidity for the traditional sperm preparation techniques and the neat sample,
476 but not for rheotaxis, which showed a slightly higher percentage of spermatozoa with
477 low membrane fluidity. The results of the current study are in line with previous reports
478 in which under non capacitating conditions, similar to ours, there was no increase in
479 membrane fluidity between the neat sample and the sperm population selected by
480 density gradient centrifugation (Buffone *et al.*, 2009) or swim up (Force *et al.*, 2001).
481 Zhang *et al.,* (2016) reported that there is no active signal transduction during human
482 sperm rheotaxis, as it is a passive process, resulting from hydrodynamic interactions
483 between the sperm flagellum and the surrounding fluid flow, where without extra
484 energy consumption, sperm can reserve energy for executing later important events
485 prior to fertilization.
486
487 Using mouse oocytes, we demonstrated that when mouse spermatozoa were selected for
488 ICSI using rheotaxis compared to the swim-up method, there was a significant increase
489 in fertilization rate. This improvement was also reflected by increased implantation and
490 development until the foetal stage after transferring the embryos to recipient females.
491 This may be due to spermatozoa with lower levels of DNA fragmentation being used in
492 the rheotaxis treatment. A similar experimental approach was recently used to
493 demonstrate an improved ICSI efficiency and a rise in the embryo quality with mouse
494 spermatozoa selected by thermotaxis (Pérez-Cerezales *et al.*, 2018), which lead us to
495 validate not only the use of the mouse model for obtaining data on fertilization,

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 fertilization 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

520 JR contributed to the design of the study, performed the experiments, analysed the data,
521 and drafted the manuscript. RL and RF carried out the ICSI and the embryo transfers in
522 mice. MS performed and analysed the flow cytometry experiments. FW contributed to
523 the design of the experiments with human sperm samples and conducted the
524 morphology evaluation at high magnification. JC, DM and PBL provided human semen
525 samples and contributed to the design of the experiments with the human sperm
526 samples. AGA designed and analysed the mouse ICSI and embryo transfer experiments
527 while AMG contributed to the interpretation of the data. DN secured funding for the
528 work, contributed to the design of the microfluidics system and the experiments. SF
529 secured funding for the work, conceived and designed the study, analysed the data, and
530 edited the manuscript. All authors read and revised the article and approved the final
531 manuscript.
532

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537

538 **REFERENCES**

539 **Adamson GD, de Mouzon J, Chambers GM, Zegers-Hochschild F, Mansour R,**
540 **Ishihara O, Banker M and Dyer S** (2018) International Committee for
541 Monitoring Assisted Reproductive Technology: world report on assisted
542 reproductive technology, 2011. *Fertility and Sterility* **110** 1067–1080.
543 **Ajina T, Ammar O, Haouas Z, Sallem A, Ezzi L, Grissa I, Sakly W, Jlali A and**
544 **Mehdi M** (2017) Assessment of human sperm DNA integrity using two

545 cytochemical tests: Acridine orange test and toluidine blue assay. *Andrologia* **49** 546 1–6.

547 **Antinori M, Licata E, Dani G, Cerusico F, Versaci C, d'Angelo D and Antinori S** 548 (2008) Intracytoplasmic morphologically selected sperm injection: A prospective 549 randomized trial. *Reproductive BioMedicine Online* **16** 835–841.

550 **Auger J, Jouannet P and Eustache F** (2016) Another look at human sperm 551 morphology. *Human Reproduction* **31** 10–23.

552 **Buffone MG, Doncel GF, Calamera JC and Verstraeten S V.** (2009) Capacitation-553 associated changes in membrane fluidity in asthenozoospermic human 554 spermatozoa. *International Journal of Andrology* **32** 360–375.

555 **Bungum M, Humaidan P, Spano M, Jepson K, Bungum L and Giwercman A** 556 (2004) The predictive value of sperm chromatin structure assay (SCSA) 557 parameters for the outcome of intrauterine insemination, IVF and ICSI. *Human* 558 *Reproduction* **19** 1401–1408.

559 **Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J and** 560 **Giwercman A** (2007) Sperm DNA integrity assessment in prediction of assisted 561 reproduction technology outcome. *Human Reproduction* **22** 174–179.

562 **Cassuto NG, Bouret D, Plouchart JM, Jellad S, Vanderzwalmen P, Balet R, Larue** 563 **L and Barak Y** (2009) A new real-time morphology classification for human 564 spermatozoa: a link for fertilization and improved embryo quality. *Fertility and* 565 *Sterility* **92** 1616–1625.

566 **Chi HJ, Kim SG, Kim YY, Park JY, Yoo CS, Park IH, Sun HG, Kim JW, Lee KH**
567 **and Park HD** (2017) ICSI significantly improved the pregnancy rate of patients
568 with a high sperm DNA fragmentation index. *Clinical and Experimental*
569 *Reproductive Medicine* **44** 132–140.
570 **Chinnasamy T, Kingsley JL, Inci F, Turek PJ, Rosen MP, Behr B, Tüzel E and**
571 **Demirci U** (2018) Guidance and Self-Sorting of Active Swimmers: 3D Periodic
572 Arrays Increase Persistence Length of Human Sperm Selecting for the Fittest.
573 *Advanced Science* **5**.
574 **Cissen M, Van Wely M, Scholten I, Mansell S, De Bruin JP, Mol BW, Braat D,**
575 **Repping S and Hamer G** (2016) Measuring sperm DNA fragmentation and
576 clinical outcomes of medically assisted reproduction: A systematic review and
577 meta analysis. *PLoS ONE* **11**.
578 **De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko T,**
579 **Scaravelli G, Smeenk J, Vidakovic S, Goossens V *et al.*** (2020) ART in Europe,
580 2015: results generated from European registries by ESHRE†. *Human*
581 *Reproduction Open* **2020** 1–17.
582 **De Martin H, Cocuzza MS, Tiseo BC, Wood GJA, Miranda EP, Monteleone PAA,**
583 **Soares JM, Serafini PC, Srougi M and Baracat EC** (2017) Positive rheotaxis
584 extended drop: a one-step procedure to select and recover sperm with mature
585 chromatin for intracytoplasmic sperm injection. *Journal of Assisted Reproduction*
586 *and Genetics* **34** 1699–1708.
587 **Fernández-Gonzalez R, Moreira PN, Pérez-Crespo M, Sánchez-Martín M,**
588 **Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, Hourcade JDD, De**
589 **Fonseca FR *et al.*** (2008) Long-term effects of mouse intracytoplasmic sperm
590 injection with DNA-fragmented sperm on health and behavior of adult offspring.

591 *Biology of Reproduction* **78** 761–772.

592 **Force A, Grizard G, Giraud MN, Motta C, Sion B and Boucher D** (2001)
593 Membrane fluidity and lipid content of human spermatozoa selected by swim-up
594 method. *International Journal of Andrology* **24** 327–334.

595 **Hazout A, Dumont-Hassan M, Junca AM, Bacrie PC and Tesarik J** (2006) High-
596 magnification ICSI overcomes paternal effect resistant to conventional ICSI.
597 *Reproductive BioMedicine Online* **12** 19–25.

598 **Highland HN, Rishika AS, Almira SS and Kanthi PB** (2016) Ficoll-400 density
599 gradient method as an effective sperm preparation technique for assisted
600 reproductive techniques. *Journal of Human Reproductive Sciences* **9** 194–199.

601 **Jiang H, He RB, Wang CL and Zhu J** (2011) The relationship of sperm DNA
602 fragmentation index with the outcomes of in-vitro fertilisation-embryo transfer and
603 intracytoplasmic sperm injection. *Journal of Obstetrics and Gynaecology* **31** 636–
604 639.

605 **Kantsler V, Dunkel J, Blayney M and Goldstein RE** (2014) Rheotaxis facilitates
606 upstream navigation of mammalian sperm cells. *ELife* **3** e02403.

607 **Kishi K, Ogata H, Ogata S, Mizusawa Y, Okamoto E, Matsumoto Y, Kokeguchi S**
608 **and Shiotani M** (2015) Frequency of sperm DNA fragmentation according to
609 selection method: Comparison and relevance of a microfluidic device and a swim-
610 up procedure. *Journal of Clinical and Diagnostic Research* **9** QC14–QC16.

611 **Lacham-Kaplan O, Shaw J, Sanchez-Partida LG and Trounson A** (2003) Oocyte
612 Activation after Intracytoplasmic Injection with Sperm Frozen Without
613 Cryoprotectants Results in Live Offspring from Inbred and Hybrid Mouse Strains.
614 *Biology of Reproduction* **69** 1683–1689.

615 **Leahy T and Gadella BM** (2015) New insights into the regulation of cholesterol efflux

616 from the sperm membrane. *Asian Journal of Andrology* **17** 561–567.

617 **Lyu QF, Deng L, Xue SG, Cao SF, Liu XY, Jin W, Wu LQ and Kuang YP** (2010)
618 New technique for mouse oocyte injection via a modified holding pipette.
619 *Reproductive BioMedicine Online* **21** 663–666.

620 **Ma S, Wang P, Zhou W, Chu D, Zhao S, Fu L and Li Y** (2020) A modified holding
621 pipette for mouse oocyte fertilization. *Theriogenology* **141** 142–145.

622 **Miller D, Brinkworth M and Iles D** (2010) Paternal DNA packaging in spermatozoa:
623 More than the sum of its parts? DNA, histones, protamines and epigenetics.
624 *Reproduction* **139** 287–301.

625 **Moreira PN, Pérez-Crespo M, Ramírez MA, Pozueta J, Montoliu L and Gutiérrez-**
626 **Adán A** (2007) Effect of transgene concentration, flanking matrix attachment
627 regions, and RecA-coating on the efficiency of mouse transgenesis mediated by
628 intracytoplasmic sperm injection. *Biology of Reproduction* **76** 336–343.

629 **Nakao S, Takeo T, Watanabe H, Kondoh G and Nakagata N** (2020) Successful
630 selection of mouse sperm with high viability and fertility using microfluidics chip
631 cell sorter. *Scientific Reports* **10** 8862.

632 **Oguz Y, Guler I, Erdem A, Mutlu MF, Gumuslu S, Oktem M, Bozkurt N and**
633 **Erdem M** (2018) The effect of swim-up and gradient sperm preparation
634 techniques on deoxyribonucleic acid (DNA) fragmentation in subfertile patients.
635 *Journal of Assisted Reproduction and Genetics* **35** 1083–1089.

636 **Oseguera-López I, Ruiz-Díaz S, Ramos-Ibeas P and Pérez-Cerezales S** (2019)
637 Novel Techniques of Sperm Selection for Improving IVF and ICSI Outcomes.
638 *Frontiers in Cell and Developmental Biology* **7**.

639 **Palermo G, Joris H, Devroey P and Van Steirteghem AC** (1992) Pregnancies after
640 intracytoplasmic injection of single spermatozoon into an oocyte. *The Lancet* **340**

641 17–18.

642 **Palermo GD, O'Neill CL, Chow S, Cheung S, Parrella A, Pereira N and**
643 **Rosenwaks Z** (2017) Intracytoplasmic sperm injection: State of the art in humans.
644 *Reproduction* **154** F93–F110.

645 **Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Ciampaglia W and Filicori M**
646 (2010) 'Physiologic ICSI': Hyaluronic acid (HA) favors selection of spermatozoa
647 without DNA fragmentation and with normal nucleus, resulting in improvement of
648 embryo quality. *Fertility and Sterility* **93** 598–604.

649 **Pérez-Cerezales S, Boryshpolets S and Eisenbach M** (2015) Behavioral mechanisms
650 of mammalian sperm guidance. *Asian Journal of Andrology* **17** 628–632.

651 **Pérez-Cerezales S, Laguna-Barraza R, De Castro AC, Sánchez-Calabuig MJ,**
652 **Cano-Oliva E, De Castro-Pita FJ, Montoro-Buils L, Pericuesta E, Fernández-**
653 **González R and Gutiérrez-Adán A** (2018) Sperm selection by thermotaxis
654 improves ICSI outcome in mice. *Scientific Reports* **8** 2902.

655 **Quinn MM, Jalalian L, Ribeiro S, Ona K, Demirci U, Cedars MI and Rosen MP**
656 (2018) Microfluidic sorting selects sperm for clinical use with reduced DNA
657 damage compared to density gradient centrifugation with swim-up in split semen
658 samples. *Human Reproduction* **33** 1388–1393.

659 **Ricci G, Perticarari S, Boscolo R, Montico M, Guaschino S and Presani G** (2009)
660 Semen preparation methods and sperm apoptosis: swim-up versus gradient-density
661 centrifugation technique. *Fertility and Sterility* **91** 632–638.

662 **Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, Kirkman-**
663 **Brown J and Coomarasamy A** (2012) The effect of sperm DNA fragmentation
664 on miscarriage rates: A systematic review and meta-analysis. *Human Reproduction*
665 **27** 2908–2917.

666 **Shirota K, Yotsumoto F, Itoh H, Obama H, Hidaka N, Nakajima K and Miyamoto**
667 **S** (2016) Separation efficiency of a microfluidic sperm sorter to minimize sperm
668 DNA damage. *Fertility and Sterility* **105** 315-321.e1.

669 **Sikka SC and Hellstrom WJG** (2016) Current updates on laboratory techniques for
670 the diagnosis of male reproductive failure. *Asian Journal of Andrology* **18** 392–
671 401.

672 **Vanderzwalmen P, Hiemer A, Rubner P, Bach M, Neyer A, Stecher A, Uher P,**
673 **Zintz M, Lejeune B, Vanderzwalmen S *et al.*** (2008) Blastocyst development
674 after sperm selection at high magnification is associated with size and number of
675 nuclear vacuoles. *Reproductive BioMedicine Online* **17** 617–627.

676 **Volpes A, Sammartano F, Rizzari S, Gullo S, Marino A and Allegra A** (2016) The
677 pellet swim-up is the best technique for sperm preparation during in vitro
678 fertilization procedures. *Journal of Assisted Reproduction and Genetics* **33** 765–
679 770.

680 **Wdowiak A, Bakalczuk S and Bakalczuk G** (2015) The effect of sperm DNA
681 fragmentation on the dynamics of the embryonic development in
682 intracytoplasmatic sperm injection. *Reproductive Biology* **15** 94–100.

683 **World Health Organization** (2010) WHO laboratory manual for the examination and
684 processing of human semen.

685 **Worrilow KC, Eid S, Woodhouse D, Perloe M, Smith S, Witmyer J, Ivani K,**
686 **Khoury C, Ball GD, Elliot T *et al.*** (2013) Use of hyaluronan in the selection of
687 sperm for intracytoplasmic sperm injection (ICSI): Significant improvement in
688 clinical outcomes-multicenter, double-blinded and randomized controlled trial.
689 *Human Reproduction* **28** 306–314.

690 **Yamanaka M, Tomita K, Hashimoto S, Matsumoto H, Satoh M, Kato H, Hosoi Y,**
691 **Inoue M, Nakaoka Y and Morimoto Y** (2016) Combination of density gradient
692 centrifugation and swim-up methods effectively decreases morphologically
693 abnormal sperms. **62** 16–18.
694 **Yanagimachi R** (1998) Intracytoplasmic sperm injection experiments using the mouse
695 as a model. *Human Reproduction* **13** 87–98.
696 **Zhang Z, Liu J, Meriano J, Ru C, Xie S, Luo J and Sun Y** (2016) Human sperm
697 rheotaxis: A passive physical process. *Scientific Reports* **6** 23553.
698 **Ziarati N, Tavalaee M, Bahadorani M and Nasr Esfahani MH** (2019) Clinical
699 outcomes of magnetic activated sperm sorting in infertile men candidate for ICSI.
700 *Human Fertility* **22** 118–125.
701
702

703 **FIGURE LEGENDS**

704

705 **Figure 1. Microfluidic chip.**

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

720

721 **Figure 2. Motility, DNA fragmentation and morphology of frozen-thawed and**
722 **fresh human spermatozoa by the method of processing.**

723 Total motility (a and b), DNA fragmentation index (DFI; c and d) and normal
724 morphology (e and f) of frozen-thawed (a, c and e) and fresh (b, d and f) human
725 spermatozoa selected by density gradient (gradient), swim-up or rheotaxis. Non selected
726 (control) treatment is also represented. Each bar is a mean ± s.e.m of the 10
727 replicates/donors. ^{abc}Differing superscripts differ significantly.

728

729 **Figure 3. Frozen-thawed human sperm morphology evaluated at high**
730 **magnification (6000X).**

731 Spermatozoa selected by density gradient (gradient), swim-up, rheotaxis, or non-
732 selected (control) spermatozoa were graded and classified into four groups according to
733 the presence or size of vacuoles following the criteria described by Vanderzwalmen *et*
734 *al*., (2008). As only grade I and II are suitable for use in ICSI, the percentage of Grade I
735 + Grade II are shown together. Samples from three different ejaculates of each donor
736 were thawed and pooled, the experiment was replicated 4 times with different donors,
737 and at least 200 spermatozoa were assessed per donor/sample. Each bar is a mean ±
738 s.e.m of the 4 replicates. [abc]Differing superscripts differ significantly ($P < 0.001$).

739

740 **Figure 4. Viability, acrosome integrity and membrane fluidity of frozen-thawed**
741 **human spermatozoa evaluated by flow cytometry.**

742 Viability (denoted a), acrosome integrity (denoted b) and membrane fluidity (denoted c)
743 of frozen-thawed human spermatozoa selected by density gradient (gradient), swim-up
744 or rheotaxis. Non selected (control) treatment is also represented. Data are expressed as
745 percentage from the total analysed population. Each bar is a mean ± s.e.m of the 12
746 replicates/donors. [abc]Differing superscripts differ significantly ($P < 0.05$).

747

748

749 **Figure 5. Cleavage, implantation, and foetal development rates following**
750 **intracytoplasmic sperm injection of mouse spermatozoa preselected by swim-up or**
751 **rheotaxis.**

752 Fertilization was evaluated as the percentage of embryos reaching the 2-cell stage
753 (Cleavage rate; denoted a). Subsequently, after transferring the embryos to recipient
754 females, the percentage of embryos implanted (Implantation rate; denoted b) and the
755 percentage of embryos developed until the foetus stage (Foetus development rate;
756 denoted c) were calculated. Twenty replicates were completed using a total of 944
757 oocytes and spermatozoa from 10 males. Embryos reaching the 2-cell stage were
758 transferred to 25 females. Each female received 10-12 embryos from both treatments
759 into either the left of right oviducts. Females were sacrificed at D14 of gestation.
760 Vertical bars represent mean ± s.e.m. **Differing superscripts differ significantly.
761

VIDEO LEGENDS

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).

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).

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).

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).

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).