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ORIGINAL ARTICLE

Microfluidic sperm sorting improves ICSI outcomes in patients with increased values of Double-Strand Breaks in sperm DNA

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Double-Strand Breaks;
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Microfluidic sperm sorting

Abstract
Background: Delays in embryo kinetics, implantation failures in ICSI treatments and recurrent miscarriages have been associated with high values of Double-Strand Breaks (DSB) in sperm DNA. While conventional methods for semen preparation have been shown to be inefficient reducing DSB values, Microfluidic Sperm Sorting (MSS) devices are promising tools to reduce this damage.
Objective: To study the clinical utility of an MSS device in ICSI treatments when the male partner presents increased DSB values, as compared to the use of conventional methods based on sperm motility.
Methods: This retrospective cohort study included 28 infertile couples undergoing ICSI treatments. Only couples where the male partner presented increased values of DSB were included. DSB values were evaluated in semen samples by the Neutral Comet assay. Couples performed a first ICSI cycle using conventional methods for semen preparation (Density Gradients and Swim-up) and a second ICSI cycle using the ZyMōt™ICSI (formerly named FertileChip®) microfluidic device. Embryology and clinical outcomes were compared between ICSI cycles.
Results: Semen parameters and the number of obtained and fertilized oocytes did not show differences between ICSI rounds. Clinical outcomes were statistically better when MSS was used: the biochemical pregnancy rate increased 28.31%; the clinical pregnancy rate increased 35.56% and the number of live births increased 35.29%, as compared to the first ICSI cycle in this group of patients.

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Conclusions: The ZyMōt™ICSI microfluidic device improved the reproductive outcomes in couples where the male partner presented increased DSB values, when compared to the use of conventional semen preparation techniques.

PALABRAS CLAVE
Selección espermática;
Roturas de cadena doble;
Fragmentación del ADN espermático;
ICSI;
Selección espermática por microfluidos

La selección espermática mediante un dispositivo microfluídico mejora los resultados de ICSI en pacientes con valores elevados de fragmentación de cadena doble en el ADN espermático

Resumen
Antecedentes: Valores elevados de fragmentación de cadena doble (DSB) en el ADN de los espermatozoides se han asociado con retrasos en la cinética embrionaria, fallos de implantación en ciclos de ICSI y con abortos de repetición. Actualmente no hay evidencias de que los métodos convencionales para la preparación del semen puedan reducir los niveles de DSB. Por el contrario, los nuevos dispositivos microfluídicos de selección espermática (MSS) han mostrado resultados prometedores en cuanto a la reducción de la fragmentación.
Objetivo: Evaluar el uso de un dispositivo MSS en ciclos de ICSI donde el varón presenta niveles elevados de DSB, en comparación con el uso de métodos convencionales basados en la selección por motilidad.
Métodos: Este estudio retrospectivo ha incluido a 28 parejas infértiles que han realizado ciclos de ICSI y donde se han detectado valores elevados de DSB en la muestra seminal del varón. Los niveles de DSB se han analizado mediante el test Cometa Neutro. Las parejas realizaron un primer ciclo de ICSI utilizando métodos convencionales para la preparación del semen (gradientes de densidad y Swim-up). Posteriormente, las parejas realizaron un segundo ciclo de ICSI utilizando el dispositivo microfluídico ZyMōt™ICSI (antes FertileChip®). Se han comparado los resultados de embriología y los resultados clínicos entre ambos tratamientos.
Resultados: No se han encontrado diferencias entre ambos ciclos de ICSI en cuanto a parámetros seminales y el número de ovocitos obtenidos y fecundados. Los resultados clínicos fueron mejores cuando se usó el dispositivo MSS: se observó un incremento del 28,31% en la tasa de embarazo bioquímico, del 35,56% en la tasa de embarazo clínico y del 35,29% en la tasa de nacidos vivos, en comparación con el uso de métodos convencionales.
Conclusiones: El dispositivo microfluídico ZyMōt™ ICSI mejoró los resultados clínicos en ciclos de ICSI cuando donde el varón presentaba niveles elevados de DSB, en comparación con métodos convencionales de preparación del semen.

Introduction

Since the first human pregnancies were achieved through Assisted Reproductive Treatments (ART) in the 1970s, more than eight million babies have been born worldwide thanks to these techniques.[1] Different factors are responsible for the significant improvement of ART clinical outcomes, such as the standardization of the procedures and materials in the laboratories or the introduction of the Intracytoplasmic Sperm Injection (ICSI) technique in the 1990s.[2] All of these improvements lead to a mean pregnancy rate per embryo transfer of 35.0% in Europe and 35.8% in the USA, according to the last available data.[3,4]

A male factor is present in half of the infertility cases, meaning that over 30 million men worldwide may experience reproductive difficulties of different aetiologies.[5,6] The evaluation of sperm DNA fragmentation has been an emerging field during the last decade, showing that Single- and Double-Strand Breaks (SSB and DSB, respectively) have a major impact on embryos' development and reproductive outcomes.[7,8] Increased values of SSB in semen samples have been associated with high levels of Reactive Oxygen Species (ROS).[9] Moreover, ROS have also been shown to negatively affect sperm motility through membrane, axonemal and mitochondrial alterations.[10] Since SSB are produced in an extensive manner throughout all of the genome, the presence of this damage may impair first mitotic divisions in embryos.[11] In this regard, the presence of SSB in sperm DNA has been associated with lower pregnancy rates in natural conception and a higher risk of preimplantation failures in ART.[11,12] On the contrary, DSB are mainly produced in a controlled manner by enzymatic

mechanisms,[11] for example, through the action of Spo11 during the prophase I stage of spermatogenesis to allow homologous recombination.[13] Endonuclease activity, environmental toxics or alterations during histone-protamine replacement can also induce DSB.[14] Therefore, first mitotic divisions could be possible in embryos after the injection of sperm with DSB but may lead to lower embryo kinetics during *In Vitro* Fertilization (IVF) treatments, and to a major risk of pregnancy loss.[7,11]

Among different tests to analyze sperm DNA fragmentation, the Comet assay is able to discriminate between SSB and DSB by performing alkaline and neutral versions of the test, respectively.[8,15] In this assay, isolated cells are embedded in an agarose microgel and exposed to an electrophoretic current. In the alkaline version of the Comet assay, the double helix of DNA is denaturalized and breaks affecting one DNA strand are visualized (SSB). In neutral pH conditions, the double helix remains and only breaks affecting both strands are visualized (DSB).[16]

Before the microinjection of sperm during ICSI cycles, semen samples must be processed. Conventional methods for semen preparation include the Swim-up technique (SU), where the sample is centrifuged and the pellet is resuspended in media to allow motile cells to swim to the upper layer of the tube; or Density Gradients (DG), where two layers of colloidal silica at different concentrations filter centrifuged semen samples to obtain motile sperm with normal density.[17] These procedures require centrifugation steps which have been widely described to produce iatrogenic DNA damage through ROS formation in patients[18] and fertile donors.[19] In this regard, ROS may increase DNA damage, especially in sperm with previously compromised chromatin integrity.[8] Nevertheless, some authors have reported that these conventional methods partially reduce the overall value of SSB in the semen sample because immotile sperm are discarded.[20] A recent study showed that the SU procedure and the sperm selection process performed by embryologists during ICSI cycles, based on morphology and motility, significantly reduce SSB values, but did not appear to have any effect in selecting sperm with low DSB damage.[21]

New methodologies for sperm selection based on microfluidics have recently emerged.[17,22] The microchannels inside these devices imitate the natural sperm selection process that happens inside the female reproductive tract. Interestingly, these Microfluidic Sperm Sorting (MSS) devices do not require centrifugation steps, therefore, avoiding iatrogenic DNA and membrane damage.[23] Also about DNA damage, these devices have shown promising results reducing DNA fragmentation values.[20,24]

The main objective of the present study is to show the first evidence on the potential beneficial effect on reproductive outcomes of the use of an MSS device in couples with high levels of DSB in semen.

Material and methods

Samples and groups

This retrospective cohort study included two consecutive ICSI cycles from 28 infertile couples. A first ICSI cycle was performed using DG and SU for semen preparation. After the failure of this cycle, males were assessed for DSB values by the Neutral Comet assay, showing altered values (>60%, internal cut-off value). A second ICSI cycle was programmed using an MSS device with the objective of reducing DSB values. No lifestyle changes were recorded between either ICSI cycles, including nutritional or toxic consumption, for both the female and the male partners. Couples were excluded from the study when the male presented normal values of DSB (<60%) or other fertility impairments such as genitourinary infections or severe alterations of the sperm parameters[25]; or when the female presented other infertility conditions, aside from advanced age, such as hormonal disturbances, endometriosis, polycystic ovary or tubal factor. ICSI treatments were performed at the GINEFIV fertility centre (General Life IVF Group). This study was approved by the Corporació Sanitaria Parc Taulí Ethics Committee (Ref 2017902) and signed informed consents were obtained from all patients.

Ovarian stimulation and oocyte retrieval

Ovarian stimulation was carried out using a short protocol with Gonadotropin releasing hormone (GnRH) antagonists: FSHr (Gonal; MerckSerono, Germany), HMG (Menopur; Meriofert, Italy), FSHr and LHr (Pergoveris; MerckSerono, Germany). Ovulation was induced when three or more follicles reached 17 mm in diameter using GnRH analogues (Decapeptyl 0.2 mg; Ipsen Pharma, France) and hCG (Ovitrelle; MerckSerono, Germany). Oocyte retrieval was performed 34–36 h later.

Women received oestrogens to prepare the endometrium through oral administration of Progynova (Bayer, Germany) or Meriestra (Novartis, Switzerland) (4–8 mg/day). After oocyte retrieval, women started the administration of progesterone (Progeffik; Effik, Spain or Utrogestan; Seid, Spain). If a pregnancy was confirmed, the administration of oestrogens and progesterone was maintained up to a maximum of 100 days.

Oocytes were maintained in G-IVF media (Vitrolife, Sweden) and denuded using 1:9 hyaluronidase (Hyasetm-10x; Vitrolife, Sweden) and buffered media (G-MOPS Plus; Vitrolife, Sweden).

Sperm selection and ICSI

Semen samples were obtained by masturbation after 3–5 days of sexual abstinence. Semen parameters were evaluated before each ICSI treatment. Sperm DNA damage was evaluated between both cycles.

During the first ICSI treatment, semen preparation was performed using a combination of DG and SU. Gradients were prepared using 80% and 40% Sperm Grad and IVF media (Vitrolife, Sweden). The first centrifugation was performed at 1000 rpm during 20 min. The pellet was resuspended in 0.7 mL of IVF media. A final washing step was performed during 5 min at 1200 rpm. The pellet was resuspended in 150–300 μL of IVF media and a standard SU procedure was performed.[25]

During the second ICSI treatment, semen preparation was performed using an MSS device (ZyMōt™ICSI, formerly named FertileChip®; DxNow Inc., MD, USA). The five

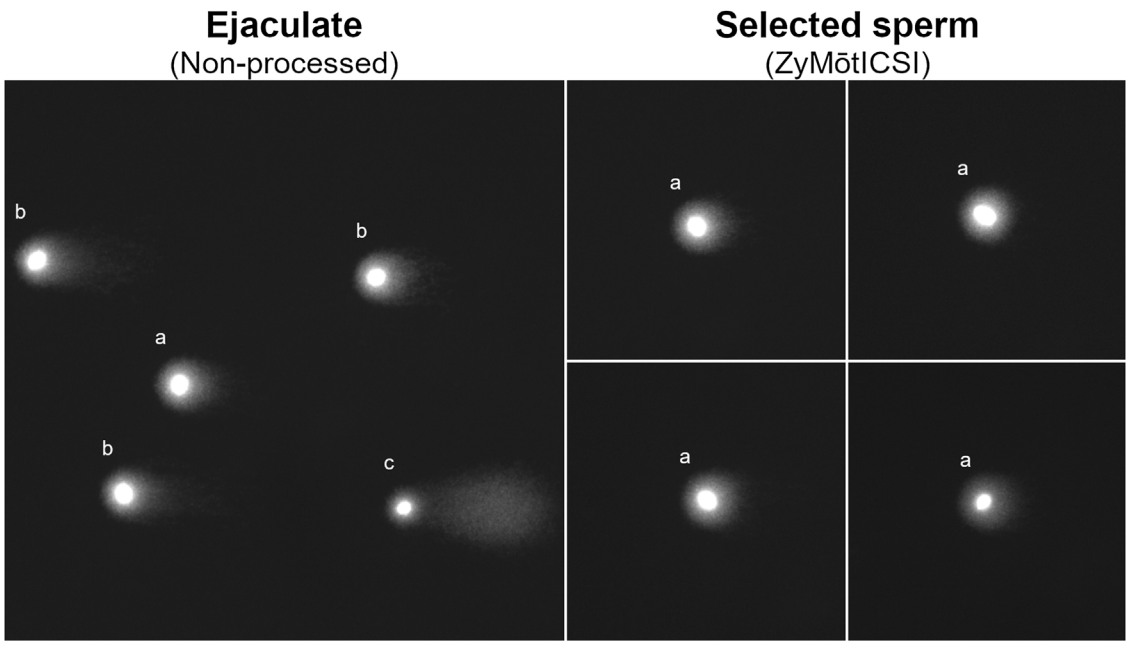

Figure 1 Microscopic images of the Neutral Comet assay performed on sperm from the raw semen sample (ejaculate) and selected sperm filtered using the ZyMōtICSI MSS device. [a] Indicates sperm with intact DNA; [b] Indicates sperm with fragmented DNA; [c] Indicates sperm with highly degraded DNA.

channels were hydrated by injecting 13 μL of IVF media. Then, 1 μL of the raw semen sample was slowly injected through the inlet port. The MSS was incubated for 30 min at 37 °C in a CO_2 atmosphere. After incubation, selected sperm were recovered from the outlet port.

Spermatozoa were microselected in a polyvinylpyrrolidone and recombinant human albumin microdrop (Vitrolife, Sweden) following the recommendations of the World Health Organization for normal morphology and progressive motility.[25] Microinjection of oocytes was performed by the same embryologist using an inverted microscope (Nikon Eclipse TE200S 40×; Japan) on a heated surface at 37 °C (Tokai Hit Termo Plate; Olympus; Japan). Microinjected oocytes were cultured in G-TL™v5 PLUS media microdrops (Vitrolife, Sweden) and incubated in a low concentration of oxygen conditions (K-System; Origio, Denmark or Embrycope; Vitrolife, Sweden).

Fertilization, embryo development and transfers

Embryos were evaluated after 18–20 h post-insemination. Fertilization was recorded when two pronuclei and two polar bodies were observed. Embryos were evaluated and morphologically classified according to the Spanish guidelines as A/B/C/D-grade.[26] Embryo transfers were performed using ultrasound-guidance. Only clinical results from fresh transfers are shown.

Clinical outcomes

The occurrence of a biochemical pregnancy was evaluated 14 days after the embryo transfer through the detection of β-human chorionic gonadotropin (βHCG) in peripheral blood. Clinical pregnancy was evaluated by abdominal ultrasound to detect a gestational sac and foetal heart rate. Miscarriage was diagnosed when no foetal heart rate was detected after determining a clinical pregnancy.

Sperm DNA fragmentation assessment

The Neutral Comet assay was performed under specifically designed conditions to allow the detection of DSB.[11] Briefly, sperm samples were washed twice in PBS 1×, mixed 1:2 (v/v) with 1% low-melting-point agarose (Sigma–Aldrich, USA), fixed on a slide, jellified at 4 °C and immersed in two consecutive lysis solutions. Electrophoresis was performed at 20 V for 12.5 min in TBE buffer (pH 8.5). Slides were washed in a 0.4 M Trizma Base neutralization solution (pH 7.5), dehydrated in ethanol series and horizontally dried. Samples were stained with DAPI (Diamond Antifade Mountant; Invitrogen, OR, USA) and 200 cells were classified as normal or fragmented attending to the criteria reported before[27] (Fig. 1).

Statistics

Normality was studied through the Shapiro–Wilk test. The Kruskal–Wallis test was performed to find differences between groups. The Fisher's Exact test was performed for general comparisons between groups. All tests were considered significant with a confidence interval of 95%.

Results

Men's and women's ages

Two consecutive ICSI cycles from 28 infertile couples were analyzed. The mean ages of men and women at the first

Table 1 Number of ICSI cycles performed, semen parameters, men and women's age and embryology results using conventional methods (DG + SU) or the MSS device (ZyMōtICSI) for semen preparation. All male patients presented altered DSB values (>60%).

ICSI cycle Sperm selection method	First DG+SU		Second ZyMōtICSI	
	Number	Mean ± SD	Number	Mean ± SD
Number of ICSI cycles	28		28	
Semen parameters				
Concentration ($\cdot 10^6$/mL)		35.28 ± 20.23		34.02 ± 21.77
Normal morphology (%)		2.78 ± 1.02		2.73 ± 1.45
Progressive motility (%)		38.21 ± 8.82		38.08 ± 7.64
Men's age		37.53 ± 3.28		38.33 ± 3.33
Women's age		35.67 ± 3.43		36.47 ± 3.57
Obtained oocytes	324	11.57 ± 4.30	311	11.11 ± 7.67
Inseminated oocytes	258	9.21 ± 4.31	220	7.86 ± 4.83
Fertilized oocytes (2PN)	136	4.86 ± 3.27	127	4.54 ± 4.34
Fertilization rate		0.51 ± 0.29		0.50 ± 0.27
Blastocysts	56	2.00 ± 1.56	62	2.21 ± 2.28
A grade	7	0.25 ± 0.44	20	0.71 ± 1.44
B grade	18	0.64 ± 0.87	13	0.46 ± 0.79
C grade	30	1.07 ± 1.05	23	0.82 ± 1.39
D grade	1	0.04 ± 0.19	6	0.21 ± 0.57

DG+SU: sperm selection combining Density Gradients and Swim-up procedures.
PN: pronuclei.

ICSI cycle were 37.53 ± 3.28 years and 35.67 ± 3.43 years, respectively. After a few months, couples started a second ICSI cycle, when men presented a mean of 38.33 ± 3.33 years and women a mean of 36.47 ± 3.57 years, respectively (Table 1). These ages were not statistically different ($p = 0.143$ and $p = 0.143$, respectively).

Seminal parameters and DSB values

All semen samples were assessed for concentration, morphology and progressive motility before each ICSI cycle (Table 1). No statistically significant differences were found between ICSI cycles ($p = 0.270$; $p = 0.900$ and $p = 0.900$, respectively). Males showed a mean value of DSB of 71.18% ± 9.88% between the first and the second ICSI rounds (>60% in all cases).

Oocyte retrieval and fertilization

After ovarian stimulation, similar numbers of oocytes were obtained during both ICSI rounds ($p = 0.336$). Also, similar numbers of oocytes could be finally microinjected ($p = 0.152$). After 18–20 h post-insemination, a similar number of oocytes was fertilized ($p = 0.365$). Fertilization rates did not show statistical differences ($p = 0.870$) (Table 1).

Embryo quality

Blastocyst morphology was analyzed and embryos were classified as A/B/C/D-grade. No significant differences were detected between ICSI rounds for each grade ($p = 0.097$; $p = 0.445$; $p = 0.429$ and $p = 0.134$, respectively). However, an increase of 46.0% in the A-grade blastocysts was observed when the MSS device was used (Table 1).

Clinical outcomes

A similar number of couples obtained good-quality embryos and performed a transference after using conventional methods or the MSS device for semen preparation ($p = 0.786$) (Table 2). Clinical outcomes, including biochemical pregnancy rates, clinical pregnancy rates, the number of live births and the number of miscarriages, are shown in Table 2. Results show a statistically significant increase of 28.31% in the biochemical pregnancy rate ($p = 0.041$) and an increase of 35.56% in the clinical pregnancy rate ($p = 0.070$) when the MSS device was used for semen preparation, as compared to the use of conventional methods.

Regarding live births, results show a statistically significant increase of 35.29% when the ZyMōt™ICSI device was used. A total of six live births were obtained in the second ICSI round, while no live births resulted in the same couples using conventional methods ($p = 0.039$).

When couples used conventional methods for semen preparation, two pregnancies were achieved but ended up in miscarriages during the first trimester. On the contrary, eight pregnancies were achieved using an MSS device. From that, only two ended up in miscarriages. Results show a statistically significant reduction of 75% in the miscarriage rate ($p < 0.010$).

Table 2 ICSI clinical outcomes including the biochemical pregnancy rate, the clinical pregnancy rate, the number of live births and miscarriages when using conventional methods (DG + SU) or the MSS device (ZyMōtICSI) for semen preparation. All male patients presented altered DSB values (>60%).

ICSI cycle	First		Second	
Sperm selection method	DG+SU		ZyMōtICSI	
	Number	%	Number	%
ICSI cycles with embryo transfer	16	57.14	17	60.71
Biochemical pregnancies (per transfer)	3	18.75	8*	47.06*
Clinical pregnancies (per transfer)	2	12.50	8	47.06
Live births (per transfer)	0	0.00	6*	35.29*
Miscarriages (per clinical pregnancy)	2	100.00	2*	25.00*

DG+SU: sperm selection combining Density Gradients and Swim-up procedures.
* Statistical differences compared to the first ICSI cycle ($p < 0.05$).

Discussion

The presence of high values of DSB in sperm DNA have been associated with delays in embryo kinetics and recurrent pregnancy loss.[7,11] There is no evidence that conventional semen preparation techniques reduce DSB,[21] but the ZyMōt™ICSI microfluidic device is a promising tool to reduce this damage.[20,24] In this sense, the main objective of this retrospective cohort study was to provide the first evidence on the clinical utility of this microfluidic device for semen preparation, as compared to the use of conventional methods, when patients present high values of DSB in the semen sample.

The ICSI procedure was originally conceived to treat the severe male factor.[2] Due to its reasonably easy standardization and the possibility to control laboratory timings, the use of this technique increased to 71.3% of fresh ART cycles worldwide.[28] However, ICSI outcomes are not as optimal as one could imagine.[1] Some authors suggest that these rates are limited due to the bypass of the natural sperm selection process that happens through the female genital tract and during natural fertilization.[29] Although it is not fully understood, this natural selection process may select spermatozoa with higher genetic integrity to reach the oocyte.[30]

In order to select the best spermatozoon to fertilize the oocyte, conventional semen preparation techniques aim to discard dead or immotile cells.[17] After that, embryologists select spermatozoa with optimal morphology and motility for microinjection in ICSI cycles.[30] It has been described that ROS may cause both poor motility and SSB.[9,10] Therefore, those semen preparation techniques based on sperm motility could reduce the risk of selecting spermatozoa with high values of SSB. On the contrary, sperm motility and DSB have different origins and do not appear to be associated. This may explain, at least in part, why the use of conventional selection techniques, such as the SU, combined with ICSI sperm selection, does not appear to select spermatozoa with low values of DSB.[21] Moreover, multiple studies have shown that apparently normal selected sperm may present morphologic alterations, such as vacuoles,[31] and high rates of sperm DNA damage when observed under high magnification.[32] Consequently, altered sperm with an apparently normal morphology could be finally injected into the oocyte. In this regard, Ramos et al. described in 2004 that only 55% of sperm injected in ICSI cycles contain intact DNA[33]. In these cases, genetic, epigenetic or chromatin alterations could be transmitted to the embryo, resulting in implantation failures, miscarriages and, more importantly, could lead to a liveborn with genetic alterations. In this regard, the ICSI procedure has been related to higher rates of chromosomal and epigenetic abnormalities, intellectual disabilities or autism, when compared to natural conception.[34]

In the present study, high DSB values were detected in 28 males from infertile couples undergoing ICSI treatments. A first cycle using conventional methods (a combination of DG and SU) obtained poor results, even though women were not of evident advanced age (mean age of 35.67 years) (Table 1). From the 28 couples, only 16 obtained good-quality embryos to be transferred. On the contrary, 12 couples did not obtain any transferable embryo. In these cases, the presence of a high rate of sperm DNA damage could be compromising embryo's development.[7] Other factors, such as maternal age, oocyte quality after ovarian stimulation or the activation of oocytes could also be related to the lack of transferable embryos in these cases.[35] After embryo transfer, patients presenting altered DSB values obtained a significantly lower pregnancy rate (12.5%), as compared to the average pregnancy rates reported in IVF clinics from Europe (35.0%) and the USA (35.8%).[3,4] This result highlights the critical role of sperm DNA integrity, especially DSB, to obtain healthy embryos that conclude in successful pregnancies.[7]

In order to specifically reduce DSB values, the use of the ZyMōt™ICSI microfluidic device offer different advantages: it is a cost-effective and standardized methodology able to filter small volumes of semen; it avoids iatrogenic DNA and membrane damage produced by the rise of ROS during sperm centrifugation[18,19]; it reduces sample manipulation[17]; the microfluidic technology inside the channels somehow imitates the natural selection process that takes place throughout the female genital tract[17] and, also, it has been also shown to reduce by almost 50% of DSB values in a semen sample (Fig. 1).[20,24]

To evaluate the potential beneficial effect of the ZyMōt™ICSI microfluidic device, the same group of patients underwent a second ICSI cycle using this technology for semen preparation.

The mean age of patients and semen parameters of males were not statistically different between the first and the second ICSI treatment (Table 1). Also, the number of oocytes obtained after ovarian stimulation, the number of oocytes microinjected and the number of embryos obtained did not show statistical differences (Table 1). Therefore, ICSI rounds were considered to be compared. It was previously shown that high SSB values in semen samples could impair fertilization.[11] A recent study showed that conventional methods for semen preparation such as the SU procedure, and the sperm selection performed by embryologists during ICSI cycles attending to sperm motility, entail a major reduction of SSB values.[21] Consistently, the fertilization rates obtained in this study when conventional methods and the MSS device were used for semen preparation did not show statistical differences (Table 1). As motile sperm were selected, SSB damage might have been avoided during both ICSI treatments.[21]

The presence of high values of DSB in a semen sample has been related to a delay in embryo kinetics[7] and a major risk of pregnancy loss.[11] As mentioned before, while conventional methods and the ICSI sperm selection process were not shown to reduce DSB values,[21] the ZyMōt™ICSI is a promising tool to reduce this damage.[24] Consistently, results show that the use of the MSS device in the second ICSI cycle from these couples resulted in a major proportion of A-grade embryos with normal morphokinetics (Table 1). During the second ICSI cycle, 17 couples obtained transferable embryos, while 11 did not (Table 2). As in the first ICSI cycle, other maternal or paternal factors could be affecting these couples.[35] Further studies analysing a larger cohort of patients could help in confirming this increase in A-grade embryos.

Among couples who performed a transfer, results show better clinical outcomes during the second ICSI treatment, as compared to the first one (Table 2). Also, about 28% more couples achieved a biochemical pregnancy in the second ICSI cycle, when compared to the previous cycle. About 36% more couples achieved a clinical pregnancy detecting the gestational sac and foetal heart rate. Moreover, about 35% more live births were recorded after the use of the MSS device. These results suggest that using the ZyMōt™ICSI microfluidic device to reduce high DSB values in the semen sample avoids the detrimental effect of this damage in embryos, which leads to better clinical outcomes (Table 2).

Concerning miscarriages, from two pregnancies obtained in the first ICSI cycle, both ended up in a miscarriage. Only two out of eight pregnancies were loss in the second ICSI cycle (Table 2). In agreement with previous results, the reduction of DSB resulted in lower miscarriage rates.[11] As described before, the repair of DSB in the embryo is error-prone because there is not a complementary chain to assure the integrity of the sequence.[35,36] In this sense, the repair of two non-consecutive DSB can lead to structural alterations such as inversions or translocations that could not be tolerated by the embryo leading to a pregnancy loss. Moreover, the DSB can affect a critical gene or a regulatory sequence.[36] This observation has been named the ''late paternal effect'' by some authors.[8]

In order to prevent miscarriages, the Preimplantation Genetic Testing for Aneuploidy (PGT-A) evaluates the euploidy of the obtained embryos and allows the discarding of those with chromosomal alterations.[37] However, this procedure in itself does not allow couples to obtain more euploid embryos during an ICSI cycle. Chromosomal alterations, such as translocations, could be related to the presence of DSB in the paternal genome.[36] Therefore, the specific reduction of this damage using MSS could lead to the obtaining of more euploid embryos and, therefore, to a higher pregnancy rate and higher cumulative live birth rate. Although it is still under discussion, this could be related to the increase in the proportion of A-grade embryos which showed a good morphokinetic development in this study.[38] These embryos could not carry chromosomal alterations derived from paternal DSB which would allow a proper cell-division rate.[7]

Interestingly, the pregnancy rate obtained in this study using the MSS device (47.06%) was higher than the average rates observed in Europe (35.0%) and the USA (35.8%).[3,4] Considering that general rates include heterogeneous infertility cases, the comparison between both ICSI rounds indicate that the specific detection of DSB using the Neutral Comet assay and its specific reduction using the ZyMōt™ICSI MSS device significantly improve ICSI pregnancy rates, as compared to use of conventional methods (12.5%). This increase reaches values above worldwide mean rates (Table 2).

As far as we are concerned, this is the first study analysing the potential benefit of using MSS technologies for semen preparation in ICSI cycles, specifically addressed to reduce DSB values in the semen sample. In order to avoid interferences from unknown female or male factors affecting fertility, the same couples were studied using conventional methods and the ZyMōt™ICSI device in two consecutive ICSI cycles performed by the same laboratory and embryologists. Future studies including a larger number of ICSI cycles should help to confirm these results. One limitation of this study is the impossibility to assess the clinical result of all obtained embryos from A- to D-grades. Results are based on transferable A/B-grade embryos and also, when a pregnancy and a live birth were obtained, the other possible good-quality embryos from the couple were not transferred. If this MSS device could be adapted, animal models could be used to perform an extensive comparison between sperm selection methods to understand the global effect of sperm DNA damage, especially DSB, in embryo's development and pregnancy outcomes.

Conclusions

The detection of DSB damage in infertile males has been shown to be effective in the identification of those patients that can benefit from the use of the ZyMōt™ICSI device. This MSS methodology shows a statistically significant improvement in clinical outcomes, when compared to conventional sperm selection techniques (DG and SU) in patients with high DSB values.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the ethical standards of the responsible human

experimentation committee and in accordance with the World Medical Association and the Declaration of Helsinki.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Conflict of interest

PhD Agustín García-Peiró has commercial interest in the ZyMōt™ICSI microfluidic sperm sorting device and the Comet Assay for sperm DNA fragmentation testing. None of the other authors have a conflict of interest to declare.

References

1. Esteves SC, Humaidan P, Roque M, Agarwal A. Female infertility and assisted reproductive technology. Panminer Med. 2019;61:1–2, http://dx.doi.org/10.23736/S0031-0808.18.03553-X.
2. Palermo GD, Cohen J, Rosenwaks Z. Intracytoplasmic sperm injection: a powerful tool to overcome fertilization failure. Fertil Steril. 1996;65:899–908, http://dx.doi.org/10.1016/s0015-0282(16)58257-0.
3. Calhaz-Jorge C, De Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, et al. Assisted reproductive technology in Europe, 2012: results generated from European registers by ESHRE. Hum Reprod. 2016;31:1638–52, http://dx.doi.org/10.1093/humrep/dew151.
4. US Dept of Health and Human Services. Assisted reproductive technology manual – fertility clinic success rates report 2017; 2019. doi:10.1111/j.1548-7466.2009.01061.x.105.
5. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reprod Biol Endocrinol. 2015;13:37, http://dx.doi.org/10.1186/s12958-015-0032-1.
6. Gumus E, Kati B, Pelit ES, Ordek E, Ciftci H. A different look at genetic factors in individuals with non-obstructive azoospermia or oligospermia in our research study: to whom, which threshold, when, in what way? Rev Int Androl. 2021;19:41–8, http://dx.doi.org/10.1016/j.androl.2019.08.001.
7. Casanovas A, Ribas-Maynou J, Lara-Cerrillo S, Jimenez-Macedo AR, Hortal O, Benet J, et al. Double-stranded sperm DNA damage is a cause of delay in embryo development and can impair implantation rates. Fertil Steril. 2019;111:699–707, http://dx.doi.org/10.1016/j.fertnstert.2018.11.035, e1.
8. Agarwal A, Barbăroșie C, Ambar R, Finelli R. The impact of single- and double-strand DNA breaks in human spermatozoa on assisted reproduction. Int J Mol Sci. 2020;21:1–14, http://dx.doi.org/10.3390/ijms21113882.
9. Wright C, Milne S, Leeson H. Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. Reprod Biomed Online. 2014;28:684–703, http://dx.doi.org/10.1016/j.rbmo.2014.02.004.
10. Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. World J Mens Health. 2014;32:1, http://dx.doi.org/10.5534/wjmh.2014.32.1.1.
11. Ribas-Maynou J, García-Peiró A, Fernandez-Encinas A, Amengual MJ, Prada E, Corte P, et al. Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. PLoS ONE. 2012;7, http://dx.doi.org/10.1371/journal.pone.0044679.
12. Simon L, Proutski I, Stevenson M, Jennings D, McManus J, Lutton D, et al. Sperm DNA damage has a negative association with live-birth rates after IVF. Reprod Biomed Online. 2013;26:68–78, http://dx.doi.org/10.1016/j.rbmo.2012.09.019.
13. Lange J, Pan J, Cole F, Thelen MP, Jasin M, Keeney S. ATM controls meiotic double-strand break formation. Nature. 2012;479:237–40, http://dx.doi.org/10.1038/nature10508.ATM.
14. Oliva R. Protamines and male infertility. Hum Reprod Update. 2006;12:417–35, http://dx.doi.org/10.1093/humupd/dml009.
15. Ribas-Maynou J, García-Peiró A, Fernández-Encinas A, Abad C, Amengual MJ, Prada E, et al. Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. Andrology. 2013;1:715–22, http://dx.doi.org/10.1111/j.2047-2927.2013.00111.x.
16. Singh NP. The comet assay: reflections on its development, evolution and applications. Mutat Res Rev Mutat Res. 2016;767:23–30, http://dx.doi.org/10.1016/j.mrrev.2015.05.004.
17. Marzano G, Chiriacò MS, Primiceri E, dell'Aquila ME, Ramalho-Santos J, Zara V, et al. Sperm selection in assisted reproduction: a review of established methods and cutting-edge possibilities. Biotechnol Adv. 2020;40:107498, http://dx.doi.org/10.1016/j.biotechadv.2019.107498.
18. Balaban B, Sakkas D, Gardner DK. Laboratory procedures for human in vitro fertilization. Semin Reprod Med. 2014;32:272–82, http://dx.doi.org/10.1055/s-0034-1375179.
19. Gosálvez J, López-Fernández C, Fernández JL, Gouraud A, Holt WV. Relationships between the dynamics of iatrogenic DNA damage and genomic design in mammalian spermatozoa from eleven species. Mol Reprod Dev. 2011;78:951–61, http://dx.doi.org/10.1002/mrd.21394.
20. Quinn MM, Jalalian L, Ribeiro S, Ona K, Demirci U, Cedars MI, et al. Microfluidic sorting selects sperm for clinical use with reduced DNA damage compared to density gradient centrifugation with swim-up in split semen samples. Hum Reprod. 2018;33:1388–93, http://dx.doi.org/10.1093/humrep/dey239.
21. Lara-Cerrillo S, Ribas-Maynou J, Rosado-Iglesias C, Lacruz-Ruiz T, Benet J, García-Peiró A. Sperm selection during ICSI treatments reduces single-but not double-strand DNA break values compared to the semen sample. J Assist Reprod Genet. 2021;38:1187–96, http://dx.doi.org/10.1007/s10815-021-02129-w.
22. Samuel R, Feng H, Jafek A, Despain D, Jenkins T, Gale B. Microfluidic-based sperm sorting & analysis for treatment of male infertility. Transl Androl Urol. 2018;7:S336–47, http://dx.doi.org/10.21037/tau.2018.05.08.
23. Göde F, Gürbüz AS, Tamer B, Pala I, Isik AZ. The effects of microfluidic sperm sorting, density gradient and swim-up methods on semen oxidation reduction potential. Urol J. 2020;17:397–401, http://dx.doi.org/10.22037/uj.v0i0.5639.

24. Pujol Masana A, García-Peiró A, Ribas-Maynou J, Lafuente R, Mataró D, Vassena R. A microfluidic sperm sorting device reduces the proportion of sperm with double strand DNA fragmentation. Zygote. 2021:1–6, http://dx.doi.org/10.1016/j.fertnstert.2019.07.356.
25. WHO. WHO Laboratory Manual for the examination and processing of human semen. *World Heal Organ Press*; 5th ed. 2010.
26. ASEBIR. Cuadernos de embriología clínica: criterios ASEBIR de valoración morfológica de oocitos, embriones tempranos y blastocistos humanos. *Cuad Embriol clínica ASEBIR*. 2015:9–20.
27. Ribas-Maynou J, García-Peiró A, Abad C, Amengual MJ, Navarro J, Benet J. Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. Hum Reprod. 2012;27:652–8, http://dx.doi.org/10.1093/humrep/der461.
28. De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko T, et al. ART in Europe, 2014: Results generated from European registries by ESHRE. Hum Reprod. 2018;33:1586–601, http://dx.doi.org/10.1093/humrep/dey242.
29. Xiong X, Dickey RP, Buekens P, Shaffer JG, Pridjian G. Use of intracytoplasmic sperm injection and birth outcomes in women conceiving through in vitro fertilization. Paediatr Perinat Epidemiol. 2017;31:108–15, http://dx.doi.org/10.1111/ppe.12339.
30. Sakkas D, Ramalingam M, Garrido N, Barratt CLR. Sperm selection in natural conception: what can we learn from mother nature to improve assisted reproduction outcomes? Hum Reprod Update. 2015;21:711–26, http://dx.doi.org/10.1093/humupd/dmv042.
31. Perdrix A, Rives N. Motile sperm organelle morphology examination (MSOME) and sperm head vacuoles: state of the art in 2013. Hum Reprod Update. 2013;19:527–41, http://dx.doi.org/10.1093/humupd/dmt021.
32. Pastuszek E, Kiewisz J, Skowronska P, Liss J, Lukaszuk M, Bruszczynska A, et al. An investigation of the potential effect of sperm nuclear vacuoles in human spermatozoa on DNA fragmentation using a neutral and alkaline Comet assay. Andrology. 2017;5:392–8, http://dx.doi.org/10.1111/andr.12324.
33. Ramos L, De Boer P, Meuleman EJH, Braat DDM, Wetzels AMM. Evaluation of ICSI-selected epididymal sperm samples of obstructive azoospermic males by the CKIA system. J Androl. 2004;25:406–11, http://dx.doi.org/10.1002/j.1939-4640.2004.tb02807.x.
34. Davies MJ, Moore VM, Willson KJ, Essen PV, Priest K, Scott H, et al. Reproductive technologies and the risk of birth defects. N Engl J Med. 2012;366:1803–13, http://dx.doi.org/10.1056/NEJMoa1008095.
35. Hartshorne GM. Factors controlling embryo viability. Hum Fertil. 2001;4:225–34, http://dx.doi.org/10.1080/1464727012000199571.
36. Middelkamp S, van Tol HTA, Spierings DCJ, Boymans S, Guryev V, Roelen BAJ, et al. Sperm DNA damage causes genomic instability in early embryonic development. bioRxiv. 2019:1–12, http://dx.doi.org/10.1101/681296.
37. Griffin DK, Ogur C. Chromosomal analysis in IVF: just how useful is it? Reproduction. 2018;156:F29–50, http://dx.doi.org/10.1530/REP-17-0683.
38. Pennetta F, Lagalla C, Borini A. Embryo morphokinetic characteristics and euploidy. Curr Opin Obstet Gynecol. 2018;30:185–96, http://dx.doi.org/10.1097/GCO.0000000000000453.