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Can a sperm selection technique improve embryo ploidy?

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Abstract

Background: Spermatozoa with the highest motility retain a superior genomic integrity; elevated sperm chromatin fragmentation (SCF) has been linked to a lower ability of the conceptus to develop and implant. Therefore, the utilization of a sperm selection method, such as microfluidic sperm selection (MFSS), is capable of reducing the SCF by yielding the most motile fraction of spermatozoa with highest embryo developmental competence. What remains unclear, however, is the causal mechanism that links SCF to an impaired embryo development.

Objectives: To identify a relationship between SCF and an unexpectedly high proportion of embryo aneuploidy, while addressing treatment options.

Materials and methods: We identified couples with a high incidence of embryo aneuploidy in a previous intracytoplasmic sperm injection (ICSI) cycle with pre-implantation genetic testing for aneuploidy (PGT-A) utilizing spermatozoa selected by density gradient (DG). Terminal deoxynucleotidyl dUTP transferase nick-end labeling (TUNEL) and neutral Comet assays were carried out on the semen specimens to assess total SCF and, specifically, double-stranded DNA (dsDNA) fragmentation. These couples underwent subsequent ICSI/PGT-A cycles with MFSS. Total SCF and dsDNA fragmentation were compared between the two sperm selection methods. Embryo aneuploidy, implantation, clinical pregnancy, delivery, and pregnancy loss rates were compared between the couples' historical DG and subsequent MFSS cycles.

Results: In 57 couples undergoing 71 ICSI/PGT-A cycles, where DG sperm selection was carried out, a high incidence of aneuploid embryos (74.7%) resulted in poor implantation and no viable pregnancies. Testing for SCF, inclusive of dsDNA breaks, evidenced a SCF of 26.2% and dsDNA break of 3.6% in the raw specimen, that decreased to 18.0% ($p < 0.001$) and 3.1%, respectively, in the DG processed specimen. Following MFSS, total SCF and dsDNA fragmentation decreased to 1.9% and 0.3%, respectively ($p < 0.001$). The embryo euploidy rate improved from 25.3% in the DG cycles to 42.9% in the MFSS cycles ($p < 0.001$). The 6.7% implantation rate in the DG cycles increased to 65.5% in the MFSS cycles ($p < 0.001$). Similarly, the clinical pregnancy rate rose from 10.5% (DG) to 64.6% (MFSS), resulting in a 62.5% delivery rate ($p < 0.001$).

Discussion and conclusions: In couples with a relatively young female partner with a negative infertility workup, and a male partner with semen parameters adequate for ICSI presenting with a high rate of embryo aneuploidy, an additional subtle male factor component may be the culprit. Thus, it is crucial to assess the SCF and test for the dsDNA breaks, which can eventually contribute to embryo chromosomal abnormalities. Given the inverse relationship between SCF and motility, a selection of the most motile gamete by MFSS enhanced the proportion of spermatozoa with an intact genome contributing to the generation of more euploid embryos that are capable of implanting and yielding increased term pregnancies.

KEYWORDS
sperm chromatin fragmentation, dsDNA breaks, microfluidics, ICSI, PGT-A, embryo aneuploidy

1 | INTRODUCTION

It is not unusual to encounter couples who fail to achieve a pregnancy, despite a negative female infertility workup and a male partner with normal semen parameters. This phenomenon can be as high as 30%, and these couples are often treated in the most cost effective and conservative manner, with a superovulation protocol combined with programed intercourse (PI) or intrauterine insemination (IUI).[1] If this approach fails, the reproductive physician can only attempt other treatment options without any specific guidance.

With promising tests, such as the Cap-Score,[2] and the more commonly used sperm chromatin fragmentation (SCF) assays, it is possible to recognize male factor infertility. Indeed, a treatment algorithm based on the latter has been proposed in couples with unexplained infertility and poor pregnancy outcome with PI or IUI, as they were screened for SCF in order to guide them through the proper assisted reproductive technology (ART) treatment to fulfill their childwish.[3] Nonetheless, in the presence of high SCF, couples treated by intracytoplasmic sperm injection (ICSI) may still fail to achieve a pregnancy, suggesting the use of testicular spermatozoa as the last resort. The utilization of surgically retrieved spermatozoa is based on the observation that gametes retrieved from proximal sections of the male genital tract present a healthier genome.[4] Although this approach is somewhat successful, it is invasive, costly, and limited by the lower fertilization rate inherent to the utilization of testicular spermatozoa.[4, 30]

There are several factors that influence SCF, one of those being the paternal age,[5] and the other being the length of the ejaculatory abstinence.[6] However, the foremost indicator of elevated SCF is represented by a compromised sperm motility. It has been shown that the sperm portion with the highest motility also retains a superior genomic integrity.[7] From this, a more palatable alternative is the utilization of a sperm processing method, such as microfluidic sperm selection (MFSS), capable of reducing the SCF by yielding the most motile fraction of spermatozoa.[8]

Elevated SCF has been linked to a lower ability of the conceptus to develop with a consequent impairment of a successful pregnancy, contributing to a higher pregnancy loss.[9] What remains unclear, however, is the causal mechanism that links SCF to an impaired embryo development. In fact, a recent study[10] indicated that the SCF has two components: the single-stranded sperm chromatin fragmentation (ssSCF) and the double-stranded sperm chromatin fragmentation (dsSCF). Although not all the assays can distinguish between the two, the most common tests measure the ensemble (ssSCF and dsSCF). Nonetheless, there are tests, such as the sperm chromatin structure assay[11] or the neutral Comet assay,[12] which can identify the dsSCF indeed linked to structural chromosomal abnormalities, suggesting the direct relationship between elevated SCF and embryo aneuploidy. The presence of gamete euploidy is inherent to human conception and is considered the bottleneck of ART treatment, as oocyte aneuploidy is directly correlated to advancing maternal age.[13] Embryonic aneuploidy is so relevant in the treatment of the infertile patient that several clinics in the United States offer couples to undergo ART with genetic assessment of all conceptuses and transfer the chromosomally normal conceptus in a subsequent programed or natural cycle.[14] It has become increasingly evident that a couple's aneuploidy is not only contributed by the oocyte, but can also occur in couples with a young female partner and a male partner with a normal semen analysis suggesting that the male gamete also plays a role.

In this study, we assess couples, plagued by a high incidence of embryo aneuploidy, for SCF. Those with elevated SCF were treated by subsequent ICSI and pre-implantation genetic testing for aneuploidy (PGT-A) cycles with spermatozoa selected by MFSS, in order to increase their chances of obtaining euploid embryos and the ability to sustain term pregnancy.

2 | MATERIALS AND METHODS

2.1 | Inclusion criteria and study design

Over a 4-year period, we included 57 couples (maternal age 36.5 ± 5 years, paternal age 40.9 ± 6 years) undergoing ART treatment at our

center. These couples underwent initial ICSI/PGT-A cycles, in which spermatozoa were selected routinely by density gradient (DG), and resulted in an embryo aneuploidy proportion of >50% of the cohort of embryos biopsied.

All couples had a normal infertility work-up and a normal BMI with no history of smoking, excess drinking, or use of recreational drugs. To minimize confounding factors, all patients were Caucasian and had comparable hormonal profiles, stimulation responses, as well as duration and indications of infertility.

The components of an initial infertility evaluation for the female partner consisted of a comprehensive review of the medical, reproductive, family, and social history, as well as a physical examination to detect for pathology that could impact fertility or reproductive potential. Additional infertility tests evaluating ovulatory function, ovarian reserve, cervical and peritoneal factors, uterine abnormalities, and tubal patency were also performed. Male partners underwent a semen analysis according to tWorld Health Organization (WHO) standards. Those with severe oligo-/astheno-zoospermia were excluded, defined as $\leq 1 \times 10^6$/ml and ≤20%, respectively.

An SCF assessment was carried out by terminal deoxynucleotidyl dUTP transferase nick-end labeling (TUNEL) and neutral Comet on spermatozoa from the raw and post-DG specimens from all participants. After an elevated SCF was confirmed, the same couples underwent subsequent ICSI and PGT-A cycles with MFSS, of which aliquots were also assessed for SCF. Clinical outcomes of the MFSS cycles, including fertilization, embryo euploidy, implantation, pregnancy, and delivery rates were compared with those from the couples' historical DG cycles.

2.2 | Spermatozoa collection and preparation

Ejaculates were obtained by masturbation with an average abstinence of 2–4 days. Semen samples were allowed to liquify at 37°C for 15 min. Liquified semen samples were evaluated according to the WHO.[15]

2.2.1 | Density gradient centrifugation

For DG processing, liquefied semen samples were diluted with HEPES-buffered human tubal fluid medium (H-HTF; Irvine Scientific, CA, USA) supplemented with human serum albumin (HSA solution G Series culture media; Vitrolife, Goteborg, Sweden). Initial centrifugation at 600*g* for 10 min was carried out to remove seminal plasma. The resulting pellet was then resuspended by HTF medium and gently loaded onto 1 ml of DG (Enhance-S Plus Cell Isolation Media, 90%; Vitrolife), followed by a second centrifugation at 300*g* for 10 min. The DG layer with motile spermatozoa was isolated, resuspended in 3:1 HTF medium, and centrifuged at 600*g* for 10 min to remove silica particles. The supernatant was discarded, and the final pellet was resuspended in 0.5 ml HTF. Concentration, motility, and morphology were reassessed in 5 µl on a Makler chamber.[15] SCF was also measured on the DG processed specimen. The specimens were diluted to a final concentration of $1–3 \times 10^6$/ml for ICSI.

2.2.2 | Microfluidic sperm selection (MFSS)

MFSS was performed as previously described,[8] using a commercial device (ZyMōt Multi [850 µl] device; DxNow, Gaithersburg, Maryland). Briefly, 850 µl of the liquefied semen sample was loaded into the collection chamber through the inlet of the device. An equivalent amount of HTF medium was loaded to cover the membrane surface. . The loaded device was incubated for 30 min in a humidified, 37°C, gassed incubator. Following incubation, motile spermatozoa were obtained from the harvest chamber. This sorted suspension was reassessed for concentration, motility, morphology, and SCF. Sperm concentration was adjusted to $1–3 \times 10^6$/ml in preparation for ICSI.

2.3 | Sperm DNA fragmentation assessment

SCF was detected using TUNEL, which measures both single-stranded and double-stranded (ss/dsDNA) damage, and the neutral Comet assay, which unlike any other SCF test, can measure solely dsDNA damage.

TUNEL was performed using a commercially available kit (In Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Rotkreuz, Switzerland) as previously described.[8] Slides were smeared with 5 µl of the sperm specimen (raw, DG, MFSS) and dried at room temperature overnight. The specimens were fixed by 4% paraformaldehyde for 1 h, washed in phosphate buffered saline (PBS), and air-dried overnight once again. Slides were then immersed for 2 min at 4°C with 0.1% Triton X-100 and 0.1% sodium citrate in PBS to permeabilize the sperm cells. Diluted enzyme and fluorescent labels were applied onto the permeabilized slides according to manufacturer protocol and left to incubate at 37°C for 1 h. Slides were subsequently rinsed in PBS to remove excessive fluorescent labels. A DNA staining, DAPI/Antifade solution (Millipore Sigma, Billerica, MA, USA), was added to visualize sperm nuclei, which were examined under a fluorescent microscope for positive fluorescein signals, indicating DNA breakage. A minimum of 500 spermatozoa were assessed per sample. A SCF of ≤15% was considered normal based on previous studies.[4,16,17]

Neutral Comet was performed using a modified protocol.[18, 19] In brief, spermatozoa from raw, DG, and MFSS were diluted to 0.1×10^6/ml by PBS and combined with molten low melting point agarose at 1:10 volume ratio. For each slide, 50 µl of combination was smeared onto a glass slide and chilled in the dark at 4°C to promote agarose gelling. The slide was then immersed in lysis solution (0.4 M Tris–HCl, 0.8 M dithiothreitol, and 1% w:v sodium dodecyl sulfate) for 1 h. After lysis treatment, the slides were drained to remove excess lysing solution, immersed in neutral electrophoresis buffer (0.05 M Tris-base, 0.15 M Sodium Acetate, pH 7.5) for 30 min, and aligned equidistant from electrodes in the gel electrophoretic machine (Bio-Rad, Hercules, CA, USA) with following setting: 1 V/cm^2, 45 min, 4°C. After electrophoresis, the slides were gently immersed in DNA precipitation solution (1.0 mol ammonium acetate in 95% ethanol) for 30 min at room temperature, subsequently immersed in 70% ethanol for 30 min at room temperature and dried in a 37°C

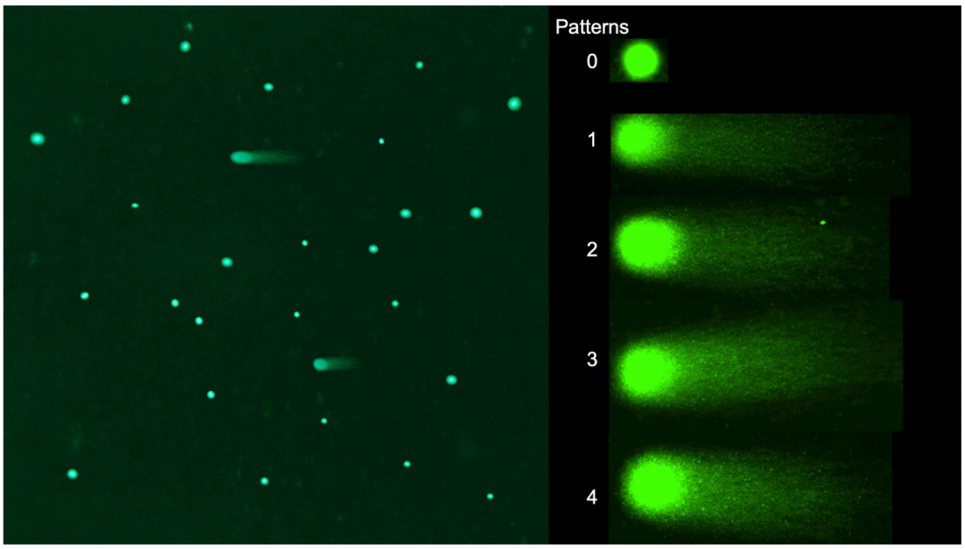

FIGURE 1 Depicted here is the Comet assay. On the left, there is an overall view of spermatozoa with different levels of fragmentation. On the right, spermatozoa 1–4 fall in the positive criteria with >50% tail moment and >120 μm tail length, compared to spermatozoon 0 without DNA fragmentation.

oven for 10–15 min. To visualize the DNA for Comet assay, 100 μl of diluted SYBR green/fluorescein (1 μl SYBR in DMSO, in 30 ml Tris–base buffer) was added onto the slide to stain the DNA for 30 min in the dark. After removal of excess solution by rinsing in nuclease-free water and drying at 37°C, the slides were visualized in a fluorescent microscope. For each assay, a minimum of 200 spermatozoa were assessed. Tail length was measured using NIS-Elements (Nikon, Minato City, Tokyo, Japan). Tail moment was calculated based on the percentage of DNA in the comet tail measured by fluorescent intensity profile and tail length using a formula previously described.[28] We considered spermatozoa to have a high level of dsSCF when the comet tail moment was >50% and comet tail length >120 μm (Figure 1).[20]

2.4 | Ovarian superovulation and oocyte collection

Similar superovulation protocols were utilized for all couples. Patients were treated with gonadotropins daily (Follistim; Merck, Kenilworth, NJ, USA; Gonal-F; EMD Serono, Geneva, Switzerland; and/or Menopur; Ferring Pharmaceuticals Inc, Parsippany, NJ, USA). GnRH-antagonist (Ganirelix acetate; Merck, Kenilworth, NJ, USA; or Cetrotide; EMD Serono In. Rockland, MA, USA) was administered to prevent precocious ovulation. Human chorionic gonadotropin (hCG) (Pregnyl Merck) was administered to trigger final oocyte maturation when the two leading follicles reached a diameter of ≥17 mm. Ultrasound-guided transvaginal oocyte retrieval was performed under anesthesia 35–37 h after hCG trigger. A period of 2 h after retrieval, oocyte-cumulus-complexes were denuded by 40 IU/ml recombinant human hyaluronidase (ICSI Cumulase; Cooper Surgical, Inc, Trumbull, CT). Oocytes were then incubated 1–2 h prior to ICSI.

2.5 | Embryo culture and morphologic and cytogenetic evaluation

Embryo evaluation, criteria, and the biopsy procedure have been described previously.[21] Good-quality blastocysts had the following morphologic grades according to a well-established grading system[22]: blastocoele, 1–3 (blastocoele expansion ≥50% the volume of the embryo); inner cell mass (ICM), A-B (distinct ICM with healthy cells); and trophectoderm (TE), A-B (healthy and cohesive cells).

The TE biopsy procedure was performed at the blastocyst stage (day 5/6 trophectoderm biopsy), due to there being less chance of mosaicism compared to earlier stages and less harm caused by biopsy at this stage.[29] The embryo was held by a holding pipette at 9 o'clock, and laser pulses (Hamilton-Thorne, Beverly, MA, USA) were applied at 3 o'clock of the embryo to breach the zona pellucida to allow a 20-μm inner diameter biopsy pipette to aspirate the TE. Embryos were rotated to place ICM at 9 o'clock to avoid damage by the laser during TE biopsy. For each TE biopsy, three-to-seven trophoblastic cells were isolated, collected in a 200 μl PCR tube with 2 μl of PBS buffer, and analyzed by 24-chromosome aneuploidy screening with next-generation sequencing (NGS). Biopsied embryos were then rinsed in culture media and vitrified.

2.6 | Embryo transfer and pregnancy assessment

To prepare for embryo replacement, a suggested daily supplementation with progesterone, starting the day after ovulation, was administered vaginally. When an endometrial lining was of at least 7 mm, the patient underwent embryo replacement. Only confirmed euploid

TABLE 1 Patient Demographics

	Selection	
	DG	MFSS
Couples	57	
Cycles	71	78
Maternal Age (M ± SD)	36.5 ± 5	37.2 ± 5
Paternal Age (M ± SD)	40.9 ± 6	41.1 ± 7

embryos were transferred. Mosaic embryos were not selected for transfer. For ovulatory women, a hormone-free option is often preferred and when unfeasible, programed.[23] To assess pregnancy status, serum hCG (βhCG) levels were measured 10–14 days after frozen embryo transfer (FET), followed by an ultrasound confirming fetal heart activity to diagnose a clinical pregnancy.

2.7 | Statistical analysis

A paired *T*-test was used to compare semen parameters between the patients' historical cycle, using spermatozoa processed by DG, and a subsequent cycle, using spermatozoa processed by MFSS. χ^2 analysis was performed to assess clinical outcomes. *p*-Value ≤0.05 were considered statistically significant.

3 | RESULTS

We recruited 57 couples with a female partner age ranging between 29 and 39 years and a male partner age ranging from 31 to 56 years at the time of their first attempt (Table 1). Semen parameters are presented in Table 2, with some men presenting with astheno-/terato-zoospermia.

These couples underwent a total of 71 ICSI cycles (1.2 cycles/patient). They obtained a satisfactory fertilization that ranged from 20% to 100%, resulting, however, in an aneuploidy of the assessed blastocysts ranging from 50% to 100%. Of the 30 euploid embryos replaced (1.9 embryos transferred/patient), only 2 implanted but resulted in pregnancy loss. Because of the unexpected poor clinical outcome of these couples, particularly considering the relatively young age of the female partners, the semen samples of the male partners were screened by terminal deoxynucleotidyl dUTP transferase nick-end labeling (TUNEL), and resulted in a SCF ranging between 20.4% and 46.0%. To quantify the proportion of double-stranded DNA (dsDNA) breaks, the male partner specimens were also tested by the neutral Comet assay with results ranging from 0.99% to 15.8%. To test whether the sperm selection methods would be able to reduce SCF, a comparison was carried out on aliquots of the specimen processed by DG and MFSS. The SCF was 26.2% ± 8% in the raw specimen, 18.0% ± 6% in the specimen processed by DG, and 1.9% ± 1% in the specimen processed by MFSS ($p < 0.001$). To quantify the component of dsDNA that may be responsible for structural chromosomal abnormalities, a sample aliquot was also tested by neutral Comet which evidenced 3.6% ± 2% fragmentation in the raw specimen, 3.1% ± 1% after DG, and only 0.3% ± 0.2% after MFSS ($p < 0.001$).

All couples underwent 78 subsequent ICSI cycles (1.4 cycles/patient) with PGT-A where the semen sample was processed by MFSS. We compared the semen parameters between the raw and the processed specimens (raw vs. DG/MFSS), and between the two processing methods themselves (DG vs. MFSS) (Table 2). Within each treatment group, whether DG or MFSS, we observed a significantly lower volume with a reduction in concentration and improvement in motility compared to the raw specimen ($p < 0.001$). The group processed by MFSS also evidenced an improvement in normal morphology compared to the raw specimen ($p < 0.05$).

When we compared the two processing methods (DG vs. MFSS), we saw that the raw semen parameters were similar as expected, but there was a remarkable improvement in motility following MFSS (96.9%), in comparison to DG (71.3%) ($p < 0.001$), as well as an enhancement in normal morphology (DG, 2.0; MFSS, 3.3) ($p < 0.01$). There was an obvious reduction in concentration that went from 36.7×10^6/ml in the raw, to 12.8×10^6/ml after DG, and 8.0×10^6/ml after MFSS ($p < 0.001$).

In the subsequent MFSS cycles, fertilization rate was remarkably higher at 76.2% versus 68.8% in the DG group ($p < 0.001$). Therefore, the number of embryos that generated blastocysts that were available for testing was much higher in the MFSS cycles ($p < 0.001$). As depicted in Figure 2, the euploid embryo proportion was 25.3% in DG, and rose to 42.9% following MFSS ($p < 0.001$).

In the historical cycles, where the specimen was processed by DG, there were only 19 FET cycles. Of the 30 embryos transferred, only 2 implanted (6.7%), however, all ended in pregnancy loss. In the subsequent MFSS cycles, because of the higher euploidy rate, the couples underwent a total of 48 FET cycles where 58 embryos were replaced (1.0 embryos transferred/patient), leading to an implantation rate of 65.5% ($p < 0.001$) and a delivery rate of 62.5% ($p < 0.001$) (Table 4).

4 | DISCUSSION

In this study, we assessed the feasibility of identifying male factor in couples where the female partner is of a relatively young age with a negative infertility workup and a male partner with adequate semen parameters. We included couples with the above-mentioned characteristics that underwent an ICSI cycle with PGT-A and that unexpectedly generated a low number of euploid embryos, that once replaced did not lead to a viable pregnancy. A stronger association—in couples trying to conceive naturally or with IUI, and less clearly in couples attempting conventional in vitro insemination (cIVI) or ICSI—between high SCF and poor reproductive outcomes has been identified. This awareness has brought us to screen these couples for male factor by assessing sperm genomic integrity by terminal deoxynucleotidyl dUTP transferase nick-end labeling (TUNEL).

It has been previously demonstrated that up to 39% of couples with idiopathic infertility have sperm DNA damage, and for those affected, a low live birth rate following IVF was observed.[24] In a later study, couples with unexplained infertility who had less than a 2% clinical

TABLE 2 Semen parameters of raw specimen and after DG and MFSS processing

	Cycles						
	History			Study			DG vs. MFSS
	Raw	DG	*P* value	Raw	MFSS	*P* value	*P* value
Volume (mL) (M ± SD)	2.0 ± 1.3	0.5 ± 0	<0.001	2.0 ± 1.2	0.6 ± 0	<0.001	–
Concentration (x106/mL) (M ± SD)	36.7 ± 32	12.8 ± 11	<0.001	35.4 ± 31	8.0 ± 13	<0.001	–
Total Motility (%) (M ± SD)	34.5 ± 15	71.3 ± 34*	<0.001	37.2 ± 11	96.9 ± 9*	<0.001	<0.001
Normal Morphology (%) (M ± SD)	2.0 ± 1	2.0 ± 1†	NS	2.2 ± 1	3.3 ± 1†	<0.05	<0.01
Total SCF	26.2 ± 8	18.0 ± 6‡	<0.001	26.2 ± 8	1.9 ± 1‡	<0.001	<0.001
dsDNA Fragmentation	3.6 ± 2	3.1 ± 1∫	NS	3.6 ± 2	0.3 ± 0.2∫	<0.001	<0.001

*Paired t-test, Effect of sperm selection method on motility.
†Paired t-test, Effect of sperm selection method on normal morphology.
‡Paired t-test, Effect of sperm selection method on total Sperm Chromatin Fragmentation (SCF).
∫Paired t-test, Effect of sperm selection method on double-stranded DNA (dsDNA) fragmentation.
DG: density gradient.
MFSS: microfluidic sperm selection.

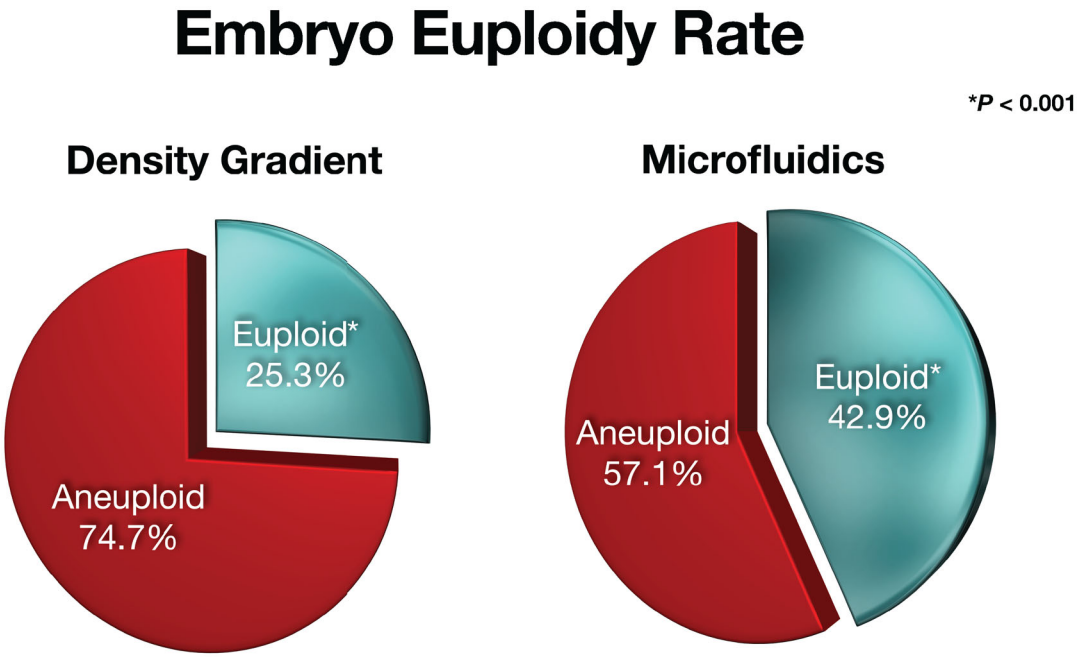

FIGURE 2 There was a significant difference in the proportion of euploid embryos between the history group, utilizing spermatozoa processed by DG, and the study group, utilizing spermatozoa processed by MFSS, at 25.3% and 42.9%, respectively. $p < 0.001$

pregnancy with IUI were recruited into a treatment algorithm that utilized SCF scores as a deciding factor of whether to escalate reproductive treatment. When the male partner was found to have a normal SCF, *cIVI* was proposed and resulted in a 12.7% clinical pregnancy rate (CPR). If an abnormal SCF was found, the couple was offered ICSI that yielded a 18.7% CPR. Couples who still failed to achieve a pregnancy with ICSI were offered a subsequent ICSI cycle utilizing surgically retrieved spermatozoa, resulting in an overall CPR of 31%.[3,4] Nonetheless, the retrieval of spermatozoa from the epididymis or the testis is an invasive and costly procedure involving anesthesia and surgical risks, and may therefore appear less palatable to some men. Hence, the observation of the strong inverse relationship between motility and SCF comes to aid.[7,25,26] In the present study, in order to verify the effect of a sperm selection method on the genomic integrity of the male gamete, we carry out a comparison where aliquots of ICSI specimen were processed by DG or MFSS and tested by TUNEL, and confirmed that MFSS was able to decrease the total SCF to 1.9% and increase the embryo euploidy rate to 42.9% ($p < 0.001$).

The couples involved in this study, in addition to an elevated SCF, were also affected by a higher incidence of embryo aneuploidy. This is difficult to explain; however, it has been reported that there may be a relationship between the dsDNA breaks and structural chromosomal abnormalities that affect all stages of embryo development and implantation.[10] In addition, a more recent study confirms the link

TABLE 3 Effect of sperm selection method on fertilization and embryo development

	Selection		
	DG	MFSS	*P* value
Couples	57		–
Cycles	71	78	–
Fertilization (%)	594/863 (68.8)*	615/807 (76.2)*	<0.001
Blastocyst Biopsied	281†	389 †	<0.001
Euploidy Rate (%)	71/281 (25.3)‡	167/389 (42.9)‡	<0.001

*χ^2, 2 x 2, 1 df, Effect of sperm selection on intracytoplasmic sperm injection (ICSI) fertilization.
†χ^2, 2 x 2, 1 df, Effect of sperm selection on blastocysts yielded for biopsy.
‡χ^2, 2 x 2, 1 df, Effect of sperm selection on euploidy rate.
DG: density gradient.
MFSS: microfluidic sperm selection.

TABLE 4 Effect of sperm selection method on clinical outcome

	Selection		
	DG	MFSS	*P* value
Couples	57		–
FET-Cycles	19	48	–
Implantation (%)	2/30 (6.7)*	38/58 (65.5)*	<0.001
Clinical Pregnancy (%)	2/19 (10.5)†	31/48 (64.6)†	<0.001
Delivered (%)	0/19 (0)‡	30/48 (62.5)‡	<0.001
Pregnancy Loss (%)	2/2 (100)ʃ	1/31 (3.2)ʃ	<0.01

*χ^2, 2 x 2, 1 df, Effect of sperm selection on implantation.
†χ^2, 2 x 2, 1 df, Effect of sperm selection on clinical pregnancy rate.
‡χ^2, 2 x 2, 1 df, Effect of sperm selection on delivery rate.
ʃχ^2, 2 x 2, 1 df, Effect of sperm selection on pregnancy loss.
DG: density gradient.
MFSS: microfluidic sperm selection.
FET: frozen embryo transfer.

between γ-radiation-induced sperm DNA damage and chromosomal fragmentation/segregation in the resulting embryos.[28]

Although TUNEL is capable of measuring total DNA fragmentation, inclusive of single-stranded DNA (ssDNA) and dsDNA breaks, to test the hypothesis of the role of dsDNA break, we used the neutral Comet assay. The dsDNA break was 3.6% in the raw semen specimen, and remained unaffected by DG, however, was remarkably reduced following MFSS at 0.3% ($p < 0.001$). Couples in a subsequent ICSI cycle, utilizing spermatozoa processed by MFSS, resulted in a remarkable improvement in motility and morphology in relation to the raw specimen, or even the sample processed by DG (Table 2). MFSS also granted a higher fertilization and a significantly higher number of blastocysts, that once biopsied yielded a higher proportion of euploid embryos (Table 3). This led to 48 FET cycles with an embryo implantation rate of 65.5% and an ongoing delivery rate of 62.5%, with a very limited pregnancy loss (Table 4). This approach confirmed that selecting for spermatozoa with a higher chromatin integrity is capable of increasing the pregnancy rate and reducing the pregnancy loss rate with ART. The identification of a dsDNA component may confirm this link , and rectifying for this may increase the number of euploid embryos. Therefore, we measured neutral comet which is solely specific for dsDNA fragmentation. Furthermore, we have preliminary data that suggest MFSS is capable of decreasing sperm aneuploidy when assessed by copy number variants using NGS. However, at this point, clinical meaning is observational. Further investigation should also be carried out to determine whether other sperm selection methods based on motility, such as the swim-up approach, would achieve the same results on PGT-A and clinical outcome as microfluidics.

The relationship between SCF and embryo aneuploidy is preliminary data and a new concept that is difficult to accept and not immediately intuitive. This study is somewhat limited because of its cohort nature, even if prospective. A study carried out on sibling oocytes would be needed to confirm the findings. Most importantly, the study does not address the issues related to ooplasmic repair mechanism that would be seen if donor eggs are used.

5 | CONCLUSIONS

The utilization of ICSI addresses the reproductive needs of many couples, and the addition of a powerful tool, such as PGT-A, would help to select euploid embryos for optimal chances of pregnancy. Nonetheless, in rare situations, couples with a relatively young female partner and a male partner with adequate semen parameters may generate a limited number of euploid embryos because of male factor. Screening for SCF may therefore guide treatment in subsequent cycles with surgically retrieved spermatozoa or, preferably, by processing ejaculate specimens by MFSS. We evidenced that a relationship between dsDNA breaks and embryo aneuploidy exists, and that the selection of the most motile fraction of spermatozoa carrying a superior genomic integrity is capable of granting higher implantation and optimal pregnancy outcome for couples with a history of high embryo aneuploidy.

AUTHOR CONTRIBUTIONS

Retrieved and analyzed data; wrote the paper; performed TUNEL and Comet assays; analyzed results; and performed statistics: Olena M. Kocur. *Contributed to performing the Comet assays; writing the paper; and analyzing the data*: Philip Xie. *Contributed to writing the paper; and analyzing the data*: Stephanie Cheung. *Performed the TUNEL assay and analyzed results*: Sydney Souness. *Performed the TUNEL assay and analyzed results*: Mary McKnight. *Contributed to reviewing the data; and writing and editing the paper*: Zev Rosenwaks. *Contributed to planning the study; analyzing the data; and writing the paper*: Gianpiero D. Palermo.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available because of privacy or ethical restrictions.

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