Combined sperm selection techniques to boost the success of in Vitro Fertilization (IVF) and Intra Cytoplasmic Sperm Injection (ICSI)

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ABSTRACT

This study aimed to assess the effectiveness of combined methods of Density gradient centrifugation and swim-up (**D.G.C/S. U)** and Density gradient centrifugation and zeta potential (**D.G.C/Z**) in semen samples to treat sperm abnormality, decrease DNA fragmentation, and choose one of the best methods for Teratozoospermia patients who have high Sperm DNA Fragmentation (SDF) undergoing assisted reproduction.

Method: 101 patients, who all have teratozoospermia with high DNA fragmentation visited the fertility clinic. Semen features were examined using established criteria According to Recent World Health Organization (WHO), DGC /Z and DGC/ SU methods were carried out on semen samples After that, the samples were evaluated by Sperm Chromatin Dispersion (SCD) testing was used by the WHO to detect DNA damage recently.

Result: The first combined method used showed statistically significant higher motility with DGC/su ($p<0.001$), while the other method showed statistically significant, lower DNA fragmentation and fewer abnormalities was DGC/Z ($p<0.001$**)**. We discovered no statistically significant connection between defective sperm morphology and DNA damage. There is no link between sperm motility and DNA damage, indicating that defective sperm are more likely to be normal DNA.

Conclusion: According to the results of the current study, the combined use of the DGC/Z and DGC/swim-up procedures improved the motility, morphology, and DNA integrity of semen samples, which may increase the probability of a successful pregnancy.

Keywords: DNA Fragmentation, Sperm selection, Zeta method. Swim-up. Density gradient.

1. INTRODUCTION

The couple's failure to have children within a year of having regular sexual relations without taking contraception is the main criterion of primary infertility (Duarte *et al.*, 2017). Infertility affects up to 15% of couples who are of reproductive age, making it a somewhat prevalent condition. Around 30 % of partners who struggle to conceive are thought to be totally at blame, and male factors are also known to play a role in about 50% of all cases of infertility(Nixon *et al*., 2023). The standard examination of sperm does not accurately predict fertility; it is merely one of many

variables. Determining sperm quality is thus one aspect of sperm research that may have an impact on how embryos develop. Sperm processing provides the most comprehensive insight into male infertility and the effectiveness of In Vitro Fertilization (IVF) treatment (Zaha *et al*., 2023). A doctor may use assisted reproductive techniques (ARTs), which involve managing human sperm, oocytes, and embryos in vitro and transferring them to females to induce pregnancy if medical treatment and surgical intervention are unsuccessful in assisting an infertile woman to conceive (Dyer *et al*., 2016).

The way infertility is controlled has totally altered because of assisted reproductive technologies (ARTs). Researchers have been working on more complex methods to separate functional spermatozoa from spermatozoa that are immotile, have weak morphology, or are not able to fertilize oocytes as assisted techniques have shifted over time from proofs that are more gynecological to more andrological proofs (Jayaraman *et al*., 2012). These studies also show a higher risk of miscarriage when using sperm from raw, unprocessed semen with mixed cell types as opposed to treated samples that have been considerably cleaned of sperm of lower quality(West *et al*., 2022) . Although some consider sperm selection essential, others differ on the most effective method. Some studies suggest that conventional processing methods, such as density gradient centrifugation (DGC) or swim-up, are adequate. On the other hand, ways to improve sperm DNA integrity are additional advanced selection strategies that have been tested(Hozyen *et al*., 2022).

The ability of motile spermatozoa to swim from seminal fluid (pellet) to sperm culture medium is the technique of swim-up. Contrarily, the density gradient chooses sperm cells with proper morphology based on their particular density (Raad *et al*., 2021). According to a recent study, combining DGC with SU (DGC/SU) may be one of the strategies that work best. DGC or SU are mostly used in Chinese reproductive places to improve sperm for in vitro fertilization. (Dai *et al*., 2020) . 50% of sperm with normal morphology may contain DNA fragmentation in infertile persons, according to (Avendaño et al., 2009). To overcome these in vitro constraints, a unique sperm selection approach based on the ability to bind surface charge has been created. Considering that sperm have the Zeta potential, which is based on an electric charge of -16 to -20 mV (Zarei-Kheirabadi *et al*., 2012), The combined use of the ζ-potential method and DGC recovers sperm with improved maturity, DNA integrity, morphology, hyper activation and progressive motility compared with sperm processed only with DGC. The combined method also enhanced the levels of protamine and sperm, phospholipase C in the isolated sperm (Leung *et al*., 2021). The fractionation of sperm DNA into smaller pieces is known as sperm DNA fragmentation and is steadily worsening in sperm cells. (Li *et al*., 2006). Sperm DNA fragmentation can be carried on by both extrinsic (such as heat exposure, smoking, environmental toxins, and chemotherapy medicines) and intrinsic (such as faulty germ cell maturation, abortive apoptosis, and oxidative stress) causes. (Agarwal et al., 2020).

2. MATERIALS AND METHODS

This blind Randomized clinical study was done at Fertility Centre between May 2021 and October 2022, samples from 101 patients were used to obtain the data. The Animal Biotechnology Department Ethics Committee, GEBRI, the University of Sadat

City and Queens Fertility Centre all gave approval for the study. Patients were told about fresh spermatozoa selection methods and other possible consequences as ethical considerations. Following that, all patients were given a written consent agreement to sign.

2.1. Inclusion Criteria:

One hundred and one patients were assigned to undergo three groups of techniques: DGC, DGC/Zeta, and DGC/SU procedures after standard semen analysis. This study included individuals who fulfilled World Health Organization (WHO, 2021) criteria for male factor infertility and at least one unacceptable sperm parameter (sperm motility, concentration, and morphology). Semen samples were collected from a group of males who were sent to the Andrology Unit for both semen analysis and ICSI between the age of 26 and 60, and samples were taken through masturbation after 2 to 7 days of abstinence. All patients with a high DNA fragmentation index (more than 25%). Four groups of the sperm samples' concentration, total motility, morphology, and DNA fragmentation were examined and compared between these methods: raw, DGC, DGC + SU, and DGC + Zeta potential.

2.2. Sample collection and preparation

Masturbation was used to extract semen samples, which were then allowed to liquefy for 30 minutes at 37°C following 2 to 7 days of ejaculatory abstinence, the analysis of the semen was done to determine sperm concentration, motility and morphology in accordance to the most recent WHO recommendations. Then DGC only and combined with Swim-up and Zeta were performed after high SDF diagnosis in raw sample.

2.3. Density Gradient Centrifugation (DGC)

A 15-ml falcon tube was used to apply layers of the discontinuous gradients of the 40/80 density gradient (Pureception, SAGE, USA). 1.0 mL of the seminal fluid was put on a gradient, which layered upper 40% and lower 80%. Every process was completed in sterile environments. The initial centrifugation was performed for 20 minutes at 1200 rpm. The three layers were eliminated, preserving the pellet's integrity. The pellet was twice washed for 8 minutes at 1200 rpm with 2 ml of preheated "sperm wash media" (Origio, Denmark). An aliquot was then collected for a rough estimation of total motility, progressive motility, morphology, and sperm DNA fragmentation then the end pellet was resuspended in 0.5 ml and split into two equal portions, one 250 µl for swim-up and the other 250 µl for Zeta.

2.4. Density gradient centrifugation and swim up (D.G.C/SU)

Along the inner tube wall, 250 µl of the DGC-produced sperm suspension were slowly covered with 0.5 ml of sperm wash media; the tube was oriented at 45 C to enhance the surface area of the sperm culture medium. And after that, the medium was incubated for 60 minutes at 37 C in a carbon dioxide incubator (Dai *et al*., 2020) . After incubation, the tube was carefully set upright. The highly motile sperm cells in the supernatant fractions were then removed. This sample of supernatant was concentrated by centrifuging it at 1500 rpm for five minutes, after which the supernatant was discarded. The pellet was resuspended in 0.3 ml of sperm wash media, and sperm DNA, morphology, and total motility were all measured.

2.5. Density gradient centrifugation and zeta potential (D.G.C/Z)

After Density gradient centrifugation (DGC), the Zeta technique was applied in accordance with (Chan *et al*., 2006). Findings. Simply put, 250 µl of sperm suspension with a sperm count of 5 x 10^6/ml was diluted into media without serum and applied to induce positive surface charge. The tube was swiftly taken out while being spun twice or three times to generate a positive charge after being placed into a latex glove up to the cap. Each tube was allowed to stand at room temperature for one minute prior to the charged sperm could attach to the tube wall. The caps were fitted to the tubes in order to avoid grounding of the tube. After spinning for a minute, the tubes were centrifuged at 1200 rpm for five minutes. After that, sperm and other cells were eliminated by discarding the supernatant and pellet that did not. The surface of the tube rinsed with 0.3 ml of sperm wash medium with albumin to neutralize the charge on the tube wall and separate the adhering sperm. To boost the amount of sperm recovered, the bottom-collected media from each tube was manually re-washed many times and utilized to repeatedly rinse that tube's wall. From samples of the detached sperm, the total motility, progressive motility, morphology, and sperm DNA fragmentation were identified.

2.6. Determination of DNA Fragmentation

DNA fragmentation was determined by performing a sperm chromatin dispersion (SCD) test using the Halo-sperm® G2 kit (Halo-tech DNA, Madrid, Spain), the degree of sperm DNA fragmentation was determined. 20 µl of melted agarose was mixed equally with 10 µl of sperm suspension that was diluted to 10x106/mL in phosphate-buffered saline (PBS). After that, a glass coverslip (22 mm x 22 mm) was put on a super-coated slide with a 15-µl aliquot of the cell/agarose solution on top of it. The coverslip was slowly removed after the slide was positioned on a cool surface at 4 °C for 5 minutes in the refrigeration unit to create a micro gel that contains implanted sperm cells. The slide was then immersed for 7 minutes at room temperature in an acid denaturant. The slide was then treated with a lysis solution for 20 minutes at room temperature. The slide was dehydrated in a stepwise ethanol series (70% and 100%) for 2 minutes each after a 5-minute rinsing in distilled water, and it was then air-dried. This-dehydrated slide was stained with eosine followed by thiazine then it was examined for haloes using a bright field microscope (Z2000-S, Germany). Sperm cells were categorized as having fragmented DNA if they had very small halos, no halos, or were degraded and having normal DNA if the halo large and medium.

3. RESULTS:

3.1. Statistical analysis

Each value of result was expressed as means ± S.D. With the help of the statistical program SPSS version 21, data were coded and entered. Mean and standard deviation were used for quantitative variables to summarize the data, whereas number and percent were used for qualitative factors. non-parametric Wilcoxon Sign Rank tests for quantitative variables. P values ≤ 0.05 were considered statistically significant.

The effectiveness of each technique was assessed by calculating the percentage reduction in DNA fragmentation compared to unprocessed samples.

Table (1) represent that a negative correlation exists between the Age group and sperm concentration and sperm motility (r=-0.479) (r=-0.227) respectively among the studied group

while a positive correlation exists between the Age group and sperm DNA fragmentation of the studied group (r=0.35), No correlation exists between Age and sperm Abnormal form (r=0.069).

Table (2) shows that statistically significant increase in sperm motility of DGC/Zeta (90.54 ± 5.42) compared to raw samples (44.19 ± 12.17) Respectively while the statistically significant decline in sperm concentration, Abnormal form, and sperm DNA fragmentation of DGC/Zeta (3.81± 1.25), (97.06± 1.02) and (6.10 ± 2.4) compared to raw samples (42.6 ±10.00), (98.7 ± 0.62) and (41.21 ± 11.94), respectively.

Table (4) shows that statistically significant increase in sperm motility of DGC/SU (92.9 ± 5.08) compared to raw samples (44.19 ± 12.17) while the statistically significant decline in sperm concentration, Abnormal form, and sperm DNA fragmentation (6.7±1.80), (97.6 ± 1.07) and (7.61 ± 3.6) respectively compared to raw samples (42.6 ±10.00), (98.7 ± 0.62) and (41.21 ± 11.94), respectively.

4. DISCUSSION

A considerable amount of study has been devoted to comparing various semen preparation methods in relation to sperm DNA damage. However, there is no agreement on this issue in the literature. Therefore, the goal of the current study is to explore the degree of DNA damage in an ejaculate and how various sperm preparation can eliminate DNA-damaged sperm. Researchers have been working on more complex methods to separate functional spermatozoa from immotile spermatozoa, that have poor morphology, or are unable to fertilize oocytes as assisted techniques have shifted over time from more gynecological proofs to more andrological proofs (Jayaraman et al., 2012) .

Additionally, there are disagreements about the efficacy and selection of different semen preparation techniques that could retrieve the most viable sperm. These unpopular findings reflect that sperm DNA integrity may be influenced by a variety of factors, including the semen sample procedure, the DNA quality measurement method, and the study population. To find the underlying reasons for inconsistencies, develop these procedures, and improve their use in clinical settings, a rigorous evaluation of traditional semen processing methods is therefore required. To assess the impact of three different selection strategies on the quantity of sperm, their general motility, their shape, and their DNA fragmentation in teratozoospermia samples all of which have higher DNA fragmentation rates—was the goal of the current investigation. Overall, sperm motility, morphology, and DNA fragmentation were affected by selection techniques in all semen samples. As expected, when compared to fresh semen samples, the three techniques increased sperm motility.

Density gradient centrifugation (DGC) and swim-up (SU) are the two main semen preparation techniques that are frequently used to separate sperm from semen. The best approach for sperm enrichment has been the subject of numerous investigations. Studies from various groups, though, have produced contradictory results. DGC combined with SU (DGC/SU) may be one of the most successful strategies, according to recent studies. The majority of Chinese fertility clinics use DGC/SU to enhance sperm for IVF.(Dai et al., 2020).

Our findings showed that when used to prepare sperm for IVF cycles, the techniques DGC/zeta and DGC/SU may considerably boost the sperm recovery rate

from defective semen samples, suggesting that these approaches may be a quick, efficient, and safe way to extract functional sperm. As per a previous study(Dai et al., 2020) Increases in centrifugation time or force may speed up the recovery of sperm. In agreement with this results, we found that both healthy and deficient semen samples retrieved more sperm when centrifugation periods were prolonged. more importantly, in comparison to typical samples. The results of the current study are in line with earlier studies that examined the elimination of sperms with DNA fragmentation and an immature nuclear structure. These studies also demonstrated that the motility of motile sperms in samples produced by combining DGC and swim-up was higher than that in the original semen and DGC alone.(Yamei Luo., 2012) discovered that sperm DNA fragmentation was decreased after processing by both swim-up and DGC as compared to whole semen. This conclusion was backed by several research that confirmed the current study's findings that spermatozoa produced using swim-up or Percoll density centrifugation had enhanced morphology.

According to study by (Sakkas et al., 2000), DGC is more effective than swim-up at reducing the proportion of sperm with DNA damage. On the other hand, Zini et al discovered that in nonazoospermic patients, the percentage of spermatozoa with DNA damage significantly declined after swim-up processing compared to unprocessed whole semen, although a modest increase was observed after Percoll treatment(Zini et al., 2000).

Our finding that the SDF was much lower in samples treated with DGC/zeta compared to samples treated with DGC/swim-up and DGC alone suggests that both methods of semen preparation may improve DNA integrity. Regarding how ageing and aberrant form are related This study found no statistically significant change in sperm aberrant shape between the 40 and under 40 age groups using complete semen samples, which is consistent with According to a study by(Siddighi et al., 2007)., Age-related changes to strict normal morphology were nonexistent. However, in untreated semen samples, there was no statistically significant change in sperm aberrant form. Instead, we found that the level of aberrant sperm shape was marginally lower in DGC/zeta than in DGC/swim up. In contrast to (Brahem et al., 2011), which found that there isn't a relationship between age and DNA fragmentation in people with teratozoospermia, this study found a statistically significant positive relationship between age and sperm DNA fragmentation (r=0.35). Studies examining the connection between sperm shape and DNA integrity in individuals with aberrant sperm, particularly those with teratozoospermia, are quite rare. Few studies, as far as we are aware, have looked at the connections between sperm abnormalities and DNA integrity in different age groups in teratozoospermia patients. The findings of our study showed that sperm abnormalities in total semen samples and sperm DNA fragmentation did not correlate. This outcome contradicts several findings from related studies.

We found no statistically significant correlation between DNA damage and abnormal sperm morphology. Sperm motility and DNA damage are unrelated, suggesting that dysfunctional sperm are more likely to have normal DNA.

The methods used to prepare sperm today range from the most widely used ones, such as swim-up and DGC, to some of the more complex ones, such as electrophoretic separation, high-magnification sperm morphological selection, and the utilization of hyaluronic acid binding. There is currently no ideal and

reliable method for ART in patients with teratozoospermia because each procedure has unique benefits and drawbacks. The conventional methods of semen preparation continue to be acceptable practices because there isn't yet a single contemporary strategy that can entirely replace them. Combining modern and traditional procedures could offer a seductive new solution to the complexity, efficacy, and safety issues that plague modern approaches. However, the questioned DNA integrity found in the pellet fractions of the density gradient and the combination technique in the current study requires us to reevaluate the current sperm processing techniques. We advise that in the future, studies with a large sample size be used to validate similar findings. We propose that the two procedures used in the current research are equivalent since they all produced a population of sperm with less DNA damage than the unprocessed fraction.

5. CONCLUSION:

In conclusion our results shown that in spite the Z potential technique that is a very useful tool for sperm selection in assisted reproduction treatments; it significantly reduces the sperm DNA fragmentation index gave semen samples recover more favorably in terms of morphology, DNA integrity, and motility. The standard method used, DGC/swim up, demonstrated statistically significant higher motility, while the standard method, DGC/Zeta, demonstrated statistically significant lower DNA fragmentation and fewer abnormalities. In addition, there is no clear correlation between sperm motility and DNA damage, indicating that damaged sperm was more likely to have healthy DNA. Finally, this tool could replace the diagnosis of sperm DNA fragmentation index when couples have already opted for an IVF treatment, that could reduce costs for patients by giving them a better chance to success in their treatments, with more facilities and no need to more expensive techniques are used for sperm selection.

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Table 1: Correlation between Age and raw sperm parameters of the studied group:

Raw Sperm parameters	*r	*p-value
Sperm Concentration (C)	-0.479	<0.001
Sperm Motility (%)	-0.227	0.023
Abnormal form of sperm (%)	0.069	0.496
Sperm DNA fragmentation (%)	0.349	<0.001

*r =correlation coefficient (non-parametrical correlation.)

Table 2: The effect of DGC/Zeta on raw sperm parameters (group I):

Sperm parameters	Raw(control) Mean ± S.D	DGC/ Zeta Mean+/- S.D	*P-Value
Sperm Concentration (C)	42.6 ±10.00	3.81± 1.25	<0.001
Sperm Motility (%)	44.19 ± 12.17	90.54 ± 5.42	<0.001
Abnormal form of sperm (%)	98.7 ± 0.62	97.06± 1.02	<0.001
Sperm DNA fragmentation(%)	41.21 ± 11.94	6.10 ± 2.4	<0.001

*The P-value of the Wilcoxon Signed Ranks Test was used for quantitative variables that are not normally distributed

Table 3: The effect of DGC/SU on raw sperm parameters (Group II):

Raw Sperm parameters	Raw(control) Mean ± S. D	DGC/ SU Mean+/- S. D	*P-Value
Sperm Concentration (C)	42.6 ±10.00	6.7±1.80	<0.001
Sperm Motility (%)	44.19 ± 12.17	92.9 ± 5.08	<0.001
Abnormal form of sperm (%)	98.7 ± 0.62	97.6 ± 1.07	<0.001
Sperm DNA fragmentation (%)	41.21 ± 11.94	7.61 ± 3.6	<0.001

*P-Value of the Wilcoxon Signed Ranks Test was used for quantitative variables which are not normally distributed.

Table 4: comparing percent change between zeta and swim-up within <40 age and ≥40 age groups.

	<40 age			≥40 age		
Raw sperm parameters	DGC/ Zeta	DGC / SU	**p.value	DGC/ Zeta	DGC / SU	**p.value
Sperm Concentration (C)	82.3±5.2	69.4±6.6	<0.001	78.9±6.7	63.02±8.65	<<0.001
Sperm Motility (%)	4.48±2.11	.9±2.9	<0.001	5.50±2.77	8.74±4.01	<<0.001
Abnormal form of sperm (%)	0.99±0.6	0.55±0.52	<0.001	0.99±0.48	0.37±0.55	<<0.001
Sperm DNA fragmentation (%)	54.83±10.44	48.56±15.15	<0.001	59.6±9.48	46.62±11.88	<<0.001

*P-Value of Wilcoxon Signed Ranks Test used for quantitative variables that are not normally distributed.