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The role of testicular sperm in individuals with high sperm DNA fragmentation index who had previously failed intracytoplasmic sperm injection

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ABSTRACT

The aim of this study is to investigate the results of intracytoplasmic sperm injection (ICSI) in patients with previous failure and suffering from high sperm DNA fragmentation index (SDF) when using testicular spermatozoa instead of ejaculate spermatozoa. This study involved 107 patients, all of them were with SDF >22% and previous ejaculate ICSI failure. This study examines the embryonic factors (fertilization, cleavage, and blastocyst formation) that resulted from ICSI using testicular and ejaculate sperms. In cases with high SDF that were detected using sperm chromatin dispersion assay (SCD assay) in ICSI program, testicular sperms extraction fine needle aspiration technique (FNA group) was used instead of ejaculate sperms (ejaculate group). There was no significance difference between FNA and ejaculate groups in oocyte fertilization rate and embryo cleavage rate ($P < 0.005$) while blastocysts formation rate was higher significance in FNA group than ejaculate group ($P > 0.001$), while pregnancy rate was 69.16 % positive, and 30.84 % negative, and implantation rate was 30%. Average of embryo transferred by cycle 2±1 /cycle and resulted in 91 live birth in healthy twins or singlet babies, abortion rate was 10%. In conclusion, this study suggested that improved ICSI outcomes for men with increased SDF were associated with the utilization of a testicular sperm obtained by FNA method.

Keywords: Male infertility, Sperm DNA fragmentation index, Fine needle Aspiration, Testicular sperm, ICSI outcome, ICSI failure.

1. Introduction

The inability to conceive for 12 months or more of consistent, unprotected sexual activity is known as infertility. Age of the couple, frequency of sexual activity, and duration of sexual exposure all affected the likelihood of conception. Only 5% of young, normal couples will conceive after 1.5 years, and after two, the causes are shared by men and women. After one year of unprotected sexual activity, 25% of couples experience pregnancy, 70% of couples after six months, and 90% of couples have a probability of becoming pregnant after a year (Tayebi and Ardakani, 2009).

Sperm DNA integrity is essential for the development of embryos because the right genetic information needs to be passed on to the next generation without being erased or fragmented. There are other circumstances linked to abnormal sperm DNA content, such as smoking, exposure to toxins, and high temperatures in the gonadal region, in addition to the paternal age, certain systemic disorders, and infections with reactive oxygen species (ROS) (Lewis et al., 2013).

According to Tesarik et al. (2004), an elevated sperm DNA fragmentation index (SDF) may have an impact on the activation of a male gene expression, which could raise the probability of miscarriage. Sperm DNA damage can result from post-testicular DNA fragmentation induced by oxygen free radicals during transport to the epididymis, or from strand breaks that happen during chromatin remodeling during spermatogenesis. However, DNA damage can also result from chemotherapy and radiation (Sakkas and Alvarez, 2010).

Intracytoplasmic sperm injection (ICSI) has been widely utilized to treat almost types of severe male-factor infertility, outcome of ICSI is affected by various aspects especially bad quality of injected sperms that may because of genetic reasons related to DNA damage (Esteves et al., 2014). Reactive oxygen species (ROS) that are present in excess can damage sperm on their journey from the seminiferous tubules to the epididymis. Testicular sperm can prevent this damage by utilizing ROS-free DNA during ICSI (Eve and Cristian, 2007). Even though applying testicular sperm rather than ejaculated sperm for ICSI has higher success rates and better results (Muratori et al., 2015).

There are numerous methods for detecting DNA fragmentation that rely on fluorescence, including different assays for SDF, which quantify the amount of intact and fragmented sperm DNA that is observed. Using a flow cytometer or optical microscope, probes or dyes are utilized to identify breaks in DNA. Approximately, 20% to 40% of men who are infertile have high DNA fragmentation even though their semen parameters are normal (Agarwal et al., 2016). According to Esteves et al. (2018), when infertile men undergo ICSI, the SDF test is often used as a routine test, which may enhance the procedure's outcomes and promote conception. Research suggests that the effectiveness of fertilizing an egg with sperm that has fragmented DNA may be equivalent to that of fertilizing an egg with normal sperm that has not fragmented DNA. However, by impairing embryo growth and increasing the risk of abortion, this might make artificial reproductive procedures (ART) less successful (Robinson et al., 2012).

Decreased SDF has been shown in testicular sperms compared to those of ejaculated sperms make using testicular sperm in cases with high SDF is preferred where found better ICSI outcomes (Bradley et al., 2016). Numerous techniques are present to detect and measure cellular DNA damage resulting from exposure to a suspected genotoxic agent. These techniques include the sperm chromatin dispersion (SCD) test, the sperm chromatin structure (SCS) assay, the single cell gel electrophoresis (Comet) assay, and the terminal deoxynucleotidyl transferase mediated deoxy uridine triphosphate nick end labeling (TUNEL) assay (Esteves et al., 2018).

This investigation will aim to measure the difference between ICSI outcomes when using testicular spermatozoa instead of ejaculate with high sperm DNA fragmentation as a treatment method for a male infertility. It will also study the morphogenesis of early embryonic development beginning from fertilization, cleavage, blastocyst formation and quality of embryos. Finally, the pregnancy rates for testicular embryos will be examined in this study.

2. Materials and Methods

Patient selection

This study included 107 ICSI cycles that were enrolled in El Nada Fertility Center, Mostafa El-Nahas St. Nasr City, Cairo, Egypt. Patients with a history of previous ICSI, as well as cases of severe oligoasthenoteratozoospermia and suffering from high SDF (more than 22%) were enrolled in the current study.

Confidentiality

Every patient who was admitted for the study had their privacy respected. The names of study participants will not appear in any report or publication derived from the project's data collection.

Ethical approval

Written and oral consent was obtained from all cases participating in the research, ensuring the confidentiality of the data, not mentioning any names, and stating that there is no harm according to previous research to improve their results. This study was conducted under the Registration number 126, Faculty of Medicine, Al Azhar University.

Technical approach of all male subjects

The WHO's 2021 criteria for sperm concentration, motility, and morphology, which specify the properties of semen, PH: 6-7.2, volume: > 1.5 ml, liquidation time: 20–60 min, abnormal forms: 96%, pus ≤ 0.2 million/ml, spermatogenic cell ≤ 0.2 million/ml, count: ≥ 15 million, motility: ≥ 40%, progressive motility ≥ 32%, and at least two of these three criteria were abnormal in prior testing. The SDF was determined via (SCDA) from Halo sperm® SCD technology (Martinez and Majzoub, 2021).

Semen preparation for ICSI

For ejaculate group

The ejaculate samples were processed to eliminate seminal fluid, immotile, pus, and spermatogenic cells. For that purpose, the density gradient method was used (Lachaud et al., 2004) in which semen sample (1 ml) was added above 1 ml of 90% slanted coated silica particles (Irvine Scientific, Santa Ana, CA). Centrifugation had occurred at 300 g. It was found that the round cells and immotile sperm were present in the surface layer, while healthy motile sperms were present in the precipitate layer. After removing the top layers, 2 ml of HEPES buffered medium were placed on the precipitate in a centrifuge tube. For 8 minutes, the sample was centrifuged at 300 g. After that the final pellet was resuspended in 0.2 milliliters of sperm wash medium (Irvine Scientific) (Çil et al., 2022).

For FNA group

Testicular sperms were extracted using a fine needle aspiration technique; a tiny needle was used under local anesthesia; sample of a testicular tissue was removed by the surgeon. The sample was then transferred to a dish containing HEPES buffered media (Irvine Scientific) and was squeezed using sterile slides. The supernatant was then moved to a centrifugal tube, centrifugation occurred at 300g for 4 minutes, supernatant was discarded, and precipitate was resuspended in (erythrocyte lysis buffer) to erythrocyte discarded in 1.5 ml Bio Media. Centrifugation was repeated for 8 min, supernatant was discarded, and precipitate were resuspended in 0.2 ml of washing medium (Irvine Scientific). At this moment, the samples were ready for ICSI (Sakas et al., 2023).

Oocyte retrieval

After the aspirated fluid tubes were inspected using an Olympus stereomicroscope equipped with a thermo plate unity k-system and 40× magnification, the cumulous components were identified and removed, followed by washing in clean culture media. The retrieved oocytes were kept in an incubator at 37° with 5-6% CO_2 in humidified air (Georgiou et al., 2022).

Removal of cumulus cells (denudation):

In this step, use a HEPES buffered media (life global group; 421 Ramses St. El-Abasia, Cairo, Egypt); and the surrounding cumulus cells were removed using a hyaluronidase (80 IU/mL, Irvine Scientific) (Carvalho et al., 2020).

ICSI procedure

After their yolk being removed, the recovered oocytes were evaluated for quality. ICSI was used to inject only Metaphase II (MII) oocytes using the husband's selected spermatozoa (Maggiulli et al., 2020).

Oocytes were divided into two groups (Greco et al., 2005):

Group I: (ejaculate group).

Group II: (FNA group).

Embryo scoring and morphogenesis (Popa et al., 2024):

Gardner blastocysts morphological scoring system was used including the assessment of expansion, Inner cell mass (ICM) and trophectoderm of embryos.

Blastocoel expansion grades

Grade 1: Early blastocyst, the embryo's blasto-coel cavity is less than half its total volume.

Grade 2: Blastocyst, the cavity is at least half the embryo's volume.

Grade 3: Full blastocyst, the embryo's blastocoel cavity is entirely filled.

ICM grades

Grade A: Large ICM, many small, closely spaced cells.

Grade B: less compressed, smaller, and with fewer weakly adhering cells.

Grade C: Very few cells are visible, maybe loose, and hard to differentiate from trophectoderm epithelium (TE).

Trophectoderm grades

Grade A: A densely packed, continuous epithelium is made up of numerous similar tiny cells.

Grade B: Less tiny cells and an irregular, non-continuous layer with occasional gaps.

Grade C: More gaps and a loose epithelium formed by a small number of unevenly sized cells.

Statistical analysis

Every outcome value was presented as means ± standard error, and SPSS, version 27 (IBM Corp., 2013) was used to analyze the data using one and two-way ANOVA. Data were handled in accordance with Steel et al., (1997) as a complete randomization design, where $P< 0.05$ for significance, $P< 0.01$ for high significance, and $P< 0.001$ for very highly significant.

3. Results

The clinical characteristics of patients in the study:

The clinical features of the patients in this study were represented in Table (1), including age, DNA fragmentation index, length of infertility, sperm count, motility, and morphology. In this study, 107 couples with a male partner's SDF of at least 22% were involved. ICSI was conducted using ejaculated spermatozoa and FNA spermatozoa, and all patients underwent an 18-month follow-up period until pregnancy was achieved. A variety of factors, including the age of the female and the length of infertility (defined as the period between an unprotected sexual encounter and hospitalization), as well as sperm motility and morphology, were analyzed. On the same day that the oocytes were denuded, the male partner was requested to extract a sample of semen using FNA or masturbation, which was then processed for ICSI. The presence of two pronuclei and two polar bodies was taken into consideration for normal fertilization; otherwise, it was also examined. On day five following egg retrieval or vitrification for a frozen cycle, embryo transfer was carried out. Six weeks following embryo transfer, the ultrasound-detected fetal heartbeat was further defined as clinical pregnancy. Unless they were able to conceive, all patients were advised to finish their 18-month follow-up. Pregnancy was recorded during outpatient visits or over the phone. In the 18-month follow-up, both the birth rate and the abortion rate were noted. In the end, the FNA-ICSI and ejaculated-ICSI groups were compared according to the following parameters that were recorded. Comparison of the oocyte fertilization rate, the embryo cleavage rate, and blastocyst rate formation are done between the ejaculation group and FNA group in a moderate (22-35%) and a severe SDF (>35%) and the comparison between the two groups occurred at more than (>22%) SDF (Table 1)

Table 1. The main characters of patients included in this study.

characters of patients		No.	(%)	Mean ± SE
Age	≤30 years	69	64.49	24.70 ± 0.44
	> 30 yeas	38	35.51	34.18 ± 0.41
	Total	107	100	
DNA%	Mild (=22%)	8	7.477	18.00 ± 0.00
	Moderate (22-35 %)	82	76.64	28.10 ± 0.41
	Severe (>35%)	17	15.89	44.85 ± 1.53
	Total	107	100	
Duration of Infertility (years)	≤10 ears	94	87.85	4.92 ± 0.27
	>10 years	13	12.15	14.38 ± 0.72
	Total	107	100	
Sperm count/ (million)	<5	18	16.82	2.81 ± 0.29
	5-10	20	18.69	7.55 ± 0.31
	11-15	16	14.95	13.69 ± 0.41
	>15	53	49.53	30.81 ± 1.75
	Total	107	100	
Sperm motility %	≤50	83	77.57	33.29 ± 1.59

	>50	24	22.43	57.50 ± 0.74
	Total	107	100	
Sperm Morphology%	≤96	10	9.35	94.80 ± 0.44
	>96	97	90.65	99.22 ± 0.1
	Total	107	100	
Pregnancy Rate%	Positive	74	69.16	
	Negative	33	30.84	
	Total	107	100	

All data represented as mean and (SE).

Embryonic outcomes

Table 2 illustrated the percentages of the oocyte fertilization rate, cleavage rate, and blastocyst's formation rate in a moderate and high SDF between the two studied groups. It is obvious that the differences between percentages of the oocyte fertilization rates in a moderate and severe SDF in the two studied groups were statistically non-significant (P > 0.05). This was also recorded in relation to percentages of the cleavage rate between the two groups in a moderate and severe SDF. On the other hand, the percentages of blastocyst formation rate between the ejaculation and FNA groups in case of the moderate SDF recorded a very high statistically significant difference (P < 0.001***); but recorded a significant difference (P < 0.05*) between the two groups in a severe SDF.

Table 2. Comparison of oocyte fertilization rate (%), embryo cleavage rate (%), and blastocyst rate formation (%), between ejaculation and fine needle aspiration (FNA) in a moderate and a severe sperm DNA fragmentation index (SDF).

Variables		**Ejaculation**	**FNA**	***P-value***
Moderate sperm DNA fragmentation (22-35) % (N= 90)	Fertilization rate (%)	90.87 ± 1.86	95.27 ± 1.35	0.057
	Cleavage rate (%)	96.72 ± 1.46	97.87 ± 0.92	0.505
	Blastocyst's formation rate (%)	44.88 ± 2.86	70.51 ± 2.19	P<0.001***
Severe sperm DNA fragmentation (>35) % (N=17)	Fertilization rate (%)	87.62 ± 5.75	90.49 ± 4.89	0.706
	Cleavage rate (%)	93.26 ± 3.86	97.35 ± 1.82	0.344
	Blastocyst's formation rate (%)	47.86 ± 6.84	65.46 ± 4.19	P<0.05*

Non-significant (P > 0.05), *: Significant (P < 0.05), **: High significant (P < 0.01), ***: Very high significant (P < 0.001).

Table 3 deals with the same previous issue, explaining the percentages of the fertilization rate, cleavage rate, and blastocyst's formation rate in SDF more than 22%. These results indicated that the differences between percentages of the oocyte fertilization rates in a moderate and severe SDF in the two studied groups were statistically non-significant (P > 0.05). Also, in relation to percentages of the cleavage rate between the two groups recorded a non-significant difference (P > 0.05). On the contrary, the percentages of blastocyst formation rate between the two studied groups recorded a very high statistically significant difference (P < 0.001***).

Table 3. Comparison of oocyte fertilization rate (%), embryo cleavage rate (%), and blastocyst rate formation (%), between ejaculation and fine needle aspiration (FNA) at all cases above 22% DNA fragmentation index. Data represented as Mean ± SE

Variables	Ejaculate	FNA	*P-value*
Oocyte Fertilization rate (%)	90.35 ± 1.81	94.51 ± 1.37	0.068
Embryo Cleavage rate (%)	96.17 ± 1.37	97.79 ± 0.83	0.313
Blastocyst's formation rate (%)	45.35 ± 2.63	69.71 ± 1.96	P < 0.001***

Non-significant (P > 0.05), *: Significant (P < 0.05), **: High significant (P < 0.01), ***: Very high significant (P < 0.001).

The data recorded in Table 4 had reported that the percentage of the pregnancy rate was about 69.16 % of 74 positive cases, while the percentage of the implantation rate was about 30%. Also, the average of the transferred embryo by cycle 2±1/cycle; resulted in 91 live birth from 302 transferred embryos in healthy twins or singlet babies, this leads to a low abortion rate of 10%.

Table 4. The pregnancy, implantation, and abortion rates of all patients in this study.

Reproductive clinical outcome	The percentage
Pregnancy rate (%)	69.16%
Implantation rate (%)	30%
Abortion rate (%)	10%

4. Discussion

A woman is considered infertile if, she is unable to conceive after a year of consistent sexual engagement. The length of sexual contact, frequency of encounters, and age of the couple all affected the likelihood of conception. Young, healthy couples have a 25% chance of becoming pregnant after one month of unprotected sexual activity, 70% after six months, and 90% after one year. Only 5% of couples will become pregnant after 1.5 years or two years, and both men and women are to blame for the causes (Carson and Kallen, 2021). When a couple presents with a fertility issue, most have relative subfertility, which is a decreased likelihood of conception due to one or more causes in one or both partners, rather than absolute infertility, which is no chance of conception. Only 4% of sub fertile couples will remain childless against their will because the majority will conceive naturally or respond well to treatment. A fair assessment of the additional advantage provided by treatment alternatives must consider each couple's significant possibility of conceiving naturally and compare it to the possible benefit of therapy (Taylor, 2003).

ICSI is routinely used to treat practically all types of severe male-factor infertility. Numerous factors, most notably poor- quality injected sperm that may be due to genetics, affect the result of ICSI (Esteves et al., 2014).

To produce high-quality embryos and a high fertilization rate, many difficulties have been removed; DNA fragmentation and sperm quality indicators are essential to the success of ICSI. Damage to the DNA of spermatozoa is related to early pregnancy loss, decreased rates of in vitro fertilization, lower outcomes for fertility following natural or assisted conception, and impaired embryonic development to the blastocyst stage (Robinson et al., 2012; Rafael et al., 2021). The oxidative stress during epididymal transport makes sperm more susceptible to DNA damage. This was noted by Esteves et al., (2015), who discovered that the percentage of DFI was 8.3% in testicular sperm compared to 40.7% in ejaculated sperm and this is compatible with the results of this investigation.

Greco et al. (2005) found that the DFI of the testes was significantly (P < 0.001) lower than the ejaculated sperm samples (23.6% ± 5.1%) from the same people (4.8% ± 3.6%). There were no variations in the morphological grade of the embryos or the rates of fertilization and cleavage between the ICSI efforts utilizing testicular spermatozoa and ejaculated spermatozoa. However, ICSI with testicular spermatozoa resulted in 8 clinical pregnancies (four singlets and four twins; 44.4% pregnancy rate; 20.7%

implantation rate), while ICSI with ejaculated spermatozoa resulted in only one spontaneously aborted pregnancy; these findings agreed with the study's findings.

Sperm DNA integrity is important for embryo development because proper genetic information without deletion of fragmented must be transferred to next generation, abnormality of sperm DNA content is related to a variety of conditions including smoking, gonad high temperature and toxin exposure, ROS, paternal age, and some systemic diseases (Lewis et al., 2013). Standard sperm measure may not be effective in diagnosing and predicting sperm quality as SDF, which is an independent sperm quality measure, SDF assays measure the quality of DNA in which the proportion of intact and fragmented sperm DNA is detected, many techniques used to detect DNA fragmentation that depends on florescent Probes or dyes are used to detect DNA breaks using flow cytometer or light microscope, 20% to 40% of infertile men suffering from elevated DNA fragmentation although they have normal semen parameters (Mayara et al., 2023). Using of SDF test as a routine test for infertile men that subjected to ICSI may enhance pregnancy and ICSI outcome (Esteves et al., 2011). The reproductive success of artificial reproductive techniques is decreased when sperm with fragmented DNA fertilizes an egg with the same efficiency as normal sperm without DNA fragmentation. This results in impaired embryo development and an increased risk of abortion (Robinson et al., 2012). The association of DNA damage in a sperm with a pregnancy loss agrees with the results of this study.

Ambar et al. (2021) reported that the significant heterogeneity and low quality of many studies used to justify that the use of a testicular sperm in non-azoospermic males with multiple ICSI failure cycles and an elevated DNA fragmentation. This is despite recent papers that support this practice. Testicular sperm with reduced SDF has been suggested for ICSI cycles in non-azoospermic men to successfully conceive a child because of the theory that SDF can sometimes lead to failure ICSI cycles.

The obtained results revealed that, in the terms of comparison of oocyte fertilization, embryo cleavage rate and embryos which reach to blastocyst when ICSI using sperms from ejaculation versus ICSI using testicular sperms using FNA technique, the blastocyst formation rate was significantly higher in FNA than ejaculated group in moderate and severe DNA fragmentation index and the significances were subsequently $P<0.001$ and $P<0.005$ with pregnancy rate 69.16% and implantation rate 30%. The average of transferred embryo by cycle 2 ± 1 embryos resulted in 91 healthy live birth in twins or singlet babies from 302 transferred embryos for 107 woman with low abortion rate 10%, these results approved with (Madny, 2019), and it was found that the mean TUNNEL positive sperms was 24.5% for ejaculated sperm and 4.6% for testicular sperm, using of testicular sperm in ICSI give pregnancy rates with 50% with healthy live offspring.

Therefore, the FNA technique considered the best method for pregnancy outcome and improves ICSI outcome in cases with high DNA fragmentation index, the finding of this study reported that the differences between fertilization and embryo cleavage rates recorded no significant values; although of increasing embryo cleavage rates and this approved with (Greco et al., 2005). It was concluded that the FNA technique permits to improve the assisted reproductive technologies (ART) outcomes especially in long time of infertility or repeated abortion or ICSI failure because blastocyst formation is a good indicator of clinical results after ICSI with testicular sperm and this agreed with the results of this study (Virant-Klun et al., 2003). The results of the present study revealed that the correlation analysis indicated that there is no correlation between DNA fragmentation and motility, or sperm count or sperm morphology; this finding was in concomitance with Jakubik-Uljasz et al. *(*2020) who found a negative correlation between SDF and morphologically normal sperm cells, sperm motility, and sperm vitality.

A small sample of a testicular tissue was taken using an open biopsy or needle aspiration. Testicular retrievals should be preferred over epididymal retrievals, this is because males with obstructive azoospermia have a greater reported

DFI% in epididymal sperm than in testicular sperm (Alahmar et al., 2022).

The obtained results demonstrated that the increasing blastocyst formation rate with statistically significance values in infertility patients using a testicular sperm via FNA technique, and this technique has good characteristics, and its results are acceptable, more than the traditional ICSI technique. In cases with high DNA fragmentation index, this difference may be explained as follows: Considering that poor sperm features led to the majority of ICSI cycles, it makes sense to observe a higher percentage of sperm cells with enhanced DNA fragmentation in ejaculated sperm than in sperm obtained by the FNA technique. As sperm chromatin protamines are not fully cross-linked by disulfide bonds until they reach epididymis, it is generally agreed that testicular sperm are more vulnerable to DNA damage (Esteves et al., 2015).

Wright et al. (2014) suggested that the oxidative stress is one of the mechanisms in SDF that caused DNA damage when immature sperm that produced ROS migrated through the epididymis with mature sperm. Before disulfide cross-linking occurs, mature testicular sperms exposed to ROS may be damaged by immature sperm or the epithelial cells lining the epididymis (Dalzell et al., 2004). The results of ICSI were considerably improved in a group of men who had a testicular sperm using a FNA technique compared with those with an ejaculated sperm. SDF was significantly lower in the testicular group compared with the ejaculated group. According to the current study, FNA based ICSI can effectively treat infertility in certain men who have high SDF levels in their ejaculation.

In conclusion, this data indicates that the utilization of testicular sperm by FNA technique was associated with enhanced ICSI outcomes in men with high SDF and previous ICSI failure or repeated abortion this may be good choice for obtain increased number of blastocyst and increasing pregnancy rate. ICSI technique is very important tool in ART specially when used with a definite sperm selection technique; so ICSI based on a testicular sperm with men suffering from high DNA fragmentation index and combined with decreasing motility and decreasing sperm count or increasing morphology abnormality may be useful rather than the other sperm processing technique not suitable for solving this problem.

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