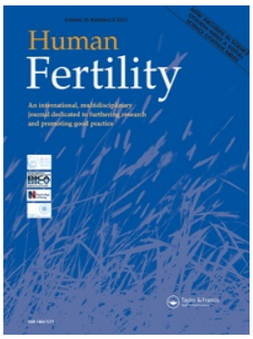

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RESEARCH ARTICLE

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Reproductive outcomes in patients with high levels of sperm DNA fragmentation using testicular sperm for intracytoplasmic injection: a retrospective analysis

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ABSTRACT

This study aims to compare the embryological and clinical parameters of intracytoplasmic sperm injection (ICSI) cycles using testicular versus ejaculated sperm in male patients with elevated sperm DNA fragmentation (SDF). A total of 73 ICSI cycles were examined in couples where the male partner exhibited high levels of SDF. ICSI was performed using either ejaculated or testicular sperm. The primary outcomes were rates of blastocyst formation, high-quality embryo development, and clinical pregnancy. The DNA fragmentation index (DFI) for testicular sperm (16.81 ± 17.51) was significantly lower than that of ejaculated sperm (56.96 ± 17.56). While the blastocyst formation rate was significantly higher in the testicular sperm group compared to the ejaculated sperm group, no statistically significant differences were noted in fertilization rate (72.15% vs. 77.23%), rate of high-quality embryo formation (47.17% vs. 46.53%), clinical pregnancy (50% vs. 56.52%), Cumulative pregnancy (70.2% vs. 55.6%), or live birth rate (43.75% vs.43.48%). Testicular spermatozoa have no additional advantage over ejaculated spermatozoa except for blastocyst quality in patients with high SDF, the use of testicular spermatozoa for the first ICSI cycle in male infertility patients with high SDF should be undertaken after much consideration at present.

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KEYWORDS
Sperm DNA fragmentation; male infertility; assisted reproductive techniques; testicular sperm; blastocyst formation rate

Introduction

The ability of routine semen analysis to differentiate between fertile and infertile men is limited, especially when evaluating IVF success, embryo abnormalities and miscarriages. Sperm DNA fragmentation is recommended as a further testing method (Alahmar et al., 2022; Elbardisi et al., 2020; Okubo et al., 2023; Robinson et al., 2012; Simon et al., 2014). Previous studies have shown that sperm DNA damage is the only abnormality among the indicators of semen analysis in some male patients (Hazout et al., 2006; Saleh et al., 2002). Several reports underscore the fundamental significance of sperm DNA integrity in the efficacy of ART treatments (Alvarez et al., 2023) and subsequent development during infancy and even adulthood (Agarwal & Said, 2003; Farkouh et al., 2022; Majzoub et al., 2019). Sperm aberrant chromatin structure and SDF have been associated with male infertility, recurrent miscarriages, and unfavourable outcomes in ART treatments (Farkouh et al., 2022). Elevated levels of sperm DNA damage have been associated with a detrimental impact on blastocyst formation (Ni et al., 2014), decreased implantation and pregnancy rates, as well as increased rates of miscarriage (Jin et al., 2015).

There are a number of treatments suggested for men with sperm DNA damage. These include: environmental and lifestyle improvements; reduced abstinence; ejaculate fractionation; antioxidant therapy; varicocelectomy; advanced sperm processing/selection techniques and ICSI using testicular spermatozoa (Marinaro & Schlegel, 2023). One of the interventions advocated is testicular sperm extraction; however, there is limited evidence. It remains uncertain if in all instances testicular sperm exhibit lower SDF compared to ejaculated sperm. Indeed, most earlier studies have

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stated that regardless of whether the source of sperm for ICSI is from the testis or ejaculation (Bukulmez et al., 2001; Ghazzawi et al., 1998), ICSI outcomes were similar, and in particular, testicular sperm do not necessarily perform better than ejaculated sperm (Nagy et al., 1995). Other reports showed that the SDF is lower in testicular sperm than in ejaculated sperm (Esteves et al., 2017; Xie et al., 2020). The use of testicular sperm instead of ejaculated sperm in men with increased SDF improved embryonic outcomes (Greco et al., 2005; Tharakan et al., 2022).

Hence, we performed a further study to evaluate whether testicular spermatozoa rather than ejaculated spermatozoa should be used in cases of elevated SDF, we detail the embryologic, clinical, and neonatal outcomes of 73 (consisting of 37 testicular sperm and 36 ejaculated sperm cycles) consecutive cycles of ART treatment.

Material and methods

Ethical considerations

Ethical clearance was obtained from the Ethics Committee of the First Hospital Affiliated with Wenzhou Medical College (Reproductive Medicine Specialty Lun Review 2019 No. 11).

Patients

This retrospective study was conducted between January 2014 and December 2018 at the Reproductive Medicine Center, The First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China. In this study, cycles involving ICSI using testicular sperm extraction were designated as the 'T-group', while cycles employing ejaculated sperm were referred to as the 'E-group'. The inclusion criteria encompassed male patients exhibiting high ($\geq$25%) SDF index (DFI) (number of sperm with fragmented DNA/total sperm $\times$ 100), with no notable anomalies in their medical history, physical examination, or endocrinological profile, and no evidence of genital infection. We only included one ART treatment cycle per patient, using only fresh sperm and only fresh embryo transfer at day 3. Exclusion criteria were as follows: obstructive azoospermia, unresolved varicocele, testicular trauma, orchiectomy, chemotherapy, or pelvic radiotherapy, any genetic abnormalities.

Sperm retrieval

For ejaculated sperm, semen samples were acquired through masturbation following 2–5 days of sexual abstinence. After liquefaction, the samples were examined for concentration and motility according to the World Health Organization (WHO) laboratory manual for the examination and processing of human semen (World Health Organization, 2010). Each SCD assay was performed using the Halosperm kit (INDAS Laboratories) with a slight modification.

In cases of surgical testicular sperm retrieval, testicular tissue was procured via testicular biopsy surgery (fine needle tissue aspiration testicular biopsy; Devroey et al., 1995). A portion of the obtained sperm was designated for ICSI, while another portion was allocated for assessment of SDF.

Sperm chromatin dispersion (SCD) test

The sperm chromatin dispersion (SCD) test was used to evaluate DNA damage, as previously described (Jin et al., 2015). In the SCD test, spermatozoa featuring DNA fragments exhibited minimal or absent dispersed DNA halos, whereas those devoid of DNA fragments extended their DNA rings, forming expansive halos. DFI values exceeding 25% were classified as indicative of high levels of DNA fragmentation (Feijó & Esteves, 2014).

Stimulation, ICSI and embryo culture

Several controlled ovarian stimulation protocols are utilized in our centre: long, short, antagonist, and other protocols. The choice of stimulation protocol was based on the patient's age and ovarian function. The patients in this study were on GnRH antagonist protocol. 150–225 IU/d of recombinant human follicle-stimulating hormone (rFSH, Gonal-F, Merck Serono, Germany) was injected from the 2–4 day of the menstrual cycle, according to the patient's ovarian function, weight and age. The dose of Gn was adjusted according to follicular development and serum hormone levels. When the dominant follicle reached 12–14 mm in diameter, the GnRH antagonist 0.25 mg/d (Cetrotide, Merck Serono, Germany) was administered until the day of final oocyte maturation. Upon the attainment of at least one follicle with a diameter $\geq$ 18 mm, recombinant human chorionic gonadotropin (rHCG) was administered (Ovidrel, Merck Serono, Germany). Follicle retrieval was performed under ultrasound guidance. After a 1–4 h cultivation period, cumulus-oocyte complexes were denuded with

hyaluronic acid. ICSI procedures were conducted 1–3 hours thereafter (Tesarik & Sousa, 1995).

On Day 1, fertilization status was assessed, with successful fertilization defined by the presence of two pronuclei and two polar bodies and cultured in fertilation medium (G 1 PLUS, Vitrolife, Sweden). Cleavage embryo and blastocyst morphological grading followed previously described methods (Balaban et al., 2011; Gardner et al., 2000; Vandervorst et al., 1998). On day 3, 1–2 high-quality cleavage stage embryos (7–9 cells with $<$20% fragmentation) were either vitrified or transferred, while the remaining embryos underwent continued culture. Blastocysts with a morphological rating of $\geq$2BC on day 5 or day 6 were vitrified. Vitrification kit and thawing kit (Kitazato, Japan) were used for vitrifying or thawing. Frozen-thawed embryo transfer (FET) was carried out for patients who failed to transfer under fresh cycle until pregnancy is achieved or the embryos are exhausted.

Outcome measures

Detailed data on embryological (rates of fertilization, embryo cleavage, high-quality day-3 embryos, blastocyst formation), clinical (rates of implantation, clinical pregnancy, singletons, twins, ectopic pregnancy, miscarriage, ongoing pregnancy, cumulative pregnancy and live-birth) and newborn (rate of newborn, time of gestation, weight) are presented. Clinical pregnancy was defined as the presence of an intrauterine gestational sac exhibiting fetal cardiac activity, as verified through transvaginal ultrasound conducted at 6 weeks of gestation (Männer, 2022). Fetal losses before reaching 28 weeks of gestation were categorized as miscarriages. Cumulative clinical pregnancy rate was calculated as the number of clinical pregnancy/number of first oocyte retrieval cycles. The live birth rate was determined by dividing the number of delivery cycles by the number of cycles involving embryo transfers.

Results

A total of 73 couples, whose male partner exhibited increased SDF, were included. Sixteen couples (16/37) in the T-ICSI group could undergo fresh embryo transfer, and 23 couples (23/36) in the E-ICSI group could undergo fresh embryo transfer. With the development of ART technology, especially with vitrification, clinical outcomes of frozen embryos transfer cycles are similar to these of fresh embryos transfer cycles with reducing risk of OHSS. Therefore, the decision for fresh embryo transfer or freeze all was dependent on clinical requirement.

In T-ICSI group a total of 33 patients had frozen embryos; 24 patients (who did not obtain a clinical pregnancy in a fresh cycle) underwent a total of 34 FET cycles. In E-ICSI group (36 patients): a total of 22 patients had frozen embryos; follow-up was 18 patients for a total of 28 FET cycles. Evaluation of male patients encompassed the DFI for both ejaculated and testicular sperm. The DFI value for testicular sperm was significantly lower than that observed for ejaculated sperm (16.81 ± 17.51 vs. 56.96 ± 17.56, $p < 0.001$).

Analysis of treatment group characteristics (ages, duration of infertility, female BMI, AMH, basic semen analysis, testicular sperm DFI and ejaculated sperm DFI) showed no significant differences between groups T-ICSI and E-ICSI (Table 1).

Analysis of the characteristics of the treatment groups (Follicles, Endometrium, Oestradiol, Progesterone) showed no significant differences between the T-ICSI and E-ICSI groups (Table 2).

Analysis of embryologic and clinical outcomes only revealed significant differences regarding the blastocyst formation rate, with group T-ICSI exhibiting higher rates than group E-ICSI (Table 3). There was no difference in any of the perinatal outcomes between the two groups (Table 4).

Discussion

In this study, although the DNA fragmentation index (DFI) of testicular spermatozoa was significantly lower than that of ejaculated spermatozoa, however, there were no statistically significant differences between the two groups in terms of fertilization rate, high-quality embryo formation rate, clinical pregnancy rate, cumulative pregnancy rate, and live birth rate.

Table 1. Treatment group characteristics of groups T-ICSI and E-ICSI.

	T-group	E-group	*p* value
No. of cycles/patients	37	36	
Female age (years)	31.54 ± 4.69	31.05 ± 4.61	>0.05
Male age (years)	34.47 ± 5.32	33.18 ± 5.89	>0.05
Duration of infertility (years)	4.38 ± 3.51	3.86 ± 3.21	>0.05
BMI of women (kg/m^2)	20.40 ± 2.39	21.56 ± 2.88	>0.05
AMH	4.91 ± 2.26	4.10 ± 2.81	>0.05
Semen DFI (%)	60.58 ± 16.41	53.25 ± 18.15	>0.05
Testicular DFI (%)	13.37 ± 14.60	20.34 ± 19.64	>0.05
No of retrieved oocytes, n	14.86 ± 6.28	13.06 ± 5.20	>0.05
Mature oocytes (MII), n	12.32 ± 6.28	10.61 ± 4.81	>0.05
Sperm concentration (10^6/ml)	35.90(12.0,88.5)	13.95(3.8,73.7)	>0.05
Total sperm number (10^6)	68.74(26.2,243.1)	34.570(14.4,182.4)	>0.05
Sexual abstinence (days)	4.00(3.0,5.0)	4.000(3.3,5.0)	>0.05
Progressive motility (%)	9.60(1.8,22.3)	10.80(4.3,23.0)	>0.05

Table 2. Stimulation characteristics of groups T-ICSI and E-ICSI.

	T-group	E-group	*p* value
No. of cycles	37	36	
Follicles (mean, range)	18.43 ± 7.59	17.65 ± 4.94	>0.05
Gonadotropin stimulation			
Day of stimulation (mean)	10.44 ± 2.06	10.83 ± 1.80	>0.05
Total dose (mean)	1876.56 ± 393.22	1991.74 ± 713.48	>0.05
Endometrium (mean, range)	12.98 ± 2.28	12.35 ± 2.57	>0.05
Oestradiol (mean)	10392.06 ± 3825.52	8307.78 ± 4058.20	>0.05
Progesterone (mean)	2.78 ± 1.15	3.00 ± 1.42	>0.05

Table 3. Comparative embryologic and clinical outcomes between T-ICSI and E-ICSI.

	T-group	E-group	*P* value
Patients (n)	37	36	
Maturation rate (MII/COC) (%)	82.91%(456/550)	81.28%(382/470)	>0.05
Fertilization rate (2PN/MII) (%)	72.15%(329/456)	77.23%(295/382)	>0.05
Embryo cleavage rate (d2/2PN) (%)	96.66%(318/329)	97.63%(288/295)	>0.05
High-quality embryo rate (%)	47.17%(150/318)	46.53%(134/288)	>0.05
Blastocyst formation rate (%)	33.85%(66/195)	21.56%(36/167)	<0.05*
Embryo transfer cycles (*n*)	16	23	
Implantation rate (sacs/ET) (*n*, %)	40%(12/30)	34.78%(16/46)	>0.05
HCG positivity rate	62.50%(10/16)	60.87%(14/23)	>0.05
Clinical pregnancy rate (/ETC) (*n*, %)	50%(8/16)	56.52%(13/23)	>0.05
Singletons (/CP) (*n*, rate)	50%(4/8)	76.92%(10/13)	>0.05
Twins (/CP) (*n*, rate)	50%(4/8)	23.1%(3/13)	>0.05
Ectopic pregnancy (/CP) (*n*, rate)	0	0	
Miscarriage (/CP) (*n*, rate)	12.5%(1/8)	23.08%(3/13)	>0.05
No. of patients have embryo suitable for freezing in each group	33	22	
FET cycles	34(34/37)	28(28/36)	>0.05
Ongoing pregnancy (*n*, rate)	87.5%(7/8)	76.92%(10/13)	>0.05
Cumulative pregnancy rate (*n*, rate)	70.2%(26/37)	55.6%(20/36)	>0.05
Stillbirth (*n*, rate)	0	0	
Live birth delivery rate (/ETC) (delivery-stillbirth) (*n*, rate)	43.75%(7/16)	43.48%(10/23)	>0.05

Table 4. Characteristics of the newborn between T-ICSI and E-ICSI.

	T-group	E-group	*P* value
Patients (*n*)	37	36	
Embryo transfer cycles (ETC) (*n*)	16	23	
Newborn (/ETC) (*n*, rate)	62.5%(10/16)	56.52%(13/23)	>0.05
Male (/NB) (*n*, rate)	30%(3/10)	30.77%(4/13)	>0.05
Female (/NB) (*n*, rate)	70%(7/10)	69.23%(9/13)	>0.05
M/F ratio	3/7	4/9	
F/M ratio	7/3	9/4	
NB malformations (/NB) (*n*, rate)	0	0	
Gestation age (weeks) (*n*, mean, range)	37.99 ± 1.00	37.51 ± 2.68	>0.05
Term (*n*, mean, rate)	100%(10/10)	69.23%(9/13)	>0.05
Preterm (PT) (*n*, mean, rate)	0	30.77%(4/13)	
Very PT (*n*, mean, rate)	0	0	
Weight (g) (*n*, mean)	2677.00 ± 309.05	3007.69 ± 682.74	>0.05
Normal weight (*n*, mean, rate)	70%(7/10)	84.62%(11/13)	>0.05
Low weight (LW) (*n*, mean, rate)	30%(3/10)	15.38%(2/13)	>0.05
Very LbW (*n*, mean, rate)	0	0	

Testicular spermatozoa had no advantage over ejaculated spermatozoa except in blastocyst formation rate for patients with high SDF.

Certain limitations must be acknowledged in the context of this study. Primarily, the study's retrospective nature and the absence of randomization introduce the potential for selection bias. While baseline data were similar between the two groups, the small sample size renders complete elimination of selection bias uncertain. Second, the study was conducted within a single-centre setting, necessitating broader multi-centre investigations to corroborate our findings.

A previous meta-analysis has corroborated that SDF levels are typically lower within the testes compared to the semen (Esteves et al., 2017). This concurs with our findings, which revealed a marked distinction between the SDF levels of testicular spermatozoa (16.81 ± 17.51) and ejaculated spermatozoa (56.96 ± 17.56), signifying significantly reduced SDF in testicular spermatozoa.

Existing reports have illuminated the association between increased SDF levels and decreased rates of

fertilization embryo quality and pregnancy, and increased rates of miscarriage (Evgeni et al., 2023; Loloi et al., 2022; Tharakan et al., 2022; Zhang et al., 2021). Notably, studies have revealed that testicular sperm tend to possess lower SDF levels than their ejaculated counterparts among patients with elevated SDF in ejaculated sperm samples (Greco et al., 2005; Moskovtsev et al., 2012). In 2005, Greco pioneered the use of testicular sperm in the treatment of men with elevated SDF levels. While no direct correlation was observed between the fertilization rate and SDF, testicular sperm was associated with significant higher pregnancy rates (44.4%) compared to ejaculated sperm (5.6%). Similarly, another study involving patients with increased SDF demonstrated that despite comparable fertilization and implantation rates, clinical pregnancy rates were significantly elevated with the application of testicular sperm (41.9%) as opposed to ejaculated sperm (20%) (Pabuccu et al., 2017). Our own study outcomes do not reveal any significant impact of employing testicular sperm on fertilization rates, embryo quality, or clinical pregnancy rates when juxtaposed with ejaculated sperm.

The observed absence of a robust correlation between SDF (sperm DNA fragmentation) and the clinical pregnancy rate might be attributed to two pivotal factors. First, during intracytoplasmic sperm injection (ICSI), embryos that are fertilized by spermatozoa with fragmented DNA might be systematically excluded from selection for transfer. This selective process potentially diminishes the predictive utility of the SDF. In contrast, a myriad of research investigations conducted on animal models—where such selective transfer is absent—have consistently demonstrated a profound impact of SDF on pregnancy outcomes (Gosálvez et al., 2014; Johnston et al., 2016). Second, an often overlooked yet critical consideration is the temporal variability associated with determining the SDF. The extent of sperm DNA fragmentation may be influenced by the elapsed time post-ejaculation. This temporal factor might indeed provide a rationale for the inconclusive findings observed in a previous IUI (intrauterine insemination) study (Muriel et al., 2006) as well as in various other studies employing diverse SDF assessment methodologies. SDF is a dynamic process, and iatrogenic DNA damage can exacerbate during prolonged in-vitro incubation preceding ART (Assisted Reproductive Technologies). This progression is contingent on the specific laboratory conditions and the meticulous processing of individual samples. In the absence of standardized and controlled conditions, the reliability and relevance of SDF evaluations could be compromised, rendering the assessments of limited clinical significance.

The use of testicular spermatozoa has no additional advantage over ejaculated spermatozoa except for blastocyst quality in patients with high SDF, for men with a sufficient number of available ejaculated sperm, clinicians should not advocate the direct use of testicular sperm in the ICSI treatment.

We strongly recommend good quality RCT with long-term follow-up of infants born after intracytoplasmic sperm injection (ICSI) treatment using testicular spermatozoa in case of high sperm DNA fragmentation compared to use of ejaculated spermatozoa.

Conclusion

While there is biological plausibility that testicular sperm may be useful in those with high DNA fragmentation, this needs to be proven in adequately powered and appropriately designed studies. The use of testicular spermatozoa for the first ICSI cycle in male infertility patients with high SDF should be carried out after comprehensive consideration.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical statement

This study was reviewed and approved by the reproductive ethics committee of the authors' hospital. Reproductive Medicine Professional Ethics Committee Review (2019) No. (11).

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