
Does Sperm DNA Fragmentation test in Cases of Male Factor Infertility Improve ICSI outcome?

Elham Hussien Madny M.D.
Department of Obstetrics
and Gynecology, Suez Canal
University, Egypt

Abstract

Background: Sperm DNA fragmentation is an uprising factor affecting male fertility and some previous results have shown significant effect of SDF on ART techniques outcomes.

Objective: To study effect of DNA fragmentation testing and treatment on ICSI outcome among infertile couples with male factor infertility.

Patients and Method: After approval of ethics committee, prospective control randomized study was conducted among a total of 106 infertile couples arranged for intracytoplasmic sperm injection (ICSI) due to male factor. Males were divided by simple random allocation into 2 groups. **Group I** included 53 males that were tested for DNA fragmentation test (Halo test). **Group II** included 53 males that were directly offered ICSI cycle. Infertile couples were evaluated as regard to fertilization rate, day 5 embryo transfer, positive pregnancy test, clinical pregnancy evaluated by visible sac with viable fetus on ultrasonographic assessment and delivery of normal viable baby.

Results: Men with initially negative DNA fragmentation test and those with positive DNA fragmentation test who responded well to treatment were found to have higher fertilization ratio of M2 ova, day 3 grade I embryo, blastocyte, positive pregnancy test and positive clinical pregnancy compared to non responders to treatment. There was no statistically significant difference between testicular and ejaculated sperm ICSI outcome among males with persistent sperm DNA fragmentation test.

Conclusion: Sperm DNA fragmentation is an important factor that significantly affects ICSI outcome. None of testicular or ejaculated sperm is superior regarding ICSI outcome among males with DNA fragmentation.

Keywords: assisted reproduction, fertility, azospermia, oligospermia.

INTRODUCTION

Infertility is a common problem affecting about 15% of couples attempting to conceive **(1)**. Up to one third of infertile cases are in part related to male factors **(2)**. Functional sperm DNA is essential for normal embryo development as the genetic information passed on to the next generation depends on sperm DNA integrity **(3)**.

Corresponding author:

Elham Hussien Madny
Tel.No: 01000552837
E-mail: elham.madny@yahoo.
com

Sperm DNA fragmentation (SDF) has emerged as potential biomarker in the assessment of male fertility. About 25% of infertile men have SDF levels higher than that found in fertile men (4, 5). SDF has been also found to be associated with early poor pregnancy outcome (recurrent idiopathic miscarriage) (6). Sperm DNA fragmentation may occur through either defective chromatin condensation that may occur during spermiogenesis; apoptosis that may occur during spermatogenesis; and oxidative stress may occur during the transit period through the male genital tract (7, 8).

Outcome of assisted reproductive techniques (ART) has been found to be affected by presence of SDF (9). Although sperm with fragmented DNA may fertilize an egg with apparently similar efficiency as sperm without DNA fragmentation, the negative impact of a damaged paternal chromatin to the integrity of embryonic genome is usually observed after implantation. This type of damage is often manifested by early pregnancy loss (10).

The Practice Committee of the American Society for Reproductive Medicine (ASRM) has stated that sperm DNA fragmentation testing might be clinically informative for in vitro fertilisation/intracytoplasmic sperm injection (IVF/ICSI) outcomes (11). The threshold of DNA fragmentation index (DFI) is quite important, as lower rates (<30%) have been strongly related to natural conception and success in intrauterine insemination (IUI), whereas higher rates (>30%) have been linked with decreased pregnancy odds in IVF (12).

Several strategies have been tried in order to overcome the presence of SDF in couples undergoing ART including varicocele repair (13), oral antioxidant therapy (14), short ejaculatory abstinence periods (15) and recurrent ejaculations (16), and laboratory sperm selection techniques such as magnetic cell sorting (MACS) (17), physiological intracytoplasmic sperm injection (PICSI) (18), and intracytoplasmic morphologically selected sperm injection (IMSI) (19).

The use of testicular rather than ejaculated sperm – with either testicular sperm aspiration (TESA) or testicular sperm extraction (TESE) – for intracytoplasmic sperm injection (ICSI) among men with high SDF was found to be associated with better pregnancy outcome (20,21). Current study was de-

signed aiming to study effect of DNA fragmentation testing and treatment on ICSI outcome among infertile couples with male factor infertility.

Patients and methods

After approval of ethics committee of Faculty of Medicine, Suez Canal University, the present prospective control randomized study was conducted among a total of 106 infertile couples arranged for intracytoplasmic sperm injection (ICSI) due to male factor. Couples age < 32 years and BMI 20 – 25 Kg/m² were included into the study. Female factor was assessed through hysterosalpingogram (HSG), 3D vaginal ultrasound, routine office hysteroscopy, and laboratory assessment. Couples were included if HSG showed normal uterine cavity and patent tubes, 3D vaginal U/S showed normal cavity, normal office hysteroscopy, and normal T3, TSH, prolactin and normal FSH/LH ratio in 3rd day of cycle. Semen analysis of males showed severe oligospermia and asthenoteratospermia.

Studied 106 males were divided by simple random allocation into 2 groups.

Group I included 53 males that were tested for DNA fragmentation test (Halo test).

Group II included 53 males that were directly offered ICSI cycle.

DNA fragmentation test:

DNA fragmentation was tested via halo test using Halosperm test kit. This kit determines the degree of DNA damage of a human spermatozoon through a process called sperm chromatin dispersion (SCD), which is responsible for male infertility. This process “involves the denaturation and controlled lysis of the sample in an appropriate medium and can be used with both fresh and frozen samples. Spermatozoa with intact DNA produce a dispersion halo as a result of the chromatin released from proteins that can be easily analysed using fluorescence or bright field microscopy. In contrast, spermatozoa with fragmented DNA will not produce this halo. The technique is as easy as a routine leucocyte count” (22). SDF level cut-off taken as high was SDF ≥ 30% using Fernandez protocol (23).

Twenty five cases of group I subjected to DNA fragmentation test were found to have negative halo test and were offered ICSI cycle (**group Ix**). Remain-

ing 28 cases of group I who showed positive halo test were offered 3 months of treatment receiving L-carnitine, vitamin E capsules daily and instructed to avoid smoking and exposure to toxins and then DNA fragmentation test was repeated after 3 months.

Post treatment DNA fragmentation test was negative among 16 of 28 treated cases (**Group Ia**) and remain positive among 12 of 28 cases (**Group Ib**). Those cases with persistent positive halo test were offered Testicular sperm extraction by TESE or fine needle aspiration for sperm to avoid oxidative free radicals in epididymis that may cause DNA fragmentation. Eight cases have refused testicular extraction and offered ICSI with ejaculate (**Group Ib1**) while four cases have been subjected to testicular extraction (**Group Ib2**).

ICSI protocol:

All cases were finally arranged to ICSI cycle using long antagonist protocol with triptorelin (decapetyl®) 0.1 subcutaneous from day 21 of the previous cycle and human menopausal gonadotropin (Fastimon®) 300 IU at second day of the cycle. Ovarian response was evaluated by serial transvaginal folliculometry and serum E2. The dose of therapeutic regimen was adjusted according to the ovarian response.

When target follicular diameter of 3 or more follicles reached 18-21 mm. HCG 1000 IU was administered and ovum pick up was done after 34-36 hours. After embryo transfer, all cases were administered luteal phase progesterone support, folic acid supplement and low dose aspirin.

Outcome measures:

Infertile couples were evaluated as regard to fertilization rate, day 5 embryo transfer, positive pregnancy test, clinical pregnancy evaluated by visible sac with viable fetus on ultrasonographic assessment and delivery of normal viable baby.

Statistical analysis:

Gathered information was processed using SPSS version 25 (SPSS Inc., chiago, IL, USA.). Quantitative data was expressed as means \pm SD while qualitative data was expressed as number and percentages (%). Unpaired t test was used to test significance of difference for quantitative variables and chi square was used to test significance of difference for qualitative variables. A probability value (p-value) <0.05 was considered statistically significant.

Results

There was no statistically significant difference between both groups of the study regarding women age, BMI, number of aspirated follicles and E2 level (**Table 1**).

Men with initially negative DNA fragmentation test and those with positive DNA fragmentation test who responded well to treatment were found to have higher fertilization ratio of M2 ova, day 3 grade I embryo, blastocyte, positive pregnancy test and positive clinical pregnancy compared to non responders to treatment. Couples who didn't perform DNA fragmentation test have no statistically significant difference compared to men with initially negative DNA fragmentation test and those with positive DNA fragmentation test who responded well to treatment regarding positive pregnancy test and clinical pregnancy while take home baby was more evident among latter groups with statistically significant difference (**Table 2**).

Table 1: Baseline characteristics among the studied patients:

Type		Group I (n=53)	Group II (n=53)	p-value
Women Age	Mean \pm SD	29.1 \pm 2.7	28.9 \pm 3.2	0.7 (NS)
	Range	25 – 32	25 – 32	
Women BMI	Mean \pm SD	24.8 \pm 3.1	24.9 \pm 2.7	0.8 (NS)
	Range	20 – 25	21 – 25	
Number of aspirated follicles	Mean \pm SD	9.3 \pm 4.3	9.1 \pm 4.8	0.8 (NS)
	Range	4 – 15	4 – 17	
E2 level	Mean \pm SD	1800.9 \pm 453.8	1850.2 \pm 604.9	0.6 (NS)
		1100 – 2300	1000 - 2600	

Table 2: Outcome parameters:

	Group IX (n=25)	Group Ia (n=16)	Group Ib (n=12)		Group II (n=53)	p-value
			Group Ib1 (n=8)	Group Ib2 (n=4)		
Fertilization ratio of M2 ova	20 (80%)a	14 (87.5%)a	2 (25%)b	3 (75%)ab	32 (60.4%)ab	0.001
Day 3 grade I embryo	5.2 ± 0.9a	5 ± 0.8a	2 ± 0.13b	4 ± 0.8c	5 ± 0.7a	0.001
Blastocyte	4 ± 1.06a	4.5 ± 0.9a	0.5 ± 0.08b	2 ± 0.7c	3 ± 0.9a	0.001
Positive pregnancy test	13 (52%)a	9 (56.25%)a	1 (12.5%)b	2 (50%)b	20 (37.7%)ab	0.001*
Positive clinical pregnancy	12 (48%)a	8 (50%)a	1 (12.5%)b	2 (50%)a	18 (33.9%)ab	0.001*
Take baby home	8 (32%)a	7 (43.75%)a	0 (0%)b	1 (25%)b	9 (16.9%)b	0.001*

GIx: men with initially negative DNA fragmentation test

GIA: Halo test became -ve after treatment

GIIb: Halo test remain +ve after treatment

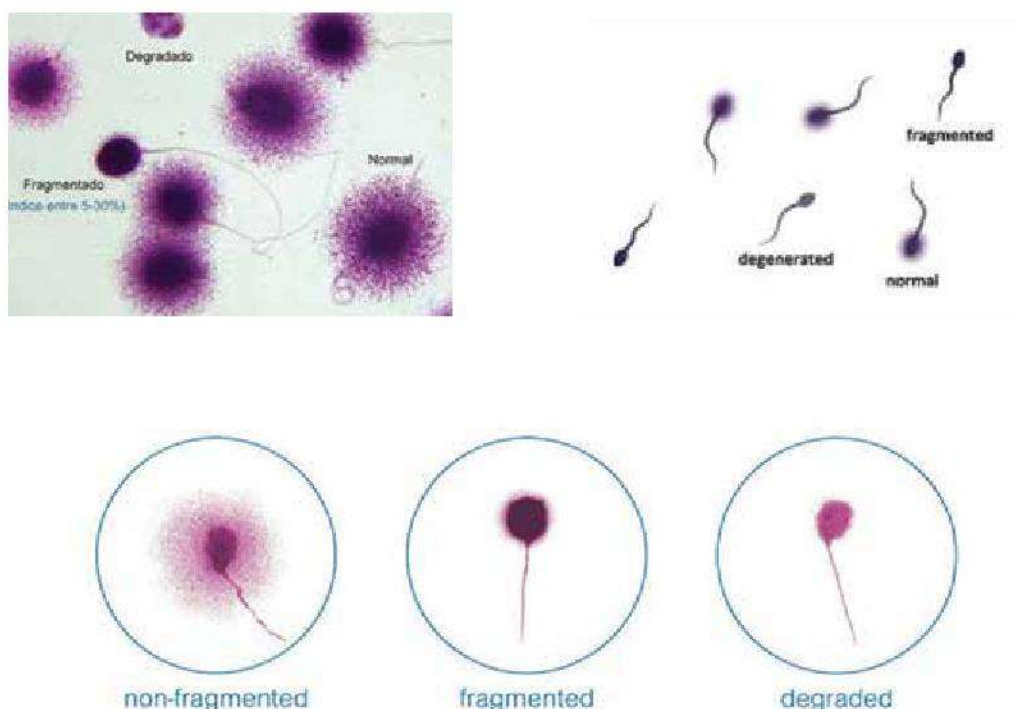
GIIb1: refused TESE and offered ICSI with ejaculate

GIIb2: subjected to testicular extraction

G2: offered ICSI directly with DNA fragmentation test

*statistically significant difference

a, b, c denote statistically significant within groups

Figure 1: Microscopic sperm morphology with DNA fragmentation

Discussion

The impact of DNA fragmentation as an important factor on male fertility and even pregnancy outcome is getting more attention recently. Studying DFI aims to add more prognostic information and planning to guide couples particularly those with repeated failure of ART attempts. The current study investigated burden of sperm DNA fragmentation on OCSI outcome and effectiveness of testicular versus ejaculated sperm on ICSI outcome among men with sperm DNA fragmentation.

Although many studies suggested that DNA fragmentation is an important indicator of successful fertilization (2), others failed to find an effect for DNA fragmentation on fertilization rate claiming that fertilization is independent of the level of the DNA damage in spermatozoa (24-26). The current study have shown higher fertilization rate, better embryo quality, higher rates of biochemical and clinical pregnancy and successful pregnancy outcome among couples with DNA fragmentation versus couples with DNA fragmentation. Successful treatment of DNA fragmentation has been found to significantly improve ICSI outcome among those couples.

Because the surgical extraction of sperm was adapted in ICSI, numerous studies have investigated the effect of sperm origin on ICSI outcomes. These studies have reported controversial results, with every study providing probable theories to support its findings. Some evidence suggested that ejaculated sperm should lead to a better outcome due to a crucial role of epididymis in the final steps of spermatogenesis, including epigenetic modification of genes (27, 28), changes in the surface proteins of spermatozoa (29), and maturation of sperm cells (30). Some epigenetic remodelling processes is necessary for the stability of DNA and its resistance to damage and have been reported to play a crucial role in early embryogenesis (31-35). Testicular sperm does not undergo these modification processes in the epididymis, and that was the explanation provided by many researchers shown higher normal fertilisation rates, implantation rates, and pregnancy rates (36 – 38), and lower abortion rates in ICSI cycles using epididymal sperm than those using testicular sperm (39, 40).

However, some studies have shown no difference in outcomes of ICSI with testicular and epididymal sperm or with testicular and ejaculated sperm (41, 42) as well as current study. Some studies have even shown better fertility outcomes with testicular sperm than with ejaculated sperm (27, 43, 44). These studies concluded that sperm selection during ICSI, significantly reduce the effect of epididymal transport on spermatogenesis and sperm quality (42, 45). Damage to the sperm DNA along the genital tract might further explain why testicular sperm leads to better fertility outcomes than does ejaculated sperm. In the ICSI outcomes, studies have suggested that damage to sperm DNA may lead to impaired sperm decondensation, which reduces fertilisation rate, produces low-quality embryos, leads to implantation failure, and causes recurrent pregnancy loss (46).

With persistent sperm DNA fragmentation despite treatment, current study has shown that there was no statistically significant difference between testicular versus ejaculated sperm regarding fertilization ratio, pregnancy rate and even successful pregnancy outcome and that only embryo quality was better with testicular sperm extraction. These findings are inconsistent with recent results by Arafa and colleagues (1). Although they found that there was no difference in the fertilization rate using ejaculate and testicular spermatozoa, clinical pregnancy was significantly higher in TESA group compared to ejaculated group. Moreover, 17 live births were documented in TESA group, and only three live births were documented in ejaculate group (1). Consistent with current findings; earlier studies showed no correlation between ICSI outcome and fertilization rate (24, 26, 47).

Arafa and colleagues (1) ascribed their findings to the fact that spermatozoa obtained from the testis exhibit lower-DNA fragmentation and as such are more likely to undergo implantation (48, 49). Furthermore, it can also be justified by results from other studies, which showed a negative correlation between clinical pregnancy and live-birth rate and high-sperm DNA damage (49-51). Inconsistent findings with current study could be explained by the fact that Arafa and co-workers have investigated only men with high DNA fragmentation index that can cause to lower pregnancy outcome with ejaculated sperm.

Inconsistent with current findings, **Greco et al. (49)** have reported a higher percentage of clinical pregnancy in testicular spermatozoa compared to ejaculate (44.4% and 5.6% respectively). Similarly, **Esteves et al., (50)** recently examined ICSI outcomes using testicular versus ejaculate spermatozoa in patients with high % of SDF. They detected higher % SDF in ejaculate spermatozoa compared to testicular spermatozoa and a significant difference in the outcomes among the two groups. Reported results showed significantly higher clinical pregnancy and live-birth rates in the testicular ICSI group (52% and 47% respectively) in comparison with the ejaculate ICSI group (40% and 26% respectively).

Testicular sperm was found to be superior to ejaculated sperm in other many studies. **Pabuccu et al., (52)** found that ICSI using testicular spermatozoa obtained by TESA seems an effective option particularly for those with repeated ART failures in terms of clinical, ongoing pregnancies and miscarriages even though conventional sperm parameters are within normal range. Similarly, **Esteves et al., (53)** have shown that testicular sperm have lower levels of SDF than ejaculated sperm, with testicular sperm for high post-testicular SDF men improving ICSI outcomes compared with ejaculated sperm.

In their recent meta-analysis and systematic review; **Kang et al., (46)** showed that the risk ratios favour fresh testicular sperm for good quality embryo rate (1.17, 95% CI 1.05–1.30, $P = 0.005$), implantation rate (95% CI 1.02–2.26, $P = 0.04$), and pregnancy rate (RR = 1.74, 95% CI 1.20–2.52, $P = 0.004$). Inconsistently with current study, they concluded that the existing evidence suggests that testicular sperm is better than ejaculated sperm for ICSI in male with cryptozoospermia. Another systematic review supported our current findings and indicated that there is no difference between testicular sperm group and ejaculated sperm group with forest plots for pregnancy rate (OR = 0.53, 95% CI 0.19–1.42, $P = 0.21$, $I^2 = 67\%$) and fertilisation rate (OR = 0.91, 95% CI 0.78–1.06, $P = 0.21$, $I^2 = 73\%$) **(54)**.

Limitation of our study: The main limitation of current study is relatively small sample size.

Conflicts of interest. None of authors have any conflict of interests

Conclusion and recommendations: Sperm DNA fragmentation is an important factor that significantly affects ICSI outcome and should be considered and adequately treated as well as it still requires further in depth investigation. Although current study has shown no significant difference between testicular and ejaculated sperm regarding ICSI outcome, divergent reports from previous studies showed that it is still controversial whether to obtain testicular sperm for better ICSI outcomes or not.

References

1. Arafa M, AlMalki A, AlBadr M, Burjaq H, Majzoub A, AlSaid S, Elbardisi H. ICSI outcome in patients with high DNA fragmentation: Testicular versus ejaculated spermatozoa. *Andrologia*. 2018;50(1): 1 – 6.
2. Simon, L., Lutton, D., McManus, J., & Lewis, S.. Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertility and Sterility*. 2011; 95(2), 652–657.
3. Gosalvez J, Lopez-Fernandez C, Fernandez JL, Esteves SC, Johnston SD. Unpacking the mysteries of sperm DNA fragmentation: ten frequently asked questions. *J Reprod Biotechnol Fertil* 2015; 4: 1–16.
4. Moustafa, M. H., Sharma, R. K., Thomson, J., Mascha, E., Abdel-Hafez, M. A., Thomas, A. J. Jr, & Agarwal, A. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Human Reproduction*. 2004; 19(1), 129–138.
5. Zini, A., Boman, J., Belzile, E., & Ciampi, A. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: Systematic review and meta-analysis. *Human Reproduction*. 2008; 23(12), 2663–2668.
6. Simon, L., Proutski, I., Stevenson, M., Jennings, D., McManus, J., Lutton, D., & Lewis, S. Sperm DNA damage has a negative association with live-birth rates after IVF. *Reproductive Biomedicine Online*. 2013; 26(1), 68–78.
7. Muratori M, Tamburrino L, Marchiani S, Cambi M, Olivito B, Azzari C, et al. Investigation on the origin of sperm DNA fragmentation: role of apoptosis, immaturity and oxidative stress. *Mol Med* 2015;21:109-22.

8. Chi HJ, Kim SG, Kim YY, Park JY, Yoo CS, Park IH, Sun HG, Kim JW, Lee KH, Park HD. ICSI significantly improved the pregnancy rate of patients with a high sperm DNA fragmentation index. *Clin Exp Reprod Med* 2017;44(3):132-140.
9. Tesarik J, Greco E, Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* 2004; 19: 611–5.
10. Esteves SC, Roque M, Garrido M. Use of testicular sperm for intracytoplasmic sperm injection in men with high sperm DNA fragmentation: a SWOT analysis. *Asian Journal of Andrology*. 2018; 20: 1–8
11. Practice Committee of the American Society for Reproductive Medicine. Diagnostic evaluation of the infertile male: a committee opinion. *Fertil Steril*. 2015; 103:e18–e25.
12. Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/ intracytoplasmic sperm injection: a systematic review and meta-analysis. *Fertil Steril*. 2014; 102:998–1005.
13. Wang YJ, Zhang RQ, Lin YJ, Zhang RG, Zhang WL. Relationship between varicocele and sperm DNA damage and the effect of varicocele repair: a meta-analysis. *Reprod Biomed Online* 2012; 25: 307–14.
14. Zini A, San Gabriel M, Baazeem A. Antioxidants and sperm DNA damage: a clinical perspective. *J Assist Reprod Genet* 2009; 26: 427–32.
15. Agarwal A, Gupta S, Du Plessis S, Sharma R, Esteves SC, et al. Abstinence time and its impact on basic and advanced semen parameters. *Urology* 2016; 94: 102–10.
16. Gosálvez J, Gonzalez-Martinez M, Lopez-Fernandez C, Fernandez JL, Sanchez-Martin P. Shorter abstinence decreases sperm deoxyribonucleic acid fragmentation in ejaculate. *Fertil Steril* 2011; 96: 1083–6.
17. Said TM, Grunewald S, Paasch U, Rasch M, Agarwal A, et al. Advantage of combining magnetic cell separation with sperm preparation techniques. *Reprod Biomed Online* 2005; 10: 740–6.
18. Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Taraborrelli S, et al. Comparison of two ready-to-use systems designed for sperm-hyaluronic acid binding selection before intracytoplasmic sperm injection: PICSi vs. sperm slow: a prospective, randomized trial. *Fertil Steril* 2012; 98: 632–7.
19. Antinori M, Licata E, Dani G, Cerusico F, Versaci C, et al. Intracytoplasmic morphologically selected sperm injection: a prospective randomized trial. *Reprod Biomed Online* 2008; 16: 835–41.
20. Esteves SC, Sanchez-Martin F, Sanchez-Martin P, Schneider DT, Gosálvez J. Comparison of reproductive outcome in oligozoospermic men with high sperm DNA fragmentation undergoing intracytoplasmic sperm injection with ejaculated and testicular sperm. *Fertil Steril* 2015; 104: 1398–405.
21. Mehta A, Bolyakov A, Schlegel PN, Paduch DA. Higher pregnancy rates using testicular sperm in men with severe oligospermia. *Fertil Steril* 2015; 104: 1382–7.
22. Chohan, K. R., Griffin, J. T., Lafromboise, M., De Jonge, C. J., & Carrell, D. T. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *Journal of Andrology*. 2006; 27(1), 53–59.
23. Fernandez, J. L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., De Jonge, C. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion (SCD) test. *Fertility and Sterility*. 2005; 84(4), 833–842.
24. Anfandis, G., Bounartzi, T., Messini, C. I., Dafopoulos, K., Markandona, R., Sotiriou, S., Messinis, I. E. Sperm DNA fragmentation measured by Halosperm does not impact on embryo quality and ongoing pregnancy rates in IVF/ICSI treatments. *Andrologia*. 2014; 47: 295–302.
25. Esbert, M., Pacheco, A., Vidal, F., Florensa, M., Riqueros, M., Ballesteros, A. Calderón, G.. Impact of sperm DNA fragmentation on the outcome of IVF with own or donated oocytes. *Reproductive Biomedicine Online*. 2011; 23(6): 704–710.
26. Gandini, L., Lombardo, F., Paoli, D., Caruso, F., Eleuteri, P., Leter, G., Spano, M. Full-term

- pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Human Reproduction*. 2004; 19(6), 1409–1417.
27. Ariel, M., Cedar, H. & McCarrey, J. Developmental changes in methylation of spermatogenesis-specific genes include reprogramming in the epididymis. *Nature genetics*. 1994; 7: 59–63.
 28. Nakanishi, Y. Study on the mammalian spermatogenic pathway. *Yakugaku zasshi: Journal of the Pharmaceutical Society of Japan*. 1995; 115: 420–430.
 29. Dacheux, J. L. et al. Role of epididymal secretory proteins in sperm maturation with particular reference to the boar. *Journal of reproduction and fertility. Supplement*. 1998; 53: 99–107.
 30. Sullivan, R. & Mieusset, R. The human epididymis: its function in sperm maturation. *Human reproduction update*. 2016; 22: 574–587.
 31. Bedford, J. M., Bent, M. J. & Calvin, H. Variations in the structural character and stability of the nuclear chromatin in morphologically normal human spermatozoa. *Journal of reproduction and fertility*. 1973; 33: 19–29.
 32. Bianchi, P. G., Manicardi, G. C., Bizzaro, D., Bianchi, U. & Sakkas, D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. *Biology of reproduction*. 1993; 49: 1083–1088.
 33. Carrell, D. T. & Hammoud, S. S. The human sperm epigenome and its potential role in embryonic development. *Molecular human reproduction*. 2010; 16: 37–47.
 34. Roux, C., Tripogney, C., Joanne, C. & Bresson, J. L. Nuclear quality of the spermatozoon: exploration tests of the chromatin of human spermatozoa (nuclear proteins). *Gynecologie, obstetrique & fertilité*. 2004; 32: 792–798.
 35. Shaoqin, G., Zhenghui, Z., Xueqian, Z. & Yuan, H. Epigenetic modifications in human spermatozoon and its potential role in embryonic development. *Yi chuan = Hereditas*. 2014; 36: 439–446.
 36. Aboulghar, M. A. et al. Fertilization and pregnancy rates after intracytoplasmic sperm injection using ejaculate semen and surgically retrieved sperm. *Fertility and sterility*. 1997; 68: 108–111.
 37. Hameed, N. & Ozturk, O. Testicular versus epididymal spermatozoa in intracytoplasmic sperm injection treatment cycles. *Journal of Ayub Medical College, Abbottabad: JAMC*. 2010; 22: 159–163.
 38. Mansour, R. T. et al. Intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia. *Human reproduction (Oxford, England)*. 1997; 12: 1974–1979.
 39. Ghazzawi, I. M., Sarraf, M. G., Taher, M. R. & Khalifa, F. A. Comparison of the fertilizing capability of spermatozoa from ejaculates, epididymal aspirates and testicular biopsies using intracytoplasmic sperm injection. *Human Reproduction*. 1998; 13: 348–352.
 40. Pasqualotto, F. F., Rossi-Ferragut, L. M., Rocha, C. C., Iaconelli, A. Jr. & Borges, E. Jr. Outcome of in vitro fertilization and intracytoplasmic injection of epididymal and testicular sperm obtained from patients with obstructive and nonobstructive azoospermia. *The Journal of urology*. 2002; 167: 1753–1756.
 41. Hourvitz, A. et al. In vitro fertilization treatment for severe male factor: a comparative study of intracytoplasmic sperm injection with testicular sperm extraction and with spermatozoa from ejaculate. *Journal of assisted reproduction and genetics*. 1998; 15: 386–389.
 42. Silber, S. J., Devroey, P., Tournaye, H. & Van Steirteghem, A. C. Fertilizing capacity of epididymal and testicular sperm using intracytoplasmic sperm injection (ICSI). *Reproduction, fertility, and development*. 1995; 7: 281–292; discussion 292–283.
 43. Weissman, A. et al. Pregnancies and live births following ICSI with testicular spermatozoa after repeated implantation failure using ejaculated spermatozoa. *Reproductive biomedicine online*. 2008; 17: 605–609.
 44. Negri, L. et al. ICSI outcome is significantly better with testicular spermatozoa in patients with necrozoospermia: a retrospective study. *Gynecological endocrinology: the official journal of the International Society of Gynecological Endocrinology*. 2014; 30: 48–52.

45. Amirjannati, N. et al. Comparison of intracytoplasmic sperm injection outcomes between spermatozoa retrieved from testicular biopsy and from ejaculation in cryptozoospermic men. *Andrologia*. 2012; 44: 704–709.
46. Kang Y, Hsiao Y, Chen C, Wu, C. Testicular sperm is superior to ejaculated sperm for ICSI in cryptozoospermia: An update systematic review and metaanalysis. *Scientific Reports*. 2018; 8:7874
47. Agarwal, A., & Allamaneni, S. S. R. The effect of sperm DNA damage on assisted reproduction outcomes. *Minerva Ginecologica*. 2004; 56: 235–245.
48. Benchaib, M., Braun, V., Lornage, J., Hadj, S., Salle, B., Lejeune, H., & Guerin, J. F. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Human Reproduction*. 2003 18(5): 1023–1028.
49. Greco, E., Scarselli, F., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S., Tesarik, J. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. *Human Reproduction*. 2005; 20(1): 226–230.
50. Esteves, S. C., Sánchez-Martín, F., Sánchez-Martín, P., Schneider, D. T., & Gosálvez, J. Comparison of Reproductive outcome in oligozoospermic men with high sperm DNA fragmentation undergoing intracytoplasmic sperm injection with ejaculated and testicular sperm. *Fertility and Sterility*. 2016; 104(6): 1398–1405.
51. Moskovtsev, S., Jarvi, K., Mullen, B., Cadesky, K. I., Hannam, T., & Lo, K. C. Testicular spermatozoa have statistically significantly lower DNA damage compared with ejaculated spermatozoa in patients with unsuccessful oral antioxidant treatment. *Fertility and Sterility*. 2010; 93(4): 1142–1146.
52. Pabuccu E, Caglar G, Tangal S, Haliloglu A, Pabuccu R. Testicular versus ejaculated spermatozoa in ICSI cycles of normozoospermic men with high sperm DNA fragmentation and previous ART failures. *Andrologia*. 2017, 49, e12609
53. Esteves SC, Roque M, Bradley C, Garrido N. Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: systematic review and meta-analysis. *Fertil Steril*. 2017;108:456–67
54. Abhyankar, N., Kathrins, M. & Niederberger, C. Use of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with cryptozoospermia: a meta-analysis. *Fertility and sterility*. 2016; 105: 1469–1475.e1461,