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Whether the Effect of Sperm DNA Fragmentation on the Clinical and Laboratory Outcomes Changes with the Paternal Age in the Intracytoplasmic Sperm Injection Cycles of Donor Oocytes

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ABSTRACT

OBJECTIVE: The present study aimed to know the effect of paternal age together with sperm DNA fragmentation on the clinical and laboratory outcomes in the donor oocyte intracytoplasmic sperm injection cycles cycles.

STUDY DESIGN: In this prospective cohort study a total of 229 patients undergoing their first intracytoplasmic sperm injection cycles and fresh blastocyst embryo transfer with donor oocytes were included in this study. All the patients of the donor oocyte intracytoplasmic sperm injection cycles were categorized into four groups based on the male age and sperm DNA fragmentation. I. Y group (young, male age <40 years) with low sperm DNA fragmentation (≤30%), II. Y group (young, male age <40 years) with high sperm DNA fragmentation (>30%), III. advanced paternal age group (male age ≥40 years) with low sperm DNA fragmentation (≤30%), and IV. advanced paternal age group (male age ≥40 years) with high sperm DNA fragmentation (>30%).

RESULTS: Clinical, as well as laboratory outcomes, were correlated among these four groups. There was no significant difference in the clinical outcomes among the groups, whereas coming to the laboratory outcomes, the advanced paternal age group with high sperm DNA fragmentation has significantly decreased good quality embryos (grade A) at day 3 rate, blastocyst rate, and good quality blastocyst rate compared to other groups (p<0.05).

CONCLUSION: In conclusion, advancing paternal age together with high sperm DNA fragmentation has no deleterious effect on the clinical outcomes in the intracytoplasmic sperm injection cycles of donor oocytes.

Keywords: Blastocyst rate, Embryo quality, Intracytoplasmic sperm injection cycles, Live birth rates, Paternal age, Sperm DNA fragmentation

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Introduction

Sperm DNA integrity is essential for the proper handover of the paternal genome. Sperm DNA integrity testing has been progressively used as an unfettered measure of sperm as it proffers more prognostic and diagnostic information than routine semen analysis (1,2). Sperm DNA fragmentation (SDF) can arise pre or post-ejaculation due to various mechanisms explained by Sakkas et al., (3). SDF affects the clinical outcomes in spontaneous and assisted reproductive techniques (ART) conceptions (2,4-6). The negative influence of SDF on the laboratory outcomes and clinical outcomes in in-vitro fertilization (IVF) cycles and/or intracytoplasmic sperm injection (ICSI) was reported by various studies and meta-analyses (7-13). Paternal age has been linked to SDF in various studies; an increase in paternal age has a positive correlation with SDF (14,15). Few studies have reported paternal age of 40 years and above has increased the risk for SDF (16,17). Males above

40 years of age group account for >25% of the males that underwent advanced infertility treatments around the globe (18).

Sperm DNA fragmentation can be measured by two different assays: those that can directly measure the extent of DNA fragmentation with the use of probes and dyes and those that measure the susceptibility of DNA to denaturation, which is higher in fragmented DNA (9). The acridine orange test (AOT) belongs to the second type of assay and differentiates the sperm with normal double-stranded DNA (green fluorescence) and abnormal denatured or single-stranded DNA (orange-red fluorescence) with the help of metachromatic shift properties of the stain (19,20). AOT is a simple and affordable test for the assessment of DNA integrity in infertile men (19,20). Clinical assessments of SDF need to be performed on the total motile fraction of sperm rather than raw ejaculate sperm by AOT, as the raw semen carries a huge number of degenerated and dead sperm with damaged DNA (21). The study is mainly aimed to know the effect of paternal age together with SDF on the clinical and laboratory outcomes in the donor oocyte ICSI cycles.

Material and Method

Study population: A prospective study with a total of 229 patients undergoing their first ICSI cycles and fresh blastocyst embryo transfer with donor oocytes from February 2017 to December 2019 were included in this study. This study got approved by the Institutional Ethics Committee (11/02/2017, IEC NO: SAIMS/IEC/2017/02/03) and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all the couples. The male patients included in this study were aged between 25 and 57 years. Patients with uterine factor infertility, male patients with surgically retrieved sperms, and severe oligozoospermia (count<1 M/ml), and patients with life-threatening diseases, ICSI with vitrified/thawed oocytes, preimplantation genetic testing, cryopreserved sperm were excluded from this study.

The causes of infertility in the patients with donor oocyte ICSI cycles were poor ovarian reserve (POR) in 123, POR with male factor in 42, POR with tubal factor in 43, and POR with mixed in 21 patients. Oocyte donation was anonymous. All the donors recruited were between the age group of 21-35 years (mean age 27.82 ± 2.44 years). The donor's recruitment, confidentiality, and screening were done according to the Indian Council for Medical Research (ICMR) guidelines (updated on Dec 10, 2018) (22).

All the patients of the donor oocyte ICSI cycles were categorized into four groups based on the male age and SDF. I. Y group (young, male age <40 years) with low SDF (SDF≤30%), II. Y group (young, male age <40 years) with high SDF (SDF>30%), III. APA Group (Advanced paternal age, male age ≥40 years) with low SDF (SDF≤30%), and IV. APA Group (advanced paternal age, male age ≥40 years) with high SDF (SDF>30%). Clinical as well as laboratory outcomes were correlated among these four groups.

Oocyte stimulation and endometrium preparation: In all donors, controlled ovarian stimulation was attained by recombinant follicle-stimulating hormone (r-FSH) (Recagon, MSD; Gonal-F, Merck) or human menopausal gonadotropin (HMG) (Gynogen, Sanzyme; Materna HMG, Emcure) starting from day 3 of the cycle. The pituitary function was suppressed either by gonadotrophin-releasing hormone (GnRH) agonists (Luprorin, Intas) in long stimulation protocol or GnRH antagonists (Cetrorelix Acetate, Emcure) in antagonist stimulation protocol. Recombinant human chorionic gonadotropin (r-HCG, Ovidrel, Merck) was administered when three or more follicles reached a diameter of ≥17mm, and appropriate serum E2 values were detected. The P4 values were <1 ng/mL. Transvaginal oocyte retrieval (TVOR) was performed 35 hours post trigger with HCG.

In recipient patients of donor oocyte cycles, oral estradiol valerate (Evadiol, Intas) was used in a step-by-step increasing dose pattern for the preparation of endometrium. Patients with optimum endometrial lining and thickness (>7mm) underwent fresh embryo transfer otherwise transfers were canceled. Micronised progesterone (Crinone 8% gel, Merck) was administered daily vaginally and intramuscularly (Hald 100mg, Intas) on alternate days from the day of donor oocyte pickup and continued till the pregnancy test was confirmed negative or continued for another 3 months if the pregnancy test was positive.

Semen analysis and processing: Patients collected semen samples in sterile, non-toxic containers by masturbation after sexual abstinence of 2-3 days. After 30 minutes of liquefaction, samples were evaluated for count, motility, and morphology according to WHO 2010 criteria (23). Semen samples were prepared by two-layer density gradient (V-Grad 80%-40%, Vitromed, Germany) centrifugation (DGC) for ICSI. The post-wash sample was used for SDF evaluation by AOT.

Acridine orange test (AOT): The SDF was assessed by the AOT method (20). Smears with 10 μL of post-wash samples were prepared and air-dried. Carnoy's solution (methanol: Glacial acetic acid, 3:1 vol/vol) was used in fixing the slides overnight. The staining solution was prepared daily from the stock solution of AO (1g/L in distilled water, stored in dark at 4°C) in the mentioned ratio of 10 mL of stock solution, 40 mL of 0.1 M citric acid, and 2.5 mL of 0.3 M Na2HPO4 7H2O and pH adjusted to 2.5. Slides were stained with the above stain for 5 minutes and rinsed in distilled water and covered with coverslips.

Slides were examined for SDF using a fluorescence microscope (Olympus C×31, Japan) under oil at ×1000 with an excitation of 450-490nm. Green fluorescence represents normal intact sperm, whereas red indicates fragmented and dena-

tured sperm. Orange or yellow heads, as well as those displaying green and red colors simultaneously, were also considered fragmented sperms (19,24). At least 400 spermatozoa were assessed in each slide of the replicates to calculate the average SDF. Slides were fixed on the same day of semen preparation and examined on the next day for SDF by AOT. One highly skilled and trained andrologist evaluated all the slides for consistency and to prevent interpersonal variability. Each stained slide was read immediately after staining to reduce the variation of fluorescence intensity.

Intracytoplasmic sperm injection: Oocytes recovered were incubated in culture media (Onestep, Vitromed, Germany) for 1-2 hours before denudation by hyaluronidase enzyme (Hyadase 80IU, Vitromed) at 37°C with 6% CO_2, 5% O_2 and the rest N_2. All the ICSI procedures were performed by a highly-skilled embryologist according to Palermo et al. (25). A morphologically normal sperm was selected and immobilized in polyvinylpyrrolidone (PVP 7%, Vitromed). The immobilized sperm was aspirated tail-first into the injection pipette and injected into the oocyte by aspirating a little cytoplasm before releasing the sperm into the oocyte. 16-18 hours post ICSI, the appearance of two pronuclei with a second polar body extrusion was noted to evaluate the fertilization. The obtained embryos were cultured till day 5 post-ICSI for embryo transfer.

Embryo grading: According to the Istanbul consensus, day 3 embryos were graded as A, B, and C based on the blastomeres number, fragmentation percentage, and multinucleation (26). Grade A indicated a good embryo with stage-specific 6-8 blastomeres, <10% fragmentation, and no multinucleation. Grade B indicated a fair embryo with stage-specific 6-8 blastomeres, 10-25% fragmentation, and no multinucleation and grade C indicated a poor embryo with non-stage specific blastomeres, severe fragmentation (>25%), presence of multinucleation.

Day 5 blastocysts were graded according to Gardner et al. (27). Expansion of the blastocysts graded as 3-6, trophectoderm (TE), and inner cell mass (ICM) graded as A, B, and C. Expansion of blastocyst graded as follows 3-full blastocyst, 4-expanded, 5-Hatching, and 6-hatched. Whereas for TE: grade A-TE with many cells forming a cohesive epithelium; grade B-TE with few cells forming a loose epithelium and grade C-TE with very few cells. Similarly, for ICM grade A-tightly packed ICM with many cells; grade B-loosely grouped ICM with many cells, and grade C-ICM with very few cells. A day 3 embryo with grade A was considered a good quality embryo. A blastocyst with a grade >3AA was considered a good quality blastocyst.

Embryo transfer and clinical follow-up: One to two embryos were transferred on day 5 using a soft catheter (Cook, Australia). Serum β HCG was observed after 14 days of the transfer to confirm the pregnancy test positive. An intrauterine sac with the presence of a fetal heartbeat was considered a clinical pregnancy. The implantation rate was calculated as the proportion of gestational sacs determined by ultrasound divided by the total number of embryos transferred. Miscarriage was defined as a pregnancy loss after an intrauterine pregnancy had been detected by ultrasound before 24 weeks of gestation. The live birth rate was calculated as the presence of a live birth (either single or multiple live births) after a fresh embryo transfer cycle.

Statistical analysis of data: Categorical variables like clinical outcomes between groups were shown as proportions and scrutinized using the chi-square test. Characteristics of patients and laboratory outcomes between groups were shown as continuous variables and scrutinized using the one-way analysis of variance (ANOVA). Kruskal-Wallis one-way ANOVA was used for data not normally distributed. Statistical significance was set at $p<0.05$. The statistical analysis was executed in the Statistical package for the social sciences (SPSS, IBM) for windows version 26.0.

Results

Characteristics of patients in different groups of donor oocyte ICSI cycles categorized according to male age and SDF: Out of 229 patients, 67 were in the Y group with low SDF, 35 in the Y group with high SDF, 80 in the APA group with low SDF, and 47 were in the APA group with high SDF. The male patient's semen characteristics like sperm count, motility, and morphology were similar except for the semen volume and SDF rates ($p<0.05$). Semen volume was significantly decreased in the APA groups compared to the Y groups and SDF rates were significantly different between the low and high SDF groups. Male age and female partner age were significantly different between the Y and APA groups ($p<0.05$) (Table I). Donor age, total FSH given, Estradiol values at the time of hCG trigger, the number of oocytes retrieved from the donor, and mature metaphase II oocytes were similar among all the groups (Table II).

Comparison of laboratory outcomes in different groups of donor oocyte ICSI cycles categorized according to male age and SDF: There was no significant difference in the fertilization and cleavage rate amongst the groups. A statistically significant difference was observed in the good quality embryos (Grade A) at day 3 rate, blastocyst rate, and good quality blastocyst rate. APA group with high SDF has significantly decreased good quality embryos (Grade A) at day 3 rate, blastocyst rate, and good quality blastocyst rate compared to other groups (Table III).

Comparison of clinical outcomes in different groups of donor oocyte ICSI cycles categorized according to male age and SDF: There was no significant difference in the clinical pregnancy rate, implantation rate, live birth rate, and miscarriage rate amongst all the groups of the donor oocyte ICSI cycles (Table IV).

Table I: *Characteristics of patients in different groups of donor oocyte intracytoplasmic sperm injection cycles categorized according to male age and sperm DNA fragmentation*

Characteristics	Y group with low SDF	Y group with High SDF	APA group with low SDF	APA group with high SDF
No of patients (n)	67	35	80	47
Male age, years	34.84±3.48[a]	35.40±3.32[a]	43.79±3.57[b]	43.85±3.87[b]
SDF rate	11.51±8.26[a]	52.52±16.62[b]	16.58±7.97[a]	53.05±18.86[b]
Volume	2.99±0.80[a]	2.85±0.80[a]	2.31±0.65[b]	2.14±0.56[b]
Sperm count X10^6 /mL	34.33±16.94	29.72±16.83	29.30±16.59	29.88±14.10
Sperm motility %	56.88±14.81	54.76±17.72	51.58±18.68	52.31±18.07
Sperm morphology %	4.53±1.05	4.24±1.26	4.37±1.24	4.45±1.44
Female partner age, years	32.33±4.58[a]	32.16±3.79[a]	38.49±3.45[b]	38.57±4.00[b]

APA: Advanced paternal age, SDF: Sperm DNA fragmentation, a,b: Values with different letters inside the line differ significantly (p<0.05). All the values are represented as mean ± standard deviation if not otherwise specified.

Table II: *Comparison of oocyte stimulation descriptive analysis among the groups categorized according to male age and sperm DNA fragmentation*

Characteristics	Y group with low SDF	Y group with high SDF	APA group with low SDF	APA group with high SDF
Donor age, years	27.46±2.27	28.12±2.47	28.13±2.57	27.54±2.64
Total FSH administered, IU	2614.66±256.36	2644.00±245.08	2676.22±214.86	2670.00±281.59
Estradiol level at HCG trigger day, pg/mL	2578.55±653.22	2598.64±688.17	2599.30±618.53	2699.40±578.11
No. of oocytes retrieved	15.00±5.11	14.00±4.71	14.43±4.37	14.80±4.76
No. of M II oocytes	12.20±4.93	11.36±3.71	11.73±4.00	12.48±4.40

APA: Advanced paternal age, SDF: Sperm DNA fragmentation, All the values are represented as mean ± standard deviation if not otherwise specified

Table III: *Comparison of laboratory outcomes in different groups of donor oocyte intracytoplasmic sperm injection cycles categorized according to male age and sperm DNA fragmentation*

Characteristics	Y group with low SDF	Y group with high SDF	APA group with low SDF	APA group with high SDF
No. of ICSI cycles (n)	67	35	80	47
Fertilization rate	86.86±14.16	81.75±17.10	86.37±14.55	83.74±12.73
Cleavage rate	85.30±14.15	79.24±17.47	81.22±17.60	80.31±17.71
Good quality embryos at day3 rate	53.80±21.98[a]	49.10±23.64[a]	48.30±26.29[a]	35.45±17.29[b]
Blastocyst rate	52.37±18.59[a]	50.39±19.34[a]	51.40±17.65[a]	43.21±19.09[b]
Good quality blastocyst rate	27.71±14.06[a]	25.12±12.31	31.34±18.60[a]	22.42±13.43[b]

APA: Advanced paternal age, SDF: Sperm DNA fragmentation, a,b: Values with different letters inside the line differ significantly (p<0.05). All the values are represented as mean ± standard deviation if not otherwise specified.

Table IV: *Comparison of clinical outcomes in different groups of donor oocyte intracytoplasmic sperm injection cycles categorized according to male age and sperm DNA fragmentation*

Characteristics	Y group with low SDF	Y group with high SDF	APA group with low SDF	APA group with high SDF
No. of transfers (n)	67	35	80	47
Clinical pregnancy rate (%)	76.11% (51/67)	68.57% (24/35)	68.75% (55/80)	82.97% (39/47)
Implantation rate*	54.44 ± 38.17	52.00 ± 42.03	45.56 ± 36.76	60.00 ± 37.96
Live birth rate (%)	59.70% (40/67)	48.57% (17/35)	52.5% (42/80)	70.21% (33/47)
Miscarriage rate (%)	21.56% (11/51)	29.16% (7/24)	23.63% (13/55)	15.38% (6/39)

*APA: Advanced paternal age, SDF: Sperm DNA fragmentation, *the values are represented as mean ± standard deviation*

Discussion

SDF's effect on the clinical outcomes mainly depends on the oocyte quality (28). So the effect of SDF in correlation with female age and oocyte quality has been studied widely (10,28). With the advances in ART, paternal age has been the least concern in the era of ICSI. Paternal age is equally important as the 50% genome of the embryo is provided by sperm. However, the importance of paternal age on embryo quality and conceptions in spontaneous and IVF treatments has gained significance recently (29).

This is the first study to classify the patients according to paternal age and the SDF rate and to correlate with the clinical and laboratory outcomes in the ICSI cycles of donor oocytes. In most of the studies, paternal age correlation was limited to semen parameters like volume, count, motility, morphology, and SDF rates (14,17,30). Female factors like age, ovarian reserve, and oocyte quality play a major role in determining the clinical and laboratory outcomes (10,28,31). In this study in order to nullify the female confounding factor only male patients who underwent ICSI cycles with donor oocytes were included.

In the current study, there was no significant difference in the clinical outcomes like clinical pregnancy rate, implantation rate, miscarriage rate, and live birth rate amongst all the groups. Whereas coming to the laboratory outcomes, APA with high SDF had remarkably decreased good quality embryos at day 3 rate and decreased blastocyst rate compared to all other three groups, and decreased good quality blastocyst rate compared to the APA with low SDF group and the Y group with low SDF group. Even with the good quality oocytes, the quality of embryos at both day 3 and day 5 and blastocyst rates remarkably went down in the high SDF APA group. This may reduce the availability of good-quality embryos for cryopreservation and consequent transfers. The group with low SDF may benefit from cumulative transfers and further cumulative live birth rates.

Both APA and SDF independently have a negative correlation to the embryo quality in donor oocytes and reported the low blastulation rates, which is in corroboration with this study (32,33). This conveys that the poor quality embryos in the APA group may be due to the effect of paternal age together with high SDF. Increased paternal age has a high risk of SDF and a higher frequency of deleterious point mutations as they underwent more mitotic replications compared to the younger males (34). The antioxidant enzyme activities within the seminal plasma and spermatozoa in older men may be reduced and so spermatozoa are more vulnerable to mutational changes in older men compared to spermatozoa of younger men (35). So high SDF with advanced paternal age may be an unfavorable combination compared to the high SDF with young male age. Also, a study reported increased paternal age was associated with decreased blastocyst rates and decreased euploid rates using 40 years as an age cutoff (36). However, the decreased number of good-quality embryos doesn't affect the clinical outcomes, possibly due to the availability and selection of the best blastocyst for embryo transfer. A study with the largest extensive data analysis concluded that SDF does not negatively affect the live birth rates in unselected patients of IVF/ICSI cycles with donor oocytes (37). This is in corroboration with this study. Further studies on APA with high SDF and preimplantation genetic testing for aneuploidy (PGT-A) correlation were planned to know the effect of APA with high SDF on the ploidy status of the embryos in ICSI cycles.

The semen parameters amongst the groups were not significantly different except for the semen volume between the Y and APA groups, which is in corroboration with some previous studies that showed a significant decrease in semen volume with the increase in the male age without any significant changes in the other parameters (38,39). The presence of abnormal semen samples in a small amount in each group does not elicit any significant changes in the semen parameters like count, motility, and morphology amongst the groups.

The AOT method is an established method for assessing the DNA integrity of the sperm of infertile men (19,20).

The inverse effect of SDF by the AOT method on pregnancy and implantation rates was perceived in the group with high SDF (>30%) in ICSI cycles (40). The AOT method is easy, simple, inexpensive, and convenient to do routinely in-house. The principle of AOT is similar to sperm chromatin structure assay (SCSA) except for the number of sperms counted. The slides after staining can be read immediately as it is done by an in-house embryologist who has been trained and technically skilled. Even though AOT is not robust as SCSA, the cells can be differentiated easily and the SDF rate can be evaluated technically. The strengths of our present study are the prospective nature of the study, only ICSI cycles with donor oocytes and blastocyst fresh transfer were included. All the related parameters like semen parameters, laboratory and clinical outcomes till live birth rate were assessed. Despite the valuable results obtained in the study, the authors are sensible of the limitations of the study. The smaller sample size due to the inclusion of patients who only underwent ICSI cycles with donor oocytes and fresh blastocyst transfer and the AOT method used may not be robust like the golden standard SCSA method but as already mentioned above the AOT method is simple, inexpensive, and comparable to the SCSA method.

In conclusion, advancing paternal age together with high SDF has no deleterious effect on the clinical outcomes in the ICSI cycles of donor oocytes. Further studies to know the effect of APA with high SDF on the ploidy status of the embryos in PGT (A) cycles are recommended.

Author's Declarations
Ethics approval and consent to participate: All participants signed informed written consent before being enrolled in the study.
Availability of data and materials: The data supporting this study is available through the corresponding author upon reasonable request.
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Author's Contributions: RD conceptualized and planned the design of the study, acquired the data, and drafted the manuscript. JC contributed to acquiring the data and revising the manuscript. KVS and SB assisted in writing the manuscript and refined the manuscript critically. All authors read and approved the final manuscript.
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References

1. Tarozzi N, Bizzaro D, Flamigni C, Borini A. Clinical relevance of sperm DNA damage in assisted reproduction. Reprod Biomed Online. 2007;14(6):746-57. Doi: 10.1016/s1472-6483(10)60678-5. PMID: 17579991.
2. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update. 2003;9(4):331-45. Doi: 10.1093/humupd/dmg027. PMID: 12926527.
3. Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil Steril. 2010;93(4):1027-36. Doi: 10.1016/j.fertnstert.2009.10.046. PMID: 20080235.
4. Giwercman A, Lindstedt L, Larsson M, Bungum M, Spano M, Levine RJ, et al. Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. Int J Androl. 2010;33(1):e221-7. Doi: 10.1111/j.1365-2605.2009.00995.x. PMID: 19840147.
5. Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod. 2007;22(1):174-9. Doi: 10.1093/humrep/del326. PMID: 16921163.
6. 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(4):998-1005.e8. Doi: 10.1016/j.fertnstert.2014.06.033. PMID: 25190048.
7. Simon L, Proutski I, Stevenson M, Jennings D, McManus J, Lutton D, et al. Sperm DNA damage has a negative association with live-birth rates after IVF. Reprod Biomed Online. 2013;26(1):68-78. Doi: 10.1016/j.rbmo.2012.09.019. PMID: 23200202.
8. Osman A, Alsomait H, Seshadri S, El-Toukhy T, Khalaf Y. The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: a systematic review and meta-analysis. Reprod Biomed Online. 2015;30(2):120-7. Doi: 10.1016/j.rbmo.2014.10.018. PMID: 25530036.
9. Borges E Jr, Zanetti BF, Setti AS, Braga DPAF, Provenza RR, Iaconelli A Jr. Sperm DNA fragmentation is correlated with poor embryo development, lower implantation rate, and higher miscarriage rate in reproductive cycles of non-male factor infertility. Fertil Steril. 2019;112(3):483-90. Doi: 10.1016/j.fertnstert.2019.04.029. PMID: 3120096 9.
10. Liang X, Mao Y, Wang Y, Liu S, Yan J. Female age affects the utility of sperm DNA fragmentation in predicting IVF and ICSI outcomes. Reprod Biomed Online. 2019;39(6):955-62. Doi: 10.1016/j.rbmo.2019.09.013. PMID: 31753711.
11. Speyer BE, Pizzey AR, Ranieri M, Joshi R, Delhanty JD, Serhal P. Fall in implantation rates following ICSI with sperm with high DNA fragmentation. Hum Reprod. 2010;25(7):1609-18. Doi: 10.1093/humrep/deq116. PMID: 20495207.
12. Simon L, Zini A, Dyachenko A, Ciampi A, Carrell DT. A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. Asian J Androl. 2017;19(1):80-90. Doi: 10.4103/1008-682X.182822. PMID: 27345006, PMCID: PMC5227680.
13. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27(10):2908-17. Doi: 10.1093/humrep/des261. PMID: 22791753.
14. Gonzalez DC, Ory J, Blachman-Braun R, Nackeeran S, Best JC, Ramasamy R. Advanced paternal age and sperm dna fragmentation: a systematic review. World J Mens Health. 2022;40(1):104-15. Doi: 10.5534/wjmh.200195. PMID: 33987998, PMCID: PMC8761235.
15. Petersen CG, Mauri AL, Vagnini LD, Renzi A, Petersen B, Mattila M, et al. The effects of male age on sperm DNA damage: an evaluation of 2,178 semen samples. JBRA Assist Reprod. 2018;22(4):323-30. Doi: 10.5935/1518-0557.20180047. PMID: 30106542, PMCID: PMC6106 22.
16. Alshahrani S, Agarwal A, Assidi M, Abuzenadah AM, Durairajanayagam D, Ayaz A, et al. Infertile men older than 40 years are at higher risk of sperm DNA damage. Reprod Biol Endocrinol. 2014;12:103. Doi: 10.1186/1477-7827-12-103. PMID: 25410314, PMCID: PMC425805 1.
17. Das M, Al-Hathal N, San-Gabriel M, Phillips S, Kadoch IJ, Bissonnette F, et al. High prevalence of isolated sperm DNA damage in infertile men with advanced paternal age. J Assist Reprod Genet. 2013;30(6):843-8. Doi: 10.1007/s10815-013-0015-0. PMID: 23722935, PMCID: PMC3696445.
18. Evenson DP, Djira G, Kasperson K, Christianson J. Relationships between the age of 25,445 men attending

infertility clinics and sperm chromatin structure assay (SCSA®) defined sperm DNA and chromatin integrity. Fertil Steril. 2020;114(2):311-20. Doi: 10.1016/j. fertnstert. 2020.03.028. PMID: 32653083.

19. Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange fluorescence. Fertil Steril 1984;42(1):87-91. Doi: 10.1016/s0015-0282(16)47963-x. PMID:6724015.
20. Ajina T, Ammar O, Haouas Z, Sallem A, Ezzi L, Grissa I, et al. Assessment of human sperm DNA integrity using two cytochemical tests: Acridine orange test and toluidine blue assay. Andrologia. 2017;49(10): e12765. Doi: 10. 1111/and.12765. PMID: 2822463.
21. Liu DY, Liu ML. Clinical value of sperm DNA damage should be assessed in motile sperm fraction rather than whole ejaculated sperm. Fertil Steril. 2013;99(2):367-71. Doi: 10.1016/j.fertnstert.2012.10.005. PMID: 23089238.
22. The Assisted Reproductive Technologies (Regulation) Rules. New Delhi: Ministry of Health & Family Welfare Govt. of India; 2010. Available from: https://www.icmr. nic.in/guidelines?field_ select_disease_tid=90.
23. World Health Organization. WHO Manual for the Examination of Human Sperm and Sperm Cervical Mucus Interaction. 5th ed. Switzerland: WHO press; 2010.
24. Erenpreiss J, Bars J, Lipatnikova V, Erenpreisa J, Zalkalns J. Comparative study of cytochemical tests for sperm chromatin integrity. J Androl. 2001;22(1):45-53. PMID: 11191087.
25. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340(8810):17-8. Doi: 10.1016/0140-6736(92)92425-f. PMID: 1351601.
26. Alpha Scientists in Reproductive M, Embryology ESIGo. The Istanbul consensus workshop on embryo assessment proceedings of an expert meeting. Hum Reprod. 2011; 26(6):1270-83. Doi: 10.1093/humrep/der037. PMID: 21 502182.
27. Gardner DK, Schoolcraft WB. In-vitro culture of human blastocyst. In: Jansen R, Mortimer D, editors. Towards reproductive certainty: infertility genetics beyond. New York: Parthenon Press; 1999:378-88.
28. Meseguer M, Santiso R, Garrido N, García-Herrero S, Remohí J, Fernandez JL. Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. Fertil Steril. 2011;95(1):124-8. Doi: 10.1016/j.fertnstert. 2010.05.055. PMID: 20643402.
29. Kumar M, Kumar K, Jain S, Hassan T, Dada R. Novel insights into the genetic and epigenetic paternal contribution to the human embryo. Clinics (Sao Paulo). 2013;68(1):5-14. Doi: 10.6061/clinics/2013(sup01)02. PMID: 235039 50, PMCID: PMC3583177.
30. Lu R, Chen X, Yu W, Jiang F, Zhou X, Xu Y, Wang F. Analysis of age-associated alternation of SCSA sperm DNA fragmentation index and semen characteristics of 1790 subfertile males in China. J Clin Lab Anal. 2020; 34(12):e23548. Doi: 10.1002/jcla.23548. PMID: 329264 80, PMCID: PMC7755769.
31. Jin J, Pan C, Fei Q, Ni W, Yang X, Zhang L, et al. Effect of sperm DNA fragmentation on the clinical outcomes for in vitro fertilization and intracytoplasmic sperm injection in women with different ovarian reserves. Fertil Steril. 2015;103(4):910-6. Doi: 10.1016/j.fertnstert.2015.01.014. PMID: 25747135.
32. Luna M, Finkler E, Barritt J, Bar-Chama N, Sandler B, Copperman AB, et al. Paternal age and assisted reproductive technology outcome in ovum recipients. Fertil Steril. 2009;92(5):1772-5. Doi: 10.1016/j.fertnstert.2009.05.036. PMID: 19539905.
33. Alvarez Sedó C, Bilinski M, Lorenzi D, Uriondo H, Noblía F, Longobucco V. et al. Effect of sperm DNA fragmentation on embryo development: clinical and biological aspects. JBRA Assist Reprod. 2017;21(4):343-50. Doi: 10.5935/1518-0557.20170061. PMID: 29116706, PMCID: PMC5714603.
34. Humm KC, Sakkas D. Role of increased male age in IVF and egg donation: is sperm DNA fragmentation responsible? Fertil Steril. 2013;99(1):30-6. Doi: 10.1016/j. fertnstert.2012.11.024. PMID: 23273987.
35. Brandt JS, Cruz Ithier MA, Rosen T, Ashkinadze E. Advanced paternal age, infertility, and reproductive risks: A review of the literature. Prenat Diagn. 2019;39(2):81-7. Doi: 10.1002/pd.5402. PMID: 30520056.
36. Hanson BM, Kim JG, Osman EK, Tiegs AW, Lathi RB, Cheng PJ, et al. Impact of paternal age on embryology and pregnancy outcomes in the setting of a euploid single-embryo transfer with ejaculated sperm: retrospective cohort study. F S Rep. 2020;1(2):99-105. Doi: 10.1016/j.xfre. 2020.06.004. PMID: 34223225, PMCID: PMC8244285.
37. Hervas I, Garrido N, Rivera-Egea R, Gonzalez-Ravina C, Amoros D, Quintana F Sr., et al. Accurate measurement of sperm DNA fragmentation effect on reproductive outcomes by cumulative live birth rates (CLBR) per embryo transferred and oocyte utilized reveals its lack of relevance in oocyte donation for unselected males. Fertil Steril. 2020;114(3):e44. Doi: 10.1016/j.fertnstert. 2020. 08.142.
38. Frattarelli JL, Miller KA, Miller BT, Elkind-Hirsch K, Scott RT Jr. Male age negatively impacts embryo development and reproductive outcome in donor oocyte assisted reproductive technology cycles. Fertil Steril. 2008;90(1):97-103. Doi: 10.1016/j.fertnstert.2007.06.009. PMID: 17765235.
39. Dain L, Auslander R, Dirnfeld M. The effect of paternal age on assisted reproduction outcome. Fertil Steril. 2011; 95(1):1-8. Doi: 10.1016/j.fertnstert.2010.08.029. PMID: 20932518.
40. Jiang H, He RB, Wang CL, Zhu J. The relationship of sperm DNA fragmentation index with the outcomes of in-vitro fertilisation-embryo transfer and intracytoplasmic sperm injection. J Obstet Gynaecol. 2011;31(7):636-9. Doi: 10. 3109/01443615.2011.590910. PMID: 21973140.