Impact of Paternal Genome with a High DNA Fragmentation Index (>60%) on Early Embryonic Development

M. Hachemi[1,2,*], A. Zoghmar[2], L. Rouabah Pr[1], L. Ounisa[1], S. Benbouhedia[2], M Bensaada[3] and A. Rouabah Pra[1]

[1]Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences de la Nature et de la Vie, Université des Frères Mentouri Constantine I, Constantine, Algérie

[2]Clinique de Chirurgie et des Sciences de la Reproduction, Ibn Rochd, Constantine, Algérie

[3]Faculté des Sciences de la Nature et de la Vie Université Abbès Laghrour, Khenchela, Algérie

Abstract: ***Objectives:*** The objective of this study is to propose thresholds of the sperm DNA fragmentation rate (IFA≤30% IFA31%-60% IFA>60%), in order to assess the clinical effects of the paternal genome on intra cytoplasmic sperm injection parameters, in particular the effect of the latter on early embryonic development. ***Materials and Methods:*** The procedure is a retrospective study, which involved 101 patients enrolled in an ICSI program with their partners. The index of spermatic DNA fragmentation rate was measured using the Sperm Chromatin Dispersion assay. ***Results:*** There is a negative correlation between high levels of the spermatic DNA fragmentation index and spermiological characteristics: Concentration P=0.002 and mobility P=0.0001. For ICSI results, there are different observations on the existence of a correlation between the spermatic DNA fragmentation index and fertility rate. On the other hand, the rate of sperm DNA fragmentation does not seem to influence early embryonic development, and even couples whose partners have a high fragmentation index manage to obtain the best quality embryos (P=0.002). We observe a decrease in the rate of implantation with an increase in the rate of alteration of the sperm genome, but this remains insignificant P > 0.05. ***Conclusion:*** ICSI remains the only alternative for men with a high rate of sperm DNA fragmentation. Moreover, the operator seems to influence the results more than is suggested. This does not exclude the paternal effect which may influence the quality of the concepltus later on.

Keywords: DNA Fragmentation Index, ICSI, Fertilization Rate, Embryos Quality.

INTRODUCTION

Analysis of the integrity of the sperm cell genome has become an important factor for estimating the fertilizing potential of a spermatozoon and its ability to carry a pregnancy to term [1]. Numerous techniques are available for the investigation of the quality of the sperm genome in another term sperm DNA fragmentation; the Sperm chromatin structure assay (SCSA) [2], Terminal deoxyribonucleotidyl transferase dUTP nick-end labelling (TUNEL) [3], the COMET technique; the single-cell gel electrophoresis assay (SCGE) [4] and the Sperm Chromatin Dispersion (SCD) technique [5]. The threshold of DNA fragmentation index (DFI) may additionally vary from one approach to another and for constant technique; different thresholds are sometimes recommended [6-8]. This lack of consensus makes it hard to determine the threshold of DNA damage from which it may also or may also not be possible for a sperm cell to carry a pregnancy to term. Moreover, the influence of the alteration of the paternal genome seems to depend on the approach used in artificial reproductive technology (ART) [9]. Studies have shown a correlation between aberrations in the sperm DNA in the arrangement of single or double strand breaks and conventional spermiological parameters such as concentration, motility and morphology [6, 10, 11]. Regarding assisted reproduction techniques, the relationship between the integrity of the sperm genome and the fertilization process. Studies diverge on the presence of a relationship between these two phenomena, some studies reveal a negative and important correlation [12-14] and others reveal a complete lack of correlation [15]. It should be noted that results may vary depending on the in vitro fertilization technique chosen [16]. In ICSI, selective biological barriers are bypassed and sperm with altered DNA has the ability to fertilize an oocyte and trigger the first stage of cell division, resulting in good quality embryos at the pre-implantation phase [6]. Several studies have shown the influence of the paternal genome on embryonic and conceptus quality could be seen later, leading to miscarriages in most cases. [17,18]. However, some authors show that; there is a possibility that a sperm with alterations in its DNA can have fertilization and normal early embryonic development followed by a full-term pregnancy if a good quality oocyte is able to repair the damage to the sperm DNA [19].

In order to assess the effects of sperm DNA fragmentation on Intracytoplasmic sperm injection (ICSI) procedure parameters and in particular on embryo quality during early embryo development, we have established new thresholds for DFI. A DFI ≤ 30% low fragmentation rate of spermatic DNA, DFI of 31%-60% medium fragmentation rate and a DFI > 60%: high damage or fragmentation rate.

MATERIALS AND METHODS

Patients

A retrospective research was carried out in an ART center. We have selected 101 couples undergoing an ICSI procedure. In This research, couples in which one or both partners had a medical history that could influence their fertility were excluded. The ethics committee of the center approved the work, and informed consent was obtained from all participants included in this study.

*Address correspondence to this author at the Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences de la Nature et de la Vie, Université des Frères Mentouri Constantine I, Constantine, Algérie; Email: marouahachemi10@gmail.com; Tel: +213 (0)6 55 69 05 64

Intracytoplasmic Sperm Injection Procedure

Semen is collected by masturbation after a period of 3 to 4 days of sexual abstinence and is analyzed according to the 2010 world health organization recommendations. The spermatozoa selection was performed with the procedure used routinely in our laboratory. A discontinuous gradient of PureSperm (PureSperm, Nicadon, Gothenburg, Sweden) constituted of two layers of PureSperm: one mL layer of PureSperm 90% and one mL layer of PureSperm 45% were used. One milliliter of sperm was placed on top of the 45% layer. After centrifugation (300 g for 20 min) at room temperature, the 90% layer was collected and washed with 2 mL of FertiCult flushing medium (FertiPro N.V., Beernem, Belgium) at 600 g for 10 min at room temperature. The pellet of sperm was resuspended in 200 µL of FertiCult IVF medium (FertiPro N. V). The semen was hold at 37°C until its use for ICSI procedure.

Ovarian stimulation is performed according to the antagonist protocol, the oocytes were obtained using ultrasound-guided, endovaginally aspiration under general anesthesia. Fertilization with microinjection is achieved according to the protocol described by Palermo [20]. At 48-72 hours, the embryos are controlled and classified according to their morphology: Grade A: embryos with 4 regular cells without cell fragmentation; Grade B: embryos with less than 10-20% cell fragmentation; Grade C: embryos with between 20% and 50% cell fragmentation; and finally Grade D: embryos with more than 50% cell fragmentation [21]. Embryo transfer is accomplished at 48 hours (Day 2) or 72 hours (Day 3) depending on the stage of embryo development. Two to three embryos are transferred, conditional on the patient's age and especially on the quality of the embryos obtained.

DNA Damage Study by SCD Technique

Sperm DNA damage has been assessed in accordance to the procedure described by Fernández et al.,2003 [8]. Briefly, 50 µL of low-melting point agarose (Halotech DNA Kit, Madrid, Spain) at 0.65% was melted in a water bath at 90°C–100°C for 5 min and then set in an oven at 37°C for 5 min for temperature equilibration. Twenty-five microliters of density gradient sperm selected containing 5–10 million spermatozoa/mL were gently mixed with the agarose. Twenty microliters of the mixture were dropped on a slide. The dropped mixture was covered by an 18 mm × 18 mm coverslip and the slides were incubated at 4°C for 5 min. The slides were immersed in denaturation HCl solution (Halotech DNA Kit Madrid, Spain) for 7 min. A lysis step was performed during 20 min in dithiothreitol (Halotech DNA Kit Madrid, Spain)+ triton X-100 (Halotech DNA Kit Madrid, Spain) solution, and then, the slides were dehydrated in increasing concentrations of ethanol (70%, 90%, and 100%) (Sigma Aldrich Saint-Louis, MO, USA) for 2 min for each bath. The sperm cells were colored using eosin (Halotech DNA Kit Madrid, Spain) for 7 min and Azure blue (Halotech DNA Kit Madrid, Spain) for 7 min. Five hundred sperm cells were counted by patient to calculate the DNA fragmentation index (DFI). The results are observing under a microscope at 400 x magnification and counting 500 cells per patient, it is possible to distinguish between spermatozoa with fragmented DNA and those with no fragmented DNA Figure 1 according to [22].

The procedure is confirmed under the same conditions as before, except that sperm nuclei are stained by the 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 2 µg/l diluted with Vectashield (Vector, Laboratories, INC). The results are observed under an epifluorescence microscope at 1000X magnification.

The fragmentation rate of spermatic DNA was considered according to 3 thresholds which are defined as follows: a DFI ≤ 30% low fragmentation rate of spermatic DNA, DFI of 31%-60% medium fragmentation rate and a DFI>60%: high damage or fragmentation rate.

The results of the SCD technique, show 3 types of chromatin halos, which according to their topologies evoke no fragmented or fragmented spermatic genomes Fernandez et al (2005) [22]. Spermatozoa with large halos (a) and medium halos (b) suggest spermatozoa with normal chromatin, while those small and without halos (c) suggest a fragmented sperm genome (Figure 1).

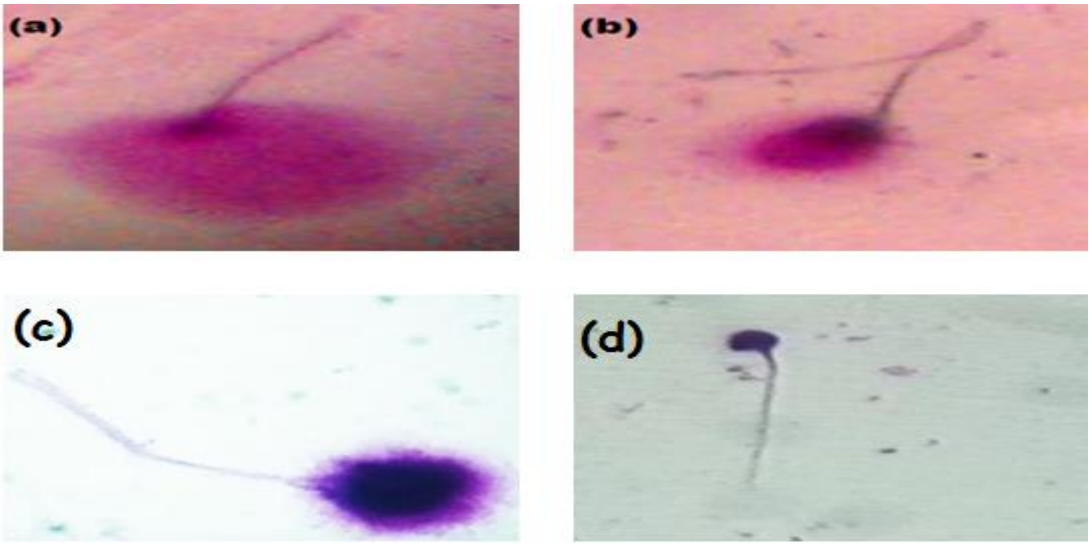

Figure 1: The types of spermatozoa obtained following treatment with the SCD technique and stained with eosin and azure blue

STATISTICAL ANALYSIS

The statistical analysis was conducted using SPSS software (SPSS 18.1, IBM, Chicago, IL, USA). The Pearson correlation coefficient, the student (t) test, and the Fisher Anova test are calculated. The variables were described as mean ± standard deviation (SD) for quantitative variables and with the distribution of percentage for categorical variables. A test was considered significant when P was less than 0.05.

RESULTS

Correlation between Sperm DNA Fragmentation Rate and ICSI Parameters

Based on the results presented in Table 1, there was no significant correlation between the rate of sperm DNA fragmentation and the age of the patient (r =0.05, P-value = 0.565). There was a negative but important association between the rate of fragmentation of sperm DNA and the sperm parameters (concentration and motility). Each time we observed an alteration of the spermatic parameters, the fragmentation rate increased. For concentration (r = -0.412, P-value = 0.0001), (r = -0.494, P-value = 0.0001) for sperm motility (Table 1).

We observed a negative and significant correlation between the sperm DNA and the fertilization rate (r = -0.217, P-value = 0.03) (Table 1); on the other hand, the sperm DNA damage did not demonstrate an influence on the embryonic cleavage rate (r = 0.04, P-value = 0.688) (Table 1).

The correlation between the sperm DNA fragmentation rate and the quality of the embryos showed us: a statistically significant association for grade A embryos (r = 0.189, P-value = 0.05), and our findings showed that there was no association between these two parameters for the other embryonic grades. For grade B embryos (r = 0.145 , P-value = 0.147), for grade C embryos (r = 0.04 , P-value = to 0.686) and finally (r = 0.08 , P-value = 0.390) for grade D (Table 1).

Table 1: Correlation between sperm DNA fragmentation rate and ICSI parameters.

	r	*P*-value
Male age (years)	0.05	0.0001
Sperm concentration (M/mL)	-0.412	0.0001
Sperm motility (%)	-0.494	0.03
Fertilization rate (%)	-0.217	0.688
Cleavage rate (%)	0.04	0.05
Embryos Grade A (%)	0.189	0.147
Embryos Grade B (%)	-0.145	0.686
Embryos Grade C (%)	0.04	0.390
Embryos Grade D (%)	-0.08	0.0001

Sperm concentration (M/ml): Million par ml

ICSI Parameters according to the Three Classes of DFI

The distribution of 101 individuals enrolled in ICSI cycles according to the three DFI thresholds enable to observe that: the assessment of the patients mean age according to the three DFI levels was not significant: for DFI ≤ 30%: 38.4±6.6 years, for intermediate DFI 31%-60%, 38.1±6.2 years and finally for DFI >60%: 40.6 ±7.0 years . P-value is equal to 0.248 (Table 2).

The sperm concentration according to the three classes of DFI showed that there is a significant difference: for the class of DFI ≤30 %; 26.4 ±22.4 million/ml, of DFI 31%-60%; 13.4±20.8 million/ml and finally for the class of DFI >60%: 9.5±7.3 million/ml, P-value: 0.002 (Table2). The evaluation of patient sperm motility according to the three DFI groups showed us a clear link between the degradation of the DFI and the reduction of the motility: DFI class ≤ 30% 38.7% ±19.7% DFI class 31%-60% 24.0% ±22.1% DFI class >60 %: 15.4% ±15.2%. P-value: 0.0001 (Table 2).

The distribution of patient characteristics (age, number of oocytes retrieved, and number of mature oocytes) according to DFI classes, has showed no significant difference in these parameters for the three DFI classes; this means that our various DFI classes have patients with the same characteristics. For age (P- value = 0.371) for the number of oocytes retrieved (P-value = 0.240), regarding the number of mature oocytes (P-value = 0.160) (Table 2).

In our cohort, no major variations in fertilization rates were found for the three groups of DFI. For the DFI class ≤ 30% the fertility rate is 86.4% ±20.3%, DFI 31%-60% the rate is 81. 9% ±23.8% and finally for the DFI class >60%, the rate is 76.1% ±16.1% (P-value = 0.155) (Table 2).

Regarding the distribution of the embryonic cleavage rate according to the various DFI groups, our findings have shown that there is no influence of the DFI rate on the cleavage rate. The number of embryos obtained (Mean ±SD) for the DFI class ≤ 30% is 4.3±1.6, from 31-% 60% 4.6±2.8 and from > 60% 4.8±2.7, the P-value = 0.622. (Table 2). The average embryo segmentation rate for the three groups of DFI (Low, Medium and High) is 90.6 %±23.7, 92.7%±14.6 and 91.3%±18.4, with a P-value equal to 0.118 (Table 2). The quality of the embryos obtained according to the three classes of DFI recommended in Table 2 shows that a significant relationship seems to be established between the rate of sperm DNA damage and the embryos quality. Furthermore, we note that embryos with good quality; grade A are in the majority in the group where sperm DNA fragmentation is high (DFI >60%).

The embryo implantation rate according to three classes of DFI has showed that there is no correlation between the DFI rate and the embryo implantation rate. For DFI ≤ 30% the rate is 74.3% ±31.1%, DFI 31%-60% the rate is 56.7% ±25.3% and for DFI >60% the rate is 66.7%±32.3%, the P-value is equal to 0.567 (Table 2). With regard to the rate of implantation, we note a decrease in the implantation rate and an increase in the rate of spermatic DNA fragmentation but this remains insignificant: DFI ≤ 30% 74.3 ± 31.1 for the DFI 31%-60% 56.7 ± 25%, DFI > 60% 66.7 ± 32.3 (P- value = 0.567) (Table2).

Table 2: ICSI parameters according to the three classes of DFI.

	DFI			*P*- value
	≤ 30 % n = 38	31-60 % n = 37	> 60 % n = 26	
Male age (years)	38.4±5.4	38.1±6.2	40.6±7.0	0.248
Sperm concentration (M/mL)	26.4±22.4	13.4±20.8	9.5±7.3	0.002
Sperm motility (%)	38.7±19.7	24.0±22.1	15.4±15.2	0.0001
Female age (years)	33.2±4.0	32.1±4.7	31.8±4.5	0.371
Oocyte retrieved (n)	7.3±2.7	8.5±3.2	7.9±3.2	0. 240
MII oocytes (n)	5.4±2.2	6.2. ±3.0	6.8±2.8	0.160
Fertilization rate (%)	86.4±20.3	81.9±23.8	76.1±16.1	0.155
Embryo obtained (n)	4.3±1.6	4.6±2.8	4.8±2.8	0.622
Cleavage rate (%)	90.6±23.7	92.7±14.6	91.3±18.4	0.118
Embryos Grade A (%)	46.4±32.7	45.6±33.5	66.3 ±32.4	0.002
Embryos Grade B (%)	35.4±26.8	37.9±31.6	23.2±25.1	0.112
Embryos Grade C (%)	9.2±15.6	15.7±26.2	9.3.1±22.0	0.357
Embryos Grade D (%)	3.7±16.8	2.7±10.0	1.1±5.6	0.705
Implantation rate (%)	74,3±31,1	56,7±25,3	66,7±32,3	0,567

DFI=DNA fragmentation index, Sperm concentration (M/ml): Million par ml MII=Metaphase II.

ICSI Parameters according to the two Extreme Groups of the DFI

In Table 3, we have performed a comparison between the two extreme classes of DFI (DFI ≤ 30% low damage / DFI > 60% high damage) with ICSI parameters. The comparison of the fertilization rate between the two extreme classes (DFI ≤ 30% / DFI > 60%), showed us a significant difference (P-value is equal to 0.03). For the cleavage rate, we did not observe any significant difference when comparing these two extreme classes P-value is equal to 0.892. Concerning embryo quality when comparing the two extreme classes of DFI. We have observed for grade A embryos, a significant difference between these two groups (DFI ≤ 30% / DFI > 60%) P-value is equal to 0.019. Note that the class of DFI >60% has earned the highest levels of embryos grade A with an average of 66.3% ±32.4% against 46.4%±32.7% for the class of DFI ≤ 30%. Alternatively, for the other embryo grades we did not observe any significant difference (Table 3). For the implantation rate we observed about this factor a decrease in the implantation rate for the group with DFI> 60% (Mean ± SD) 66.7% ± 32.3% compared to the group or the DFI ≤ 30% (Mean ± SD) 74.3% ± 31.1%, but this remains statistically insignificant P-value is equal to 0.600 (Table 3).

Table 3: ICSI parameters according to the two extreme groups of the DFI.

	DFI ≤ 30 % n = 38	DFI > 60 % n = 26	*P*-value
Fertilization rate (%)	86,4±20,3	76.1±16.1	0.035
Cleavage rate (%)	90,6±23,7	91.3±18.4	0.892
Embryos Grade A (%)	46.4±32.7	66.3 ±32.4	0,019
Embryos Grade B (%)	35.4±26.8	23.2±25.1	0,073
Embryos Grade C (%)	9.2±15.6	9.3.1±22.0	0,984
Embryos Grade D (%)	3.7±16.8	1.1±5.6	0,382
Implantation rate (%)	74,3±31,1	66,7±32,3	0.600

DFI=DNA fragmentation index

DISCUSSION

The assisted reproduction technology device an alternative to the problem of spousal infertility, the intracytoplasmic sperm microinjection is the technique offered to couples whose spouses have hypofertility with altered spermiological parameters.

Infertile men appear to have more genomes impaired than fertile males [23, 24]. Analysis of the heterogeneity of sperm DNA as an indicator of the outcome of an ART attempt (IVF or ICSI) has been extensively reported on in recent years [6, 25-27]. Indeed, several studies: such as the meta-analysis for Simon et al., 2017 [28] highlight the role of the male genome in the artificial reproductive stages, particularly during fertilization and early embryonic development. To analyze the alteration of the sperm genome numerous thresholds have been suggested and in this study three thresholds has been proposed (DFI ≤ 30 %, DFI 31%-60% DFI > 60%).

The findings showed that we did not find an association between an increase in the DFI rate and patient age while evaluating DFI at the three thresholds our findings are in agreement with those of the literature [16,29]. Even if we have not found a link between the age of the patients and the alteration of the sperm DNA, studies have proven that the age of male patients can be a risk factor for genetic disease transfer to their offspring [30,31] . Concerning the sperm parameters, our results confirm what has already been observed, the alteration of these parameters; concentration and motility, is associated with degraded sperm DNA [32-35]. With regard to the fertilization rate, we observe a difference between the fertilization rate in the three DFI groups but is not significant [26, 36]. For embryos, our results show that DFI does not influence early embryonic development (Day2-Day3) [37,38], or the quality of the embryos obtained [6,39]. We recording that the group with a high fragmentation of the sperm nucleus has obtained embryos of good quality. Concerning the implantation rate, our results show a decrease in the rate of implantation with increasing sperm DNA damage, but this remains statistically uninformative [40].

In the analysis of DFI in the extreme thresholds (<30% and >60%), our results do not show significant differences for all parameters except for the fertilization rate, which becomes statistically significant (P=0.03), as well as the embryonic quality, particularly grade A, which remains significant (P=0.019).

Indeed, for the fertility rate, our results show an increase in fertilization failure in men with a degraded sperm DNA; DFI >60%. Fertilization, which is a fundamental event involving a series of highly organized cellular and molecular processes that allow the fusion between the male and female gamete in order to edict a new cell, the zygote or unicellular embryo. This cascade of events may can be influenced by numerous episodes, such as poor oocyte or poor sperm quality, which may be morphological or genetic, thus preventing the formation of the paternal and maternal pronuclei [41]. Currently, the techniques of medically assisted procreation make it possible to respond in large part to these abnormalities. They are constantly evolving in order to improve the success rate, which unfortunately remains insufficient. In the technique of intracytoplasmic sperm microinjection, the barriers of natural selection are bypassed and fertilization with sperm carrying fragmented DNA may be possible [42]. Benchaib et al (2007) [16] indicate with an altered sperm, the embryologist have a greater proportion of spermatozoa which contains a fragmented DNA so that the probability of using an affected spermatozoon for oocyte injection is larger. Simon et al (2014) [8] described that spermatozoa with alterations in their DNA can lead to successful fertilization with pronuclei formation. Furthermore, an oocyte is capable of repairing this alteration, but the repair mechanisms fail above a certain threshold. [43,44]. Nevertheless, authors report that even if the oocytes are of good quality and manage to repair the damage present in the paternal DNA, this will hardly prevent generates of mutations that may appear in the embryo that will constitute the new individual [45-47].

The role of the male genome in this context is more often considered under two aspects; its involvement in events related to fertilization and its participation in the restoration of the diploid state after its addition with the oocyte genome. In this way, the sperm initiates the metabolic activation of the oocyte and transmits the centriole necessary for the establishment of the mitotic spindle allowing cell division. At the embryo level, the first stages of preimplantation development depend on the molecular sequences programmed by maternal transcripts. As regards the paternal genome, its influence on embryonic development only begins at a later stage [48].

Regarding our results on embryo quality, we notice that patients with DFI > 60% therefore patients with altered spermatic DNA are able to obtain better quality embryos than patients through no-altered sperm DNA with a DFI ≤ 30 %. Simon et al, (2014) observed [8]. an increase in embryo quality with increased sperm DNA damage. This result was in contrast to unexplained infertility group. This prototype is identical to the one that was used to form our cohort. Simon et al, (2014) [8] also describe a collapse in the rate of good quality embryos in the blastocyst stage in a population with degraded DFI. This phenomenon was explained by the late effect of the paternal genome. Guerin and Benchaib (2004) [49] report that for this period of preimplantation development, if the male pronuclei is altered, the embryo will develop to the 6-8 cell stage, but will not reach the blastocyst stage (64 cells). However, it is not unreasonable to assume that damaged paternal DNA may eventually allow for proper preimplantation development. Some authors have pointed out that there is a possibility of having a sperm with alterations in its genome to activate the process of fertilization and early embryonic development moreover, a sperm with an altered genome allows a pregnancy even if it has a high chance of developing into an abortion, [36,40].

Based on recent results from a previous study Hachemi et al (2019) [50], these findings confirm that even if the quality of the sperm genome does not influence the quality of the embryos during preimplantation development, the paternal genome is expressed late in order to impact the birth rate. Moreover, this genetic factor, which is the quality of the sperm DNA, remains an important element in improving the prognosis of the success rate, more precisely the rate of live births in artificial reproductive technology, especially in the technique of intra cytoplasmic sperm injection.

Also, we can assume that a patient with a sperm sample revealing degraded DNA can give live births. Only there is a risk of transmission of altered genetic material to the offspring, which may be responsible for the subsequent development of genetic diseases or even be responsible for the appearance of childhood cancers, as reported by several studies [51-53].

Our study has certain limitations. The main limitation is that no conventional IVF procedure was included. In fact, all our patients were referred to ICSI and this remains a choice of the patient himself in order to maximize the chances of success of the procedure. no transfer to the blastocyst stage has been performed.

CONCLUSION

The ICSI remains the only alternative, for infertile men with an altered spermatic DNA, Furthermore, our results show that after a microinjection with sperm containing degraded DNA, fertilization can occur and that the oocyte can repair the abnormalities present in the sperm DNA, but beyond a certain threshold, this process can be stopped. Our results confirm that the paternal genome does not affect the quality of the embryo in the first stage of embryonic development, but this would not hinder its expression in the later stages. Moreover, the ICSI technique remains a dependent operator, and it seems to influence certain outcomes more than is suggested.

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Authors Contribution

All authors have read and approved the final manuscript: all of the off them have contributed in literature search, data analysis, results interpretation and writing manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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