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RESEARCH ARTICLE

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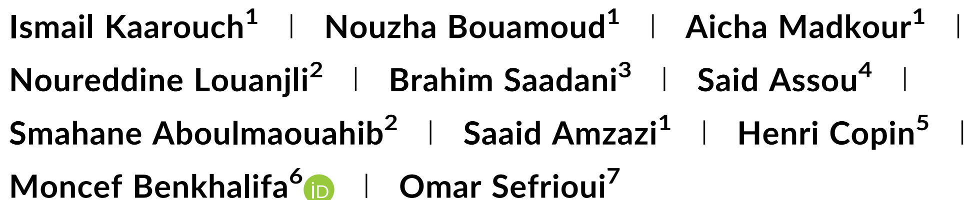
Paternal age: Negative impact on sperm genome decays and IVF outcomes after 40 years



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This study assessed sperm quality declining on relation to paternal age and its impact on in vitro fertilization (IVF) outcomes in order to estimate the APA (Advanced Paternal Age) cutoff. For this, 83 couples undergoing IVF treatment for male factor infertility were enrolled. The women age was ≤39 years, whereas the men were divided in two groups: APA ($n = 41$; age ≥ 40 years) and young (Y) ($n = 42$; age < 40 years). Conventional semen parameters (volume, concentration, motility, vitality, and morphology) were analyzed in the collected sperm samples. Furthermore, sperm genome decays (SGD) was assessed by TUNEL assay (DNA fragmentation), aniline blue staining (chromatin decondensation), and fluorescent in situ hybridization (aneuploidy). No significant difference was found concerning the conventional semen parameters between APA and Y groups. Conversely, SGD analysis showed increased DNA fragmentation; chromatin decondensation and sperm aneuploidy rates in the APA group (respectively, 41%, 43%, and 14% vs. 25%, 23%, and 4% in Y group). IVF outcomes also were affected by paternal age as indicated by the rates of cancelled embryo transfers, clinical pregnancy and miscarriage in the two groups APA and Y (29%, 17%, and 60% vs. 10%, 32%, and 42%). Finally, statistical analysis of the results suggests that the age of 40 should be considered as the APA cutoff during ART attempts.

KEYWORDS
advanced paternal age, in vitro fertilization outcomes, sperm genome decays, sperm quality

1 | INTRODUCTION

In assisted reproductive technologies (ART), the woman's age has always been considered as a determinant factor for success. Conversely, for a long time, paternal age was virtually ignored in

Abbreviations: APA, advanced paternal age; ART, assisted reproductive technology; FISH, fluorescent in situ hybridization; ICSI, intracytoplasmic sperm injection; PGS, pre-implantation genetic screening; SGD, sperm genome decays.

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studies on the effect of age on reproductive outcomes but its potential role has recently been strongly investigated. Indeed, as men continue producing sperm throughout their entire life, their biological clock has been underestimated compared with women. Furthermore, there is actually tending toward conception at an advanced paternal age with prevalence of 25% (Lawson & Fletcher, 2014). However, several recent works have shown a decline of male fertility potential over 40 years of age, particularly concerning sperm conventional parameters (Aitken, De Iuliis, & McLachlan, 2009; Alshahrani et al., 2014; De La Rochebrochard, McElreavey, & Thonneau, 2003; Kidd, Eskenazi, & Wyrobek, 2001; Sloter et al., 2006; Zini, Boman, Belzile, & Ciampi, 2008; Zini et al., 2011). Furthermore, many studies have demonstrated that the lower sperm quality linked to the advanced paternal age (APA) negatively affects embryo cleavage and in vitro fertilization (IVF) clinical outcomes. This effect was initially explained by alterations of conventional sperm parameters, such as sperm volume, concentration, motility (Belloc et al., 2009; Kidd, Eskenazi, & Wyrobek, 2001; Sobreiro et al., 2005), and morphology (Belloc et al., 2009; Cocuzza et al., 2008; Winkle, Rosenbusch, Gagsteiger, Paiss, & Zoller, 2009). Moreover, it was reported that clinical outcomes can be negatively correlated to paternal age in intracytoplasmic sperm injection (ICSI) procedures based on spermatozoa morphology selection. The last two decades many studies highlighted the importance of male fertility declining and spermatogenesis clock dysregulation (Skakkebaek et al., 2016). However, until today there is a lack of consensus concerning APA contribution on sperm parameters (Alshahrani et al., 2014; Andolz, Bielsa, & Vila, 1999; Fisch, Goluboff, & Olson, 1996; Kidd, Eskenazi, & Wyrobek, 2001), sperm DNA integrity (Belloc, Benkhalifa, Cohen-Bacrie, Dalleac, Chahine, et al., 2014; Sartorelli, Mazzucatto, & de Pina-Neto, 2001; Singh, Muller, & Berger, 2003; Sharma et al., 2015; Stone, Alex, Werlin, & Marrs, 2013), embryo development, clinical outcomes (Dain, Auslander, & Dirnfeld, 2011; Ferreira, Braga, Bonetti, Pasqualotto, & Iaconelli, 2010; Mathieu, Ecochard, & Bied, 1995) genetic and epigenetic state of fetus (Jyothy, Kumar, & Mallikarjuna, 2001), and/or of offspring (Sharma et al., 2015). It seems important to elucidate the risks to develop epigenetic diseases with paternal origin including autism and schizophrenia (Gingrich, 2016). For this reason completing conventional sperm analysis with minimal SGD tests including sperm DNA fragmentation, chromatin decondensation, and aneuploidy can be informative to assess risks (Belloc, Benkhalifa, Cohen-Bacrie, Dalleac, A., Amar, et al., 2014; Belloc, Benkhalifa, Cohen-Bacrie, Dalleac, Chahine, et al., 2014).

Studies suggest a link between male age and changes in the testicular environment, particularly the increase of reactive oxygen species production by mitochondria (Agarwal et al., 2016; Wiener-Megnazi, Auslender, & Dirnfeld, 2012), and this phenomenon is known to affect the integrity of the sperm genome and epigenome (Belloc, Hazout, et al., 2014; Benkhalifa et al., 2014; Lowe et al., 2001; Tanemura, Kurohmaru, Kuramoto, Matsumoto, & Hayashi, 1994). As the sperm genome is activated during the first embryonic cleavage stages, sperm DNA alterations could lead to embryonic development arrest due to elevated errors in post-meiotic chromatin remodeling and increased embryonic aneuploidy rate (Alio et al., 2012; Tesarik, Greco, & Mendoza, 2004). Moreover, sperm genome decays (SGD) can compromise embryo viability, resulting in pregnancy loss (Borini et al., 2006; Benkhalifa et al., 2014). Therefore, SDG is the most probable explanation for the negative impact of APA on reproductive outcomes.

Although SGD is considered as a consistent predictor of IVF outcomes, more investigations are necessary to understand the negative impact of APA on SGD and IVF outcomes. The purpose of this study was to evaluate SGD contribution in infertile male population undergoing ICSI and from which age this contribution become more significant.

2 | RESULTS

In this current study, the selected couples showed comparable clinical features of female patients to compare sperm quality between APA and Y groups (Table 1). Sperm conventional parameters including volume, concentration, morphology, motility, and vitality were not significantly different in the APA and Y groups. Conversely, semen molecular analyses revealed significantly higher rates of DNA fragmentation, decondensation and sperm aneuploidy in the APA group than in the Y group (41%, 43%, and 14% vs. 25%, 23%, and 4%, respectively) (Table 2).

Comparison of the IVF outcomes in the two groups showed that the fertilization, cleavage, blastulation,and clinical pregnancy rates were all lower in the APA group than in the Y group (56%, 94%, 24%, and 9% vs. 65%, 97%, 33%, and 16%, respectively). Moreover, the rates of cancelled transfers and of miscarriage were higher in the APA group than in the Y group (29% and 60% vs, 10% and 42%, respectively). Conversely, the implantation rate was not significantly affected by the paternal age (Figures 1 and 2).

The results obtained in both Y and APA groups (Table 3) allowed us to establish a positive correlation between paternal age and SGD. Furthermore, paternal age was also positively correlated with the miscarriage and cancelled embryo transfers rates and remained negatively correlated with the embryological outcomes and clinical pregnancy rate (except for the implantation rate). Paternal age was not correlated with conventional sperm parameters in this study.

Finally, statistical analysis depending on the linear increase of paternal age, allowed us to determine an APA cutoff for each parameter included in Table 3 with significant *r*-value. Our results suggest that SGD begins to be detectable at around 38 years of age, while IVF outcomes are meanly affected when sperm of men over 40 years is used for ICSI. APA effect on sperm conventional parameters, appeared later, at about 43–44 years of age.

3 | DISCUSSION

Although it is well established that maternal age over 35 years increases the risk of infertility and childhood genomes disorders risks. The possible negative effects of paternal age on IVF outcomes and risk of birth defects is still poorly investigated and estimated. The aim of this study was to assess advanced paternal age APA effect on

TABLE 1 Comparison of the women's characteristics

	Y ($n = 42$)	APA ($n = 41$)	p-value
Age (years)	33 ± 4.28	35 ± 3	0.06 (ns)
AMH (ng/ml)	3.2 ± 2.4	2 ± 3.3	0.07 (ns)
Estradiol (pg/ml)	33 ± 19	32 ± 21	0.29 (ns)
Progesterone (pg/ml)	0.46 ± 0.2	0.46 ± 0.21	0.12 (ns)
Endometrium thickness (mm)	9.5 ± 2.2	9.1 ± 1.7	0.06 (ns)
Oocytes per patient	11 ± 4.9	9 ± 4.1	0.10 (ns)
MII per patient	8 ± 4.1	6.5 ± 3	0.08 (ns)
Embryo transfers per patient	2 ± 1	2 ± 1	0.10 (ns)
Total embryo transfer cycles	38	29	0.13 (ns)

AMH, estradiol and progesterone were measured on day 2 of the cycle. Results are expressed as the mean ± standard deviation (SD). MII: mature oocyte metaphase II. A difference was considered significant (s) when $p < 0.05$; (ns), not significant.

conventional sperm parameters, Sperm Genome decays (SGD), and IVF outcomes in order to determine an APA cutoff that could be considered during ART procedures. In this work, the female partners were normally fertile and all younger than 40 years of age.

We did not find any significant difference in conventional semen parameters (sperm volume, concentration, morphology, motility, and vitality) in the two groups (Y vs. APA) (Table 2). This finding cannot totally refute the hypothesis that advanced age affects the semen conventional parameters because our results could have been influenced by the heterogeneous nature of the APA cohort. In the other hand, it is possible that the age-related male infertility becomes phenotypically diagnosed once APA exceeds 45–50 years (Eskenazi et al., 2003; Frattarelli, Miller, Miller, Elkind-Hirsch, & Scott, 2008; Hellstrom et al., 2006; Kidd, Eskenazi, & Wyrobek, 2011; Levitas, Lunenfeld, Weisz, Friger, & Potashnik, 2007; Schwartz & Mayaux, 1982; Sloter et al., 2006; Zini & Sigman, 2009). The observed decline in semen volume, sperm motility and morphology with APA in some studies (Johnson, 1986; Schneider, 1978) was explained by degenerative changes to the prostate and to the germinal epithelium, such as a decrease in protein and water content contributing relatively to the decrease in seminal volume and sperm motility and impacts generally the sperm morphology. Others, suggested that age-related sperm morphology defects are certainly a consequences of higher frequency of numerical and structural aberrations in sperm chromosomes (Sartorelli et al., 2001) with damaged DNA caused by double-strand DNA breaks and decreased apoptosis during spermatogenesis (Singh, Muller, & Berger, 2003).

In our study, despite the lack of APA impact on conventional sperm parameters, our results revealed a significant SGD increase including sperm DNA fragmentation, chromatin decondensation, and sperm aneuploidy rate comparing the Y with APA group (Table 2). These findings are consistent with many studies reporting a positive correlation between increasing male age and sperm DNA damage (Moskovtsev, Willis, & Mullen, 2006; Ramasamy et al., 2015; Sharma et al., 2015; Zitzmann, 2013) doubling from 25 to 55 years of age (Spanò et al., 2000). Indeed, APA could cause sperm DNA fragmentation

TABLE 2 Comparison of sperm parameters and sperm genome decays in the two paternal age groups

Characteristic	Y ($n = 42$)	APA ($n = 41$)	p-value	Power value
Age (years)	33 ± 4.28	44.8 ± 4.11	**0.01** (s)	-
Sperm volume (ml)	2.6 ± 1.1	2.4 ± 1.5	0.37 (ns)	17%
Sperm concentration (×10^6/ml)	24.3 ± 2.97	18.4 ± 2.64	0.34 (ns)	24%
Normal forms (%)	23%	21%	0.45 (ns)	13%
Motility (%)	33%	26%	0.16 (ns)	**97%**
Vitality (%)	60%	55%	0.24 (ns)	**99%**
DNA fragmentation (%)	**25%**	**41%**	**0.01 (s)**	**38%**
Chromatin decondensation (%)	**23%**	**43%**	**0.01 (s)**	**99%**
Aneuploidy (%)	**4%**	**14%**	**0.04 (s)**	**99%**

Bold values are presenting those with significant differences and high power value.
Results are expressed as n, n(%) or mean ± standard deviation (SD). DNA fragmentation, chromatin decondensation and aneuploidy were assessed, respectively, by TUNEL assay, aniline blue staining and FISH (13, 18, 21, X, and Y chromosome). A difference was considered significant (s) when $p < 0.05$; (ns), not significant.

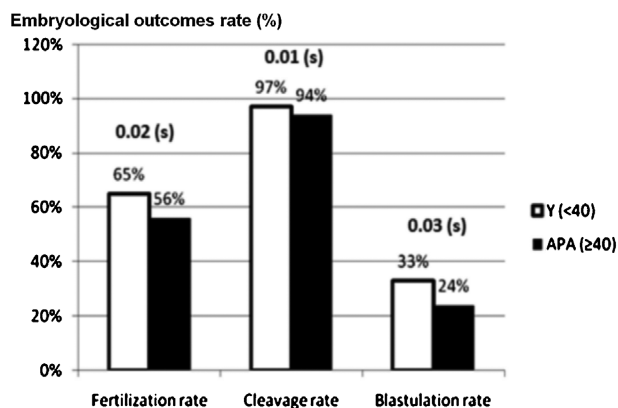

FIGURE 1 Comparison of the embryological outcomes in the APA and Y groups. Results are expressed as *n*(%). A difference was considered significant when $p < 0.05$ (s). The power value was considered important when above 80%; the mean power value of the embryological outcomes in this study was 84%

(Agarwal, Makker, & Sharm, 2008; Belloc, Benkhalifa, Cohen-Bacrie, Dalleac, Chahine, et al., 2014; Johnson et al., 2011; Nijs et al., 2011; Singh, Muller, & Berger, 2003; Wyrobek et al., 2006) and chromatin decondensation (Belloc et al., 2009; Nijs et al., 2011; Wyrobek et al., 2006). Moreover, there is relative linear correlation between APA and sperm aneuploidy rate (Agarwal et al., 2008; Sloter, Nath, Eskenazi, & Wyrobek, 2004; Sloter, Marchetti, et al., 2007; Wyrobek et al., 2006).

SGD is possibly due to an abnormal epigenetic reprogramming influenced by APA and affecting both spermatogenesis and spermiogenesis processes (El et al., 2011; Sharma et al., 2015) and resulting to global hyper-methylation of DNA with age ($r = 0.42$; $p = 0.006$) (Timothy et al., 2013) or specific methylation loss of paternal imprinted genes such as H19-DMR (El et al., 2011) and PEG 1/MEST-DMR

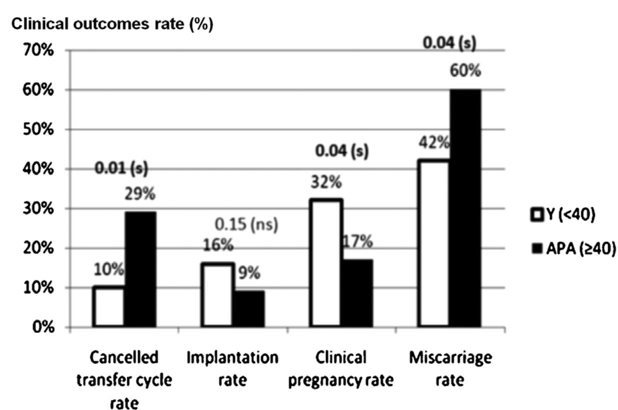

FIGURE 2 Comparison of the IVF clinical outcomes in the APA and Y groups. Results are expressed as *n*(%).The implantation rate is expressed as the ratio between the number of embryonic sacs and the total number of transferred embryos; the miscarriage rate is expressed relative to the number of clinical pregnancies. A difference was considered significant when $p < 0.05$ (s); ns, not significant. The power value was considered important when above 80%; the mean power value of the IVF clinical outcomes in this study was 95%

(Montjean et al., 2013). It was reported that there is a risk of negative correlation between age and genomic stability or expression levels of some genes, especially with decreased expression levels of Prm1 Prm2, Smcp (Benchaib et al., 2005)—and miRNAs expression changes (Belleannee, Legare, Calvo, Thimon, & Sullivan, 2013; Harries, 2014; Kotaja, 2014; Rossiello, Herbig, Longhese, Fumagalli, & Adda di Fagagna, 2014; Yan, Salazar, & Nguyen, 2013) which were dropped to 31% in the aged patients (Zhang et al., 2010). The mechanism through which this change in miRNAs expression and some gene methylation variations is suggested to be related to an increase of vulnerability with male age of selfish genetic elements (SGE) with an implicit role in evolution (Werren, 2011) or more known by selfish genes hypothesis of Dawkins (2016). The SGE effect is recently elucidated by an accumulation of de novo point mutations in the spermatozoa coding sequence, as well as significant methylation alterations with paternal aging (Katz-Jaffe, Parks, McCallie, Tignanelli, & Schoolcraft, 2017).

Indeed, all these genetic and/or epigenetic alterations of aged sperm involving the genomic instability as aforementioned could explain our embryological and clinical outcomes variations in the two groups. We observed decreased rates of fertilization, cleavage, blastulation and clinical pregnancy, and increased rates of cancelled transfer cycles and miscarriage in the APA group (Figures 1 and 2). Studies on the effects of paternal age on ART outcomes are still limited and their results are controversial. Some works suggested a negative correlation between paternal age and fertilization rate (Aboulghar et al., 2007; Katib, Al-Hawsawi, Motair, & Bawa, 2014; Luna et al., 2009), embryo quality, implantation rate, and pregnancy rate (Belloc et al., 2008; Bellver, Garrido, Remohí, Pellicer, & Meseguer, 2008; Frattarelli et al., 2008; Katib et al., 2014; Klonoff-Cohen & Natarajan, 2004; Luna et al., 2009; Robertshaw, Khoury, Abdallah, Warikoo, & Hofmann, 2013; Sharma et al., 2015). Conversely, others did not find any effect of paternal age on the rates of pregnancy, miscarriage, and live births (Alfaraj & Yunus, 2015; Alshahrani et al., 2014; Fernandez-Gonzalez et al., 2008; Jaleel & Khan, 2013; Kong et al., 2012; Meijerink et al., 2016; Pérez-Crespo, Moreira, et al., 2008; Pérez-Crespo, Pintado, et al., 2008; Whitcomb et al., 2011) especially in first IVF/ICSI cycles (Meijerink et al., 2016). As many different factors, such as maternal age, ART procedure and semen quality, should be considered when assessing the effects of paternal age on IVF outcomes, these discrepancies could be partially explained by the different study protocols/populations.

Differently from what we observed for fertilization, cleavage, blastulation, and clinical pregnancy rate, we did not find any difference in the implantation rate for the two groups (Figure 2). Ferreira, Braga, Bonetti, Pasqualotto, and Iaconelli, (2010) showed that paternal age negatively influences the implantation and pregnancy rates only in couples where the sperm concentration was lower than 20×10^6/ml. Similarly, Borini et al., (2006) found a close relationship between post-implantation development after ICSI and sperm DNA fragmentation that increased with paternal age. However, the effect of paternal age on the implantation rate is not easily comparable in different studies because it is strongly influenced by the number of transferred embryos that varies greatly, depending on the guidelines followed by the ART center.

TABLE 3 Correlation of sperm parameters and IVF outcomes with paternal age (Y and APA groups)

Parameters	Mean	R-correlation	*p*-value	APA cutoff
Sperm volume (ml)	2.5 ± 1.3	-0.11	0.29 (ns)	**44**
Sperm concentration (x10^6/ml)	21.4 ± 2.81	-0.14	0.20 (ns)	**43**
Normal forms (%)	22% ± 0.14	-0.30	0.30 (ns)	**42**
Motility (%)	30% ± 0.22	-0.16	0.35 (ns)	**43**
Vitality (%)	58% ± 0.20	-0.11	0.15 (ns)	**43**
DNA fragmentation (%)	33% ± 0.20	**0.49**	**0.001 (s)**	**38**
Chromatin decondensation (%)	33% ± 0.22	**0.55**	**0.001 (s)**	**38**
Aneuploidy (%)	9% ± 0.22	**0.22**	**0.04 (s)**	**40**
Fertilization rate (%)	61% ± 0.27	**-0.28**	**0.02 (s)**	**40**
Cleavage rate (%)	96% ± 0.19	**-0.27**	**0.02 (s)**	**40**
Blastulation rate (%)	29% ± 0.29	**-0.26**	**0.02 (s)**	**40**
Cancelled cycle transfer rate (%)	18% ± 0.4	**0.22**	**0.04 (s)**	**40**
Implantation rate (%)	12% ± 0.11	-0.18	0.29 (ns)	**-**
Clinical pregnancy rate (%)	25% ± 0.1	**-0.22**	**0.04 (s)**	**40**
Miscarriage rate (%)	47% ± 0.1	**0.47**	**0.001 (s)**	**39**

Bold values are presenting those with significant differences and high power value.
Results are expressed as n, *n*(%) or mean ± standard deviation (SD) in the total population. DNA fragmentation, chromatin decondensation and aneuploidy were assessed, respectively, by TUNEL assay, aniline blue staining and FISH (13, 18, 21, X, and Y chromosome). R-correlations were considered significant (s) when $p < 0.05$; (ns), not significant.

SGD results in an abnormal early embryonic development (Borini et al., 2006), embryo aneuploidy (Kaarouch et al., 2015) and affecting early embryo genome activation (EGA), that necessary for embryo genetic integrity maintaining toward genetic stability (Aitken et al., 2009; Benkhalifa et al., 2014; Emery & Carrell, 2006; Robertshaw et al., 2013; Tesarik et al., 2004; Simon et al., 2014; Zini et al., 2011). Whatever, there is possible sperm DNA damage repair or rescue by oocyte at fertilization stage or at later embryonic stage involving genetic repair systems generally producing embryo mosaicisms (Jaroudi et al., 2009; Kaarouch et al., 2015). However, repair failure of SGD can lead to de novo mutations and structural chromosomal alterations knowing that majority of them arise in male germline with APA. These mutations are generally transmitted to offspring with unknow nonviability risks of zygotes and early embryos (Beal, Yauk, & Marchetti, 2017). These negative effects can explain the increased miscarriage rate and decreased clinical pregnancy rates in APA (Bungum et al., 2007; Lewis et al., 2013; Priskorn et al., 2014;Robinson et al., 2012; Simon, Lutton, McManus, & Lewis, 2011; Zini et al., 2011).

Moreover, SGD is strongly associated with reduced fertility and lower probability of conception (Giwercman et al., 2010; Loft et al., 2003), but the most important thing is the involvement of SGD effect related to APA in genetic and epigenetic state of offspring (Sharma et al., 2015). Indeed, APA patients present an increased 5-mc and 5-hmc levels (methylated forms of cytosine) by 1.76% every year and causing evident gene silencing risk. This issue was proved to be consequence of several diseases as angelman Syndrome (neurogenetic disorder associated with both developmental and intellectual disability), Bechwith-Wiedmann Syndrome (genetic disorder which is usually associated with overgrowth and increased risk of childhood cancer), Apert's syndrome and achondroplasia diseases (Sharma et al., 2015; Whelan, Nwala, Osgood, & Olariu, 2016). Moreover, an accumulation of some sperm de novo point mutations and methylation alterations caused by APA, are specifically touching some genes related to neuropsychiatric disorders in offspring as Pex7 related to autism (Katz-Jaffe et al., 2017), DRD4, RDMR_2, TBKBP1, TNXB, and DMPK related to schizophrenia and bipolar disorder as well as many others (Timothy et al., 2013). Regarding oncological transmission in the offspring, Urhoj et al. (2017) revealed a presence of association between APA and higher rate of childhood acute lymphoblastic leukemia with 13% of hazard rate each 5 years in APA. Indeed, non-negligible autosomal dominant disorders are in increase due to selfish mutations tolerated at APA. In deeper evolutionary view, as it was established by Lewis et al. (2008) there are links between sperm competition and a range of issues not directly related to reproduction including SGE that are responsible of several epigenetic pathologies in offspring. As reported by Whelan, Nwala, Osgood, and Olariu, (2016), these SGE present selective advantage in the testis of the father despite a deleterious effect in offspring.

As our results indicate that APA can negatively impacts IVF outcomes even when conventional sperm parameters seem normal (but SGD are already present), we estimated the paternal age cutoff for IVF based on the different variables assessed in our study. This approach revealed that although SGD is detected from the age of 38, IVF outcomes are significantly affected only after the age 40, while conventional semen parameters begin to significantly decline after 42–44 years of age (Table 3). Interestingly, a retrospective study of Stone, Alex, Werlin, and Marrs, (2013) analyzing 5,081 sperm samples collected from men aged 16–72 years, The author's was able to establish APA threshold at 40 years old confirming relatively our results highlighting a clear declining of sperm concentration and sperm normal morphology after this APA value. Sperm motility and progressive

parameters of motile sperm fell after 43 years and ejaculate volume after 45 years.

In the other hand, we hypothesized that minimal the earlier SGD can be repaired by the oocyte (Jaroudi et al., 2009; Kaarouch et al., 2015; Ménézo, 2006; Perry, 2000) and that after the age of 40 its repair becomes difficult due to the extent of damage (Voyel et al., 2011). Our results suggest that 40 years can be considered the cutoff age for male patients undergoing IVF procedures. Whatever, APA necessitates a clear definition whereas almost are define it as 35–50 years of age (Wu, Lipshultz, & Kovac, 2016) or stratifying APA into 5-year brackets (Ménézo et al., 2007; Wu, Lipshultz, & Kovac, 2016), and our found APA cutoff at 40 years calls in need further studies.

Nevertheless, above this age, sperm quality may compromise fertilization and affect IVF success rate though the practice of sperm freezing at a younger age will no doubt be getting further attention in the future (Wu, Lipshultz, & Kovac, 2016). In the other hand, a large European multicenter study showed that the risk of infertility is significantly increased in male patients older than 40, when the female partner age is over 39 years of age (De La Rochebrochard & Thonneau, 2002; Wu, Kang, et al., 2016).

In summary, like maternal age over 35 years, paternal age over 40 years is a major risk factor for infertility. Therefore, couples should be cautioned about the potential IVF failure risks and SGD testing should be systematically performed to assess whether sperm DNA integrity needs to be improved through specific treatments. Although no treatment actually (in our knowledge) can fully restore the age-related decline in male fertility, various measures, such as exogenous antioxidant supplementation, may improve male fertility by minimizing age-related oxidative stress (Cocuzza et al., 2008; Cummins, Jequier, & Kan, 1994) and the related to SGD (Agarwal et al., 2016, 2008; Belloc, Benkhalifa, Cohen-Bacrie, Dalleac, Chahine, et al., 2014; Benkhalifa et al., 2014; Ménézo et al., 2007; Shamsi et al., 2012).

The age of parenthood has been steadily increasing over recent decades. It is widely acknowledge that fertility decrease with age in women. The impact of male age is now investigated. Although it seems smaller than that of female age, it is generally accepted that older men reduce the fertility and fecundity in a couple, especially when the female partner is with an advanced maternal age. Thus, APA could contribute to IVF failure, post-IVF miscarriages, and probable epigenetic disorders in offspring, even when conventional sperm parameters seem normal. Our analysis indicates that in couples undergoing IVF procedures, men older than 40 should undergo systematic SGD testing. Different factors such as life style changes, endocrine disruptor's, pollutants, and oxidative stress can be a major player of this fertility potential declining. Traditionally, only women are considered to possess a biological clock dysregulation; we now know that men do so as well from 40 years old.

4 | MATERIALS AND METHODS

4.1 | Ethical Standards

The study was approved by the ethics committee, (Comité d'Ethique pour la Recherche Biomédicale- Faculty of Medecine and Pharmacy, University Mohammed V, Rabat, Morocco) and patients provided written informed consent after being presented with the terms and issues of the study. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

4.2 | Patients' selection

This was an observational study over two years. It included firstly 204 couples who attended a reproductive medical center (IRIFIV and Anfa Fertility Center) and who were part of an IVF program for suspected male factor infertility. Thus, after selecting women younger than 40 years of age (range: 21 to 39 years) with unremarkable clinical history and comparable clinical features (Table 1), we obtained the selected population of 83 couples. Moreover, the rigorous patients' selection was based on exclusion criteria (consumption of alcohol and/or tobacco, azoospermia, cryptospermia, retrograde ejaculation, and collection problems (partial ejaculate), varicocele), patients with history of exposure to gonadotoxins such as chemotherapy, radiotherapy, or pesticides, and history of infection or fever in 3 previous months, antioxidant treatment, infertile couples with female factor). Indeed, we included couples with unexplained male infertility and previous IVF failures. All women received the same antagonist ovarian stimulation protocol (Madkour et al., 2016) to minimize the effect of other parameters. Men (24 to 56-year-old) were divided in two age groups, considering the age of 40 years as cutoff: APA group ($n = 41$; ≥40 years) and young (Y) group ($n = 42$; <40 years).

4.3 | SGD tests

Semen samples were collected from all included infertile men ($n = 83$) by masturbation after 3–5 days of sexual abstinence. Semen samples were firstly evaluated for spermogram analysis using a Makler chamber (Sefi Medical Instruments, Haifa, Israel) where it was analyzed for concentration, motility and smears of the fresh semen were stained using the Diff-Quik kit (Baxter Healthcare Corporation, Inc., Mc Gaw Park, IL) for assessment of sperm morphology. The smears were stained, rinsed in distilled water, air dried, and scored using Kruger's strict criteria (Kruger et al., 1986). After conventional sperm parameters evaluation (volume, concentration, motility, vitality, and morphology) based on WHO (2010) recommendations, samples were divided in three aliquots to assess DNA fragmentation by TUNEL assay, chromatin condensation by aniline blue staining and chromosomes aneuploidy by FISH with specific probes to chromosome 13, 18, 21, X, and Y (Aneuvysion™, Vysis, Downers Grove, III) (Kaarouch et al., 2015). These three tests were used to determine SGD level. Aliquots to assess DNA fragmentation by TUNEL assay chromatin decondensation by aniline blue staining as provided by (Kaarouch et al., 2015).

For TUNEL assay, briefly, 1.5 × 106 washed sperm cells were fixed with 2% paraformaldehyde (Sigma-Aldrich, Gillingham, UK) for 30 min at room temperature. Afterwards, cells were washed in PBS (Sigma-Aldrich) followed by permeabilization in 100 ml of a solution containing

0.1% Triton X–100 in 0.1% sodium citrate for 3 min on ice. After a washing procedure with PBS 1% BSA (500 g, 5 min), labeling was performed by incubation for 1 hr. at 37 °C with 50 ml labeling solution containing dUTP and 50 ml of enzyme solution (terminal deoxynucleotidyl transferase, TdT). Then, the sperm suspension was washed in PBS and counterstained by propidium iodide (2 mg/ml) to check on permeabilization. For each sample, a negative control was carried out by omitting the TdT enzyme from the reaction mixture. The positive control was obtained by incubating the spermatozoa with 2 IU DNase I for 15 min at 37 °C in Tris–HCl buffer before labelling. Fluorescence in sperm cells recorded as a positive for the TUNEL assay was assessed using a fluorescence microscope (Nikon Eclipse 80i) equipped with appropriate filters. At least 200 sperm cells from each sample were accounted, and the percentage of TUNEL-positive cells was calculated.

4.4 | IVF procedures

Embryos produced by ICSI (Madkour et al., 2016) were cultured up to day 3. Adequate embryo quality (good quality embryos; A + B) was defined based on the presence of uniformly sized and shaped blastomeres and fragmentation lower or equal to 10%. One or two good quality embryos were transferred in utero using a Frydman catheter (CCD Laboratories, Paris, France). The implantation success (observation of the embryo sac) was assessed by ultrasound imaging and calculated relative to the number of transferred embryos. Clinical pregnancy was confirmed by ultrasound imaging 6–8 weeks after embryo transfer and calculated relative to the number of transferred cycles. The miscarriage ratio was calculated relative to the number of clinical pregnancies after the first trimester. Each couple went through a single ICSI cycle during this study.

4.5 | Statistical analysis

Data are presented as the mean ± standard deviation (SD) or percentage of the total. Data were analyzed with the Student's t-test for comparison of mean values or with the chi squared test for comparison of percentages, using Statistical Package, version 6.0 (Statistica); $p < 0.05$ shows significant differences. Then, the mean values of each parameter's results were evaluated to calculate the study power with the post-hoc test using the G*Power software (version 3.0.10).

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