Am J Transl Res 2023;15(5):3759-3765
www.ajtr.org /ISSN:1943-8141/AJTR0147726

Original Article

The number of retrieved oocytes is correlated to sperm DNA fragmentation when predicting pregnancy outcomes

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Received November 13, 2022; Accepted April 26, 2023; Epub May 15, 2023; Published May 30, 2023

Abstract: Objectives: To analyze the correlation between age and sperm DNA fragmentation index (DFI), and determine whether the number of eggs retrieved from the female partner was associated with the impact of sperm DFI on clinical pregnancy rates. Methods: A retrospective analysis of 896 couples aged 19-58 years who were treated at our hospital between 2019 and 2021 was performed to analyse male semen parameters and to investigate the correlation between male age, semen parameters and DFI. In total, data from 330 cycles of assisted reproduction in couples over 40 years of age were analyzed, including 66 cycles with a normal DFI (≤ 15) and 264 cycles with an abnormal DFI (> 15), so as to correlate clinical outcomes based on the number of eggs retrieved per woman and DFI. In order to identify factors associated with clinical outcomes, logistic regression analysis was carried out. Results: There was no significant decrease in semen parameters (motility and concentration) with increasing age of the male partner ($P > 0.05$). DFI was positively correlated with male age and was significantly higher when age was ≥ 40 years ($P = 0.002$). A lower number of eggs retrieved (< 4) led to a reduced clinical pregnancy rate; with similar outcomes being found for a reduction in DFI. Conclusion: When male partner age exceeded 40 years, both the DFI and the number of eggs retrieved affected the clinical pregnancy rate.

Keywords: DFI, male age, clinical pregnancy rate, sperm DNA fragmentation, pregnancy outcomes

Introduction

The average age for pregnancy has gradually increased for both the male and female partner, within the last few decades [1]. Couples of advanced reproductive age are well-known to face various difficulties and challenges during pregnancy and delivery. A significant body of data would correlate increasing female age with elevated risks of infertility, fetal anomalies, pregnancy loss, stillbirths and various other obstetric complications [2]. However, comparatively much less attention has been focused on the processes and outcomes of pregnancy with regards to males of advanced reproductive age. Nevertheless, the fact remains that some studies had proven that advanced reproductive age is negatively correlated with DNA integrity and semen quality, including sperm morphology, and various semen parameters such as volume, vitality, progressive motility and concentration of peroxidase-positive cells. However, researchers have not yet investigated the relationship between male age and preterm birth [3], miscarriage [4], psychiatric disorders [5] (egg, autism, psychosis, and bipolar disorders) and malignancies [6].

To date, the correlation of paternal age with semen quality and reproductive outcomes remain controversial as there is no uniform definition of 'advanced paternal age', and also because different studies on male fertility have

presented different outcomes. Unlike females, the definition of age thresholds for men ranges from 29 to 45 years [7-10]. Meanwhile, the general consensus is that the sperm motility and count decreases with paternal age [7, 10, 11]. However, there is no general consensus on sperm concentration. Some studies reported an increase in sperm concentration with age [12], while others report the opposite findings [13]; with some researchers maintaining a neutral opinion [7, 8, 10, 14]. In many cases, standard semen analysis fails to detect any abnormalities and does not distinguish between fertile and infertile populations, and semen parameters of fertile and infertile men extensively overlap. It is therefore important to consider other potential indicators for the prediction of pregnancy rates. Indeed, it is becoming increasingly apparent that sperm function and embryonic development is strongly correlated to sperm DNA integrity. Sperm DNA fragmentation is directly related to the level of genetic damage to sperm DNA. Sometimes, failed conception may arise from the unusual genetic makeup of the sperm, despite the good sperm parameters of the male partner. Sperm DNA breakages are visible due to fragmentation. The sperm DNA fragmentation index (DFI) is known to increase with age and there is no consensus with regards to its effect on pregnancy and the threshold for DFI. Thus, this study aimed to investigate the effects of male age on semen parameters and pregnancy by evaluating a total of 892 patients in our centre between 2019 and 2021.

Materials and methods

Study population

Using data from the Reproduction Centre of Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, 896 couples aged 19-58 years, 147 of whom were ≥ 40 years old, were enrolled in the computer software "Assisted Reproduction Management System" (technical support: Shanghai Chuteng Information Technology Co., Ltd.) in the period 2019 to 2021. These couples underwent a total of 330 cycles, with 66 cycles of normal DFI (≤ 15) and 264 cycles of abnormal DFI (> 15). Female patients were excluded from this study if they had been diagnosed with various uterine pathologies, including myoma, adenomyosis, and uterine synechia. Male patients who had been treated with vitamins, carnitine, or Chinese medicine were also excluded from the study. Clinical pregnancy was defined by the presence of a foetal heartbeat in the uterus, as evaluated by ultrasound at between 4 to 5 weeks following embryo transfer. Ethical approval was granted by the Ethics committee of the Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (No. 2022-818-24-03).

Semen analysis

After two to seven days of abstinence, various semen parameters were analyzed by computer aided semen analysis (CASA) after 30 minutes of liquefaction. The main semen parameters analyzed were sperm motility, concentration, and morphology; which were all analyzed in accordance with the World Health Organization (WHO) standards (2010). The sperm chromatin dispersion (SCD) test was used to assess sperm DFI with a Halosperm Kit (Halotech, Spain). Briefly, this involved diluting the sperm concentration to 5-10 million per ml. Then an agarose gel was melted by placing it within a test tube that was immersed for 5 minutes in a water bath at 90-100°C. Subsequently, the semen samples were added and covered with coverslips. The slides were cooled in a refrigerator at 4°C for 5 min, followed by gentle removal of the coverslips and quick immersion in acidic solution for 7 min, after which they were placed for 25 min in a lysis solution. After washing for 5 min in distilled water, the slides were dehydrated in different concentrations of ethanol (70%, 90% and 100%) followed by air drying. A minimum of 400 spermatazoa per patient were examined and these were defined as DNA fragmentation-positive if they displayed very small or no halo. All semen analysis was carried out by the same observer.

Stimulation protocol

One of the following stimulation protocols was selected based on individual patients' medical conditions: downregulation of gonadotropin-releasing hormone (GnRH) antagonist suppression protocol (Cetrotide; Pierre Fabre) or luteal-phase GnRH agonist (Diphereline; Ipsen). This was followed by the administration of follicle-stimulating hormone (Gonal-F; Merch Serono) or urinary human menopausal gonadotropin

Table 1. Effects of age on semen parameters and DFI

Group	Motility	Concentration	DFI
< 30	40.26 (16.15)	75.68 (42.19)	17.00 (15.38)
30-35	40.33 (16.79)	77.12 (45.37)	17.92 (17.50)
35-40	40.70 (15.50)	82.65 (44.39)	16.05 (14.12)
> 40	36.52 (18.38)	68.95 (43.62)	21.226.25 (15.00)
P	0.488	0.268	0.002

Abbreviation: DFI: DNA fragmentation index.

(Lizhu; Zhuhai, China) during the early follicular phase of the cycle. When at least three follicles reached 17 mm in diameter, human chorionic gonadotropin (hCG) was administered at 5,000-10,000 IU followed by oocyte retrieval 34-36 hours later. Clinical pregnancy was defined as the presence of a visible intrauterine sac observed with transvaginal sonography at 7 weeks of gestation.

Statistical analysis

SPSS Statistics for Windows (Version 26.) software was utilized for data analysis. To correlate the risks of having high sperm DNA damage to clinical pregnancy rate, we divided patients into two categories based on DFI and the number of retrieved eggs from the female partner. A binary logistic regression model was used to analyse the correlation between each factor and clinical pregnancy rates, and the odds ratio (OR) was calculated and presented with 95% confidence intervals (CI) and *P*-values. Statistical significance was set at $P < 0.05$.

Results

Correlation of age with semen parameters and DFI

Upon comparing the four age-dependent groups (≤ 29 years *vs.* 30-34 years *vs.* 35-39 years *vs.* ≥ 40 years) with a total of 896 male outpatients, it was observed that there were no significant differences in terms of sperm motility or concentration. There was significantly higher DFI in men ≥ 40 years versus men < 40 years ($P = 0.002$; **Table 1**).

The clinical pregnancy rate per cycle

In the advanced age (≥ 40 years) group, monofactor analysis suggested that both the number of eggs retrieved and DFI were significantly correlated with clinical pregnancy rates ($P = 0.008$; $P < 0.0001$). Subsequently, multivariate logistic regression showed that men with an abnormal DFI were associated with a significantly reduced clinical pregnancy rate (10.6% *vs.* 30.3%, respectively; $P < 0.0001$). Additionally, there was a significantly lower clinical pregnancy rate when the number of oocytes retrieved was < 4 when compared with ≥ 4 retrieved oocytes (12.0% *vs.* 25.8%, respectively; $P = 0.028$; **Table 2**).

Discussion

Infertility is a worldwide problem involving couples of childbearing age in all countries and regions of the world, with epidemiological surveys indicating that male factor infertility constituted approximately 50% of all cases [15]. Semen analysis (SA) is a key indicator for the assessment of male infertility, but it does not cover all causes of infertility. WHO has acknowledged the limitations of SA and has emphasized the importance of sperm functional testing and the evaluation of sperm DNA fragmentation (SDF) as a further test in the 6th edition of the Laboratory Manual for the Examination and Processing of Human Semen [16]. Potential pathophysiological mechanisms leading to SDF include sperm chromatin packaging defects, apoptosis and excessive oxidative stress (OS).

We assigned male patients into four groups based on their age, and no significant differences were detected between the groups with regards to sperm motility and concentration, except for DFI in men over 40 years of age, which was significantly higher compared to the other age groups. The classification of male age as 40 years is consistent with the observations made in some studies [17-19]. The British Andrology Society and American Society for Reproductive Medicine have set 40 years as the upper age limit for sperm donors [20, 21]; which was consistent with our present results.

Meanwhile, it was observed that male semen parameters (sperm motility and concentration) did not decrease with age in couples undergoing assisted reproductive technology (ART) treatment; which may be explained by only a small fraction of these men having abnormal

Table 2. The rate of clinical pregnancy per cycle according to DFI (≤ 15% vs. > 15%) and the number of oocytes retrieved (< 4 vs. ≥ 4)

		Cycles (clinical pregnancy)	The rate of clinical pregnancy	OR (95% CI)	P
DFI	≤ 15%	66 (20)	30.3%	3.38 (1.72, 6.55)	< 0.0001
	> 15%	264 (28)	10.6%		
oocytes	< 4	267 (32)	12.0%	2.18 (1.06, 4.35)	0.028
	≥ 4	62 (16)	25.8%		

Abbreviation: DFI: DNA fragmentation index; OR: odds ratio; CI: confidence intervals.

semen parameters prior to treatment. However, this also implied that conventional semen analysis did not provide a comprehensive assessment of sperm quality and male fertility. Thus, researchers turned their attention to the DNA integrity of sperm, and used this as a parameter to determine the proportion of sperm that have abnormal genetic material. This is now deemed to be a key male infertility marker [10, 22-25]. Sperm DNA plays a key role in the developing embryo and can be damaged before or during ejaculation. The causative factors include smoking, varicocele, testicular heat, exposure to heavy metals, obesity, reactive oxygen species, testicular infections, and increasing paternal age [26].

In this study, we found that clinical pregnancy rates correlated with DFI and the number of oocytes retrieved from the female partner. The threshold for DFI was ≤ 15 when using 40 years-of-age as a threshold. Higher DFI levels are observed more commonly in infertile men [27, 28]. There is a positive correlation between sperm DNA integrity and male age [10]. Increasing sperm DNA damage is correlated with recurrent pregnancy loss (RPL) and genomic instability during early embryonic development [29]. Some studies concluded that sperm DNA damage is detrimental to clinical pregnancy outcome following *in vitro* fertilization (IVF) and/or intracytoplasmic sperm injection (ICSI) treatment [30-32]. Furthermore, for patients undergoing IVF, the rate of DFI in the male partner has a detrimental effect on cumulative live birth rates [33]. In contrast, other studies proposed that there was no correlation between DFI and pregnancy outcomes [34]. Nevertheless, some studies reported that sperm DNA single- or double-stranded breaks were associated with lower IVF pregnancy rates, but not in the case of ICSI [35]. The effects of DFI on IVF and/or ICSI remains controversial; because previous studies did not take into consideration various female factors, such as oocyte quality [36], ovarian reserve [37] or female age [31].

We found that clinical pregnancy rate for males of advanced reproductive age was not only related to DFI, but also to the number of eggs retrieved from the female partner. Critical DFI values reported in the medical literature ranged from 4% to 56%, without any definitive 'normal range' to distinguish between male infertility and infertility [38, 39]. This is because the final live birth rate is dependent on various factors, for example the SDF test, infertility factors, and the experience of the laboratory in handling semen. Our study found that when the threshold for DFI was set at 15%, there was a guideline for clinical pregnancy rates in couples undergoing ART, which is consistent with some previous studies [40, 41]. When DFI was reduced by one level (DFI ≤ 15%), the probability of pregnancy success increased by 3.379-fold. When the number of retrieved eggs was ≥ 4, the pregnancy success rate increased by 3.18-fold. To the best of our knowledge, this is the first report of the effects of DFI and the number of retrieved eggs on clinical pregnancy rate. In a previous study, Jin et al. found that DFI had a greater impact on IVF and ICSI outcomes among women with reduced ovarian reserve (ROR), whom were treated by the short flare-up protocol [37]. This was despite the fact ROR is associated with poor reproductive outcomes due to reduced quantity and quality of retrieved oocytes. However, treatment strategies are being investigated to develop methods that could increase the yield of oocytes. These options include changing the stimulation protocols, the types and dosages of gonadotropins, the use of adjuvants, dual stimulation cycles and the manipulation of ovarian tissue to activate primordial follicles. The number of oocytes retrieved would change according to these different methods. The choice of the appropriate

treatment protocol is dependent on the patient characteristics [42-47]. The real clinical scenario requires a flexible approach rather than just using a single ovulation protocol.

Furthermore, oocytes have the ability to repair damaged DNA from sperm, and the impact of SDF levels on the results of IVF and ICSI pregnancies may depend on oocyte quality [48-50]. However, there is a lack of effective means of assessing oocyte quality during treatment with assisted reproductive technologies. Oocyte quality is influenced by many factors and reduced ovarian reserve and poor response to ovarian stimulation are thought to be correlated with poor oocyte quality [51]. The current study found that patients had a lower pregnancy rate when the number of eggs retrieved per cycle was < 4. It is reasonable to hypothesize that the lower the number of eggs retrieved per egg retrieval cycle, the poorer the quality of the oocytes in that cycle is likely to be.

In conclusion, a reduced number of retrieved oocytes is a sign of diminished ovarian reserve, as well as poor ovarian response to gonadotropin stimulation, and may reflect a decline in oocyte quality. Reduced oocyte quality and poor ovarian environment is unable to overcome the DNA damage in sperm cells, ultimately leading to lower clinical pregnancy rates.

Disclosure of conflict of interest

None.

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