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ORIGINAL ARTICLE

Prolonged incubation of processed human spermatozoa will increase DNA fragmentation

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Summary

One of the causes of failure in ART is sperm DNA fragmentation which may be associated with long period of spermatozoa incubation at 37 °C. The objective was to evaluate the rate of sperm DNA fragmentation using the sperm chromatin dispersion (SCD) test after swim-up at different time intervals prior to use. In this prospective study, 21 normozoospermic specimens were analysed. The samples were incubated at 37 °C after preparation by direct swim-up. DNA fragmentation was assessed at different time intervals (0, 1, 2 and 3 h) using SCD test. Spermatozoa with no DNA fragmentation showed large- or medium-sized halos, and sperm cells with DNA fragmentation showed either a small halo or no halo. The rates of normal morphology and progressive motility after sperm processing were 72.33 ± 2.53% and 90 ± 1.02%, respectively. The rate of sperm DNA fragmentation was significantly higher after 2 h (8.81 ± 0.93%, $P = 0.004$) and 3 h (10.76 ± 0.89%, $P < 0.0001$) of incubation compared to 0 h (4.38 ± 0.8%). A positive correlation was found between the incubation time and sperm DNA damage ($P < 0.0001$). Prolonged incubation of prepared normozoospermic samples at 37 °C is associated with higher rates of sperm DNA fragmentation. Therefore, sperm samples intended for ART procedures should be used within 2 h of incubation at 37 °C.

Introduction

Assisted reproductive technologies (ARTs) have assisted infertile couples to have children of their own. Undoubtedly, one of the most important factors that may affect the success rate in treatment of infertile couples would be the quality of gametes. In all ART procedures, for sperm separation from other cells, the semen sample should be obtained and processed in *in vitro* condition, and the sperm DNA fragmentation may increase spontaneously due to long incubation (Muratori *et al.*, 2003). So, paying attention to avoiding iatrogenic injuries to spermatozoa in this critical situation is a rational issue.

After semen processing, the spermatozoa are routinely kept in 37 °C incubator for capacitation (van der Westerlaken *et al.*, 2006). However, there is no determined agreement on optimised time for incubation of sperm cells prior to use in ART. The studies regarding the effect of long-term *in vitro* incubation at 37 °C on sperm motility and viability have been shown that incubation for 24 h can impair sperm motility and viability (Calamera *et al.*, 2001; Lachaud *et al.*, 2004; Zhang *et al.*, 2011).

The conventional sperm parameters, in most clinics (e.g. sperm count, motility and morphology), were evaluated for the assessment of sperm quality. Regarding this fact that infertile men may have a normal spermogram (Guzick *et al.*, 1998), routine semen analysis does not reflect functional evaluation as well as DNA status of spermatozoa (Shamsi *et al.*, 2011). Recently, Lewis & Simon (2010) stated that there is no correlation between conventional sperm parameters and DNA damage.

The presence of abnormality in sperm DNA integrity is often associated with decreased fertilisation (Simon *et al.*, 2010, 2011). This can affect the pre-implantation embryo development. Although a spermatozoon with damaged DNA may fertilise the egg (Yamauchi *et al.*, 2012), blastulation rate would be lower in embryos derived from spermatozoa with high DNA damage (Nasr-Esfahani *et al.*, 2005). In addition, poor pregnancy outcome (Fernández-Gonzalez *et al.*, 2008), increased level in spontaneous

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abortion (Shamsi *et al.*, 2010), and increased risk of morbidity and cancer are other possible phenomenon following application of damaged sperm DNA in ART cases (Agarwal & Allamaneni, 2004; Shamsi *et al.*, 2008).

Short incubation of processed spermatozoa at 37 °C is suggested to improve sperm capacitation prior to use in ART (Marín-Briggiler *et al.* 2002). But, there is no general agreement regarding the duration of incubation period before use in ART. Our main goal was to assess the rates of sperm DNA fragmentations using the sperm chromatin dispersion (SCD) test after swim-up at different time intervals. This will show the optimal incubation period for processed spermatozoa in ART program.

Materials and methods

Patients

This study involved 21 normozoospermic specimens from men undergoing infertility work-up between July 2011 and March 2012. The samples were obtained from patients who were referred to Yazd Research and Clinical Center for Infertility. All the patients were signed the consent form. This study was approved by author's institute review board.

Semen collection and analysis

Semen specimens were collected by masturbation. The abstinence period for all participants was between 3 and 7 days. Semen analysis was carried out according to WHO guidelines (WHO, 2010). For sperm count and motility, Makler chamber was used (Khalili *et al.*, 2005). Sperm motility was evaluated by light microscopy and reported as percentage of progressive, nonprogressive and immotile spermatozoa. Sperm viability was evaluated by Eosin-Nigrosin staining test. Papanicolaou staining was applied for morphology assessment. The slides were assessed for morphological abnormality in tail, neck or head (WHO, 2010).

Sperm preparation

After conventional analysis of sperm, the samples were prepared by direct swim-up method. Ham's F10 supplemented with 5 mg ml^{-1} HSA was used as sperm culture medium. Briefly, 1 ml of semen was placed under 1.2-ml culture media gently in a sterile conical tube and put in the 37 °C incubator at the 45 angle for 1 h. Thereafter, the uppermost medium was removed and diluted with 2 ml culture medium. After centrifugation at 400 g for 5 min, the pellet was resuspended in 0.5 ml Ham's F-10 warmed to 37 °C. Approximately 0.5 ml of the culture medium was gently added to washed spermatozoa, and the tube was closed tightly and placed at 37 °C incubator. Before and after swim-up, conventional sperm parameters were evaluated. DNA fragmentation was assessed at different time intervals (0, 1, 2 and 3 h) using SCD test.

Sperm chromatin dispersion test

The SCD test as described by Fernandez *et al.* (2003) was adapted. In brief, the glass slides were coated by 0.65% standard agarose (Merck, Germany). After direct swim-up, 30 μl of sperm suspension was mixed with 70 μl low melting agarose (Roche, Germany). Aliquots of 50 μl of the mixture were put onto a precoated glass slide, and left to solidify at 4 °C for 4 min. The slides were then immersed in denaturation solution (0.08 N HCl) (Merck, Germany) for 17 min at room temperature (RT) in dark. The slides were transferred into lysing solution 1 (0.4 M Tris, 2-Mercaptoethanol, 1% SDS, and 50 mM EDTA, pH 7.5) for 20 min at RT. Then, the slides were placed in the lysing solution 2 (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) and washed in Trisborate- EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 15 min and 12 min at RT respectively.

For dehydration, the samples were dehydrated for 2 min in 70%, 90% and 100% ethanol and left to dry at RT. For staining, the Wright stain solution (Sigma-Aldrich, USA) was mixed with phosphate buffer solution (PBS) (1 : 1). The slides were covered by Wright stain for 10 min followed by rinsing in running water and left to dry for light microscopy evaluation. The sperm cells may show different halo patterns as pursue: large halos considered as the halo size is more than the minor diameter of core width, small halos size was smaller than one-third of the minor diameter of core width, and medium haloes size was considered between large and small halos. Spermatozoa with no DNA fragmentation showed large or medium-sized halos, while sperm cells with DNA fragmentation showed either a small halo or no halo (Fig. 1). At least 200 spermatozoa were checked and sperm DNA damage was calculated by dividing the abnormal to the total spermatozoa and reported as percentage.

Statistical analysis

Data were shown as mean ± SEM. Independent sample t-test was used for comparison of parameters between before and after sperm preparation. One-way ANOVA with Tukey post hoc test were applied for comparison of sperm DNA damage between different groups. Also linear (Pearson) correlation test was used to find out the correlation between the incubation time and sperm DNA damage. Significant level was set at two-side P -value<0.05.

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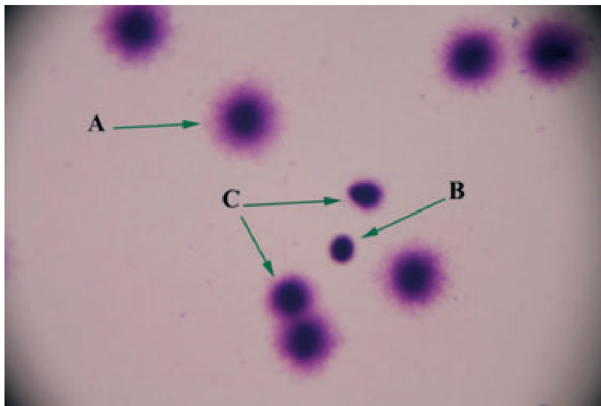

Fig. 1 Sperm chromatin dispersion (SCD) test. (a) Big halo represents no DNA fragmentation, (b) No halo and (c) Small halo show sperm with DNA fragmentation.

The Statistical Program for Social Science (SPSS 16.0, Chicago, IL) software was applied for statistical evaluation.

Results

Mean semen volume was 4.8 ± 0.34 ml, and all samples had normal viscosity. Progressive motility before and after processing was 63.71 ± 1.83% and 90 ± 1.02% respectively. Also after sperm processing, more than 90% of sperm cells were viable and over 70% had normal morphology (Table 1).

There was an increasing trend in sperm DNA fragmentation after incubation (Table 2). No significant difference was seen in percentage of sperm cells with fragmented DNA after 1 h compared to 0 h (6.14 ± 0.89 versus 4.38 ± 0.8), also 2 h compared to 1 h ($P = 0.15$) and 3 h compared to 2 h ($P = 0.4$). However, there was significant increase in sperm DNA fragmentation after 2 h (8.81 ± 0.93, $P = 0.004$) and 3 h (10.76 ± 0.89, $P < 0.0001$), also 3 h compared to 1 h ($P = 0.002$). There was positive correlation between the incubation time and sperm DNA damage (correlation coefficient (r) = 0.52, coefficient of determination (r squared) = 0.27, confidence interval (CI) 95% = 0.34–0.66, $P < 0.0001$) (Fig. 2).

Table 1 Sperm parameters before and after direct swim-up processing

Parameters	Before	After	*P*
Count (× 10^6)	97.86 ± 6.76	54.57 ± 5.29	<0.0001
Round cells (× 10^6)	0.84 ± 0.14	0	<0.0001
Progressive motility (%)	63.71 ± 1.83	90 ± 1.02	<0.0001
Viability (%)	81.86 ± 1.2	99.10 ± 0.27	<0.0001
Normal morphology (%)	49 ± 3.1	72.33 ± 2.53	<0.0001

Values are mean ± SEM.

Discussion

Incubation of processed spermatozoa at 37 °C in order to use in ART is an obligatory and routine work in infertility clinics. Evaluation of *in vitro* incubation of sperm cell has been matter of research in last decades. The early investigations were about the effect of sperm incubation on traditional sperm parameters. By elucidation of important role of sperm DNA integrity as well as introduction of new techniques for evaluation of sperm DNA status (e.g. terminal deoxynucleotidyl transferase–mediated dUTP nick end-labelling (TUNEL), sperm chromatin structure assay, sperm chromatin dispersion test, comet assay), several researchers investigated the association between various sperm incubation periods and sperm DNA integrity (Bungum M *et al.* 2010; Calamera *et al.*, 2001; Matsuura *et al.*, 2010; Muratori *et al.*, 2003; Zhang *et al.*, 2011). In this study, we tried to find the effect of incubation of spermatozoa at 37 °C on sperm DNA fragmentation using SCD test. The data generated from this study showed that there was a significant increase in sperm DNA fragmentation after incubation for 2 h.

Recently, Matsuura *et al.* (2010) evaluated the impact of different *in vitro* incubation conditions, using fresh semen, and compared the effect of incubation at 37 °C with and without CO_2 on sperm DNA fragmentation index (DFI). It was showed that after 3 h and 24 h, DFI was higher significantly at 37 °C with CO_2 compared to without CO_2 as well as in 37 °C compared to RT. Dalzell *et al.* (2004) reported increased DNA damage in testicular spermatozoa after 4 h of incubation at 37 °C. Hammadeh *et al.* (2001) showed that sperm with decondensation is increased after incubation at 37 °C. They observed a significant increase in chromatin decondensation from 25–88% during 2 h. Fernandez *et al.* (2007) investigated the dynamics of sperm DNA fragmentation in the stallion and concluded that the highest intensity of sperm DNA damage occurred up to 6 h incubation at 37 °C. Another harmful effect of incubation at 37 °C is morphological change in sperm head. Peer *et al.* (2007) showed an increase in spermatozoa with vacuolated nuclei after 2 h incubation at 37 °C compared to 21 °C.

Our findings were similar to others regarding sperm DNA fragmentation even after preparation by direct swim-up (Muratori *et al.*, 2003; Zhang *et al.*, 2011). In contrast, Zhang *et al.* (2011) showed that DNA fragmentation will increase after 4 h of incubation, whereas no significant difference was reported after 2 h. One of the probable causes of this conflict may be in the method of sperm preparation. They used indirect swim-up method

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Table 2 Rate of human sperm DNA fragmentation before and after incubation at 37 °C

	Before swim-up	After swim-up (0 h)	Incubation time (h)		
			1	2	3
Fragmented DNA (mean ± SEM)	22.24 ± 2.59	4.38 ± 0.8*	6.14 ± 0.8**	8.810 ± 0.93	10.76 ± 0.89

*$P = 0.004$ versus 2 h, $P < 0.0001$ versus 3 h.
**$P = 0.002$ versus 3 h.

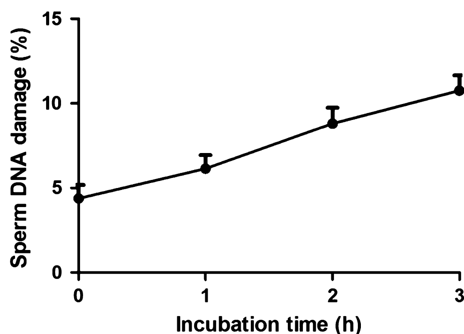

Fig. 2 Effect of different incubation times at 37 °C on the sperm DNA damage. There was positive correlation between incubation time and DNA damage in spermatozoa (P <0.0001).

for preparation of spermatozoa, whereas we used direct swim-up protocol. Also, sperm culture medium was different between two studies (Ham's F10 versus G-IVF). Another variation was incubation conditions; we incubated the processed spermatozoa at 37 °C, but they incubated prepared samples at 37 °C in 6% CO_2. Our results were in conflict with Calamera *et al.* (2001). They evaluated the effect of incubation time (immediately after swim-up up to 47 h later) on sperm parameters and DNA integrity. The authors found no significant differences at different time intervals. One possible cause maybe related to the method used for the assessment of DNA fragmentation. They used acridine orange (AO) staining method for the evaluation of sperm integrity. Both AO and SCD (method used in current study) are subjective. On the other hand, our results were similar to Bungum *et al.* (2010). They reported a significant raise in DFI in density gradient prepared spermatozoa incubated at 37 °C after 2 h compared to reference sample as well as to room temperature (23–24 °C). Yavas & Selub (2004) investigated the effect of incubation time at 37 °C on intrauterine insemination (IUI) pregnancy outcomes. It was shown a decrease in pregnancy rate when IUI procedure was carried out more than 60 min after sperm washing in hMG-IUI cycles. This study suggests that one of the causes of decease in pregnancy rate in this case could be detrimental effect of incubation at 37 °C on sperm DNA.

Lachaud *et al.* (2004) found no alterations in the sperm parameters as well as apoptosis markers after 4 h of incubation. They concluded *in vitro* prolonged incubation at 37 °C can lead to sperm cell death due to necrosis rather than by apoptosis. Also it was suggested ejaculated normal spermatozoa are unable to trigger the apoptotic pathway. One of the probable causes of sperm DNA damage may be oxidative stress. It is shown that oxidative stress, production of reactive oxygen species (ROS) is increased during *in vitro* incubation of spermatozoa (Calamera *et al.*, 2001). The spermatozoon is a sensitive and vulnerable cell to ROS because of different etiologies (e.g., presence of high unsaturated fatty acid in its membrane, lack of sufficient antioxidant in its cytoplasm). The sperm cell also produces ROS by itself. The peak ROS level was shown after 24 h of incubation (Calamera *et al.*, 2001), and it seems at least in long incubation, the role of ROS in DNA damage could be undeniable. Another group showed an increase in DNA fragmentation of sperm cells following *in vitro* incubation of spermatozoa for 4 h (Muratori *et al.*, 2003). They investigated the effect of endonuclease activity using nuclease inhibitor, aurintricarboxylic acid (ATA) on sperm DNA fragmentation at incubation times of 4 and 24 h. It was stated that treatment with ATA has no effect on sperm DNA fragmentation at different incubation times. In addition, no relationship between nuclease activity and spontaneous DNA fragmentation was reported. The authors introduced oxidative stress and endogenous production of ROS by the sperm as a possible cause for sperm DNA damage. Prolonged storage of washed spermatozoa in culture medium is another plausible cause of sperm DNA damage. Prolonged incubation of spermatozoa can lead to prolonged exposure of sperm cells to simple culture media which may have no nutrient and supporting value for sperm cells. It is, therefore, suggested to evaluate the other alternative incubation conditions on human sperm DNA damage.

In conclusion, it seems that incubation of prepared normozoospermic samples at 37 °C prior to use in ART should be less than 2 h. It would be worthwhile to elucidate the role of *in vitro* incubation of sub-normal spermatozoa on sperm DNA integrity. Although sperm with damaged DNA can fertilise the egg, numerous studies

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have shown that embryo development and pregnancy can be impaired in later stages.

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