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Sperm nuclear DNA fragmentation and chromatin structure in one-day-old ejaculated sperm

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Objective: To evaluate sperm nuclear DNA fragmentation and chromatin structure after 18 hours' incubation at room temperature.
Methods: Twenty-eight male partners who participating IVF treatment were prospectively included in this study. Ejaculated sperm count and motility were assessed. The sperm was then immediately processed by the conventional swim-up method. After utilization of some of the sample for routine clinical use, the remainder of each of the samples was divided into two aliquots. One aliquot was immediately assessed for sperm nuclear DNA fragmentation (TUNEL assay) and chromatin structure (toluidine blue [TB] staining). The other aliquot was incubated at room temperature for 18 hours and then assessed by two methods. Only dark-TB sperms were considered as having abnormal chromatin structure. Data before and after extended incubation were compared using a paired Student's *t*-test.
Results: Before and after extended culture, nuclear DNA fragmentation assessed by TUNEL was 4.9 ± 4.7% and 7.0 ± 6.4%, respectively ($p = 0.008$). The proportion of abnormal chromatin structure (dark-TB sperm) was 8.2 ± 5.6% and 10.3 ± 6.5% ($p < 0.001$), before and after incubation, respectively.
Conclusion: After 18 hours' incubation at room temperature, sperm nuclear DNA and chromatin structure were significantly affected. The IVF practitioner should bear this information in mind when performing delayed insemination, especially for *in vitro* maturation cycles.

Keywords: Spermatozoa; DNA Fragmentation; Chromatin, Chromatin; Protamination; Human

Introduction

Several studies have reported that sperm DNA fragmentation may affect the outcome of assisted reproduction in various ways [1-8]. It has also been reported that aberrant sperm chromatin structure is related to the outcome of assisted reproduction [9-12]. Sperm chromatin structure may be critical for the establishment and maintenance of proper epigenetic patterns during spermatogenesis [13]. Sperm chromatin/DNA integrity may be an essential part of the accurate transmission of paternal genetic information [14]. The causes of this type of DNA damage are still uncertain, but oxidative stress and aberrant apoptosis are considered to be the most likely [15]. Oxidative stress has been shown to affect the integrity of sperm chromatin [16].

During stimulated IVF cycles, up to 30% of oocytes are recovered as immature ones that can be rescued via *in vitro* maturation [17]. Hence many IVF laboratories attempt delayed insemination using one-day-old sperms if the oocytes are matured the next day. Although extended incubation of human sperm *in vitro* could result in increased nuclear DNA fragmentation and changed chromatin structure, there has been no published study directly addressing this issue.

In conventional IVF or ICSI cycles, extended incubation of sperm has routinely been performed at the physiologic temperature of 37°C

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[18,19]. However, a recent report noted that *in vitro* incubation of sperm for 2 or more hours at 37°C may cause morphologic impairment of the sperm nuclei, contributed by the appearance of large nuclear vacuoles, which did not occur at 21°C [20]. Based on this observation, sperm has routinely been incubated at room temperature in our IVF laboratory.

Here we have assessed changes in nuclear DNA fragmentation and chromatin structure in ejaculated sperms before and after 18 hours' incubation at room temperature.

Methods

Twenty-eight male partners participating in IVF treatment between 2009 and 2010 were recruited in this prospective study. The mean age of the males was 36.7±4.3 years. All of the subjects had no history of genital inflammation or surgery. No subjective symptoms or self-reported medical risk factors were identified. No participants had taken prescribed medications. Informed consent was obtained from all subjects and the use of human semen for this study was approved by the Institutional Review Board of the Seoul National University Bundang Hospital.

After avoiding coitus for at least three days, semen samples were obtained by masturbation at the time of oocyte pick-up. After liquefaction for 30 minutes at room temperature, sperm quality was routinely assessed by using computer-assisted semen analysis (SAIS-PLUS 10.1; Medical Supply Co., Seoul, Korea). The semen samples were then processed by the conventional swim-up method. After utilization of a portion of the samples for fertilization, the remainder of each of the samples was divided into two aliquots. One aliquot was immediately processed for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and toluidine blue (TB) staining. The other aliquot was incubated for 18 hours at room temperature and then assayed for TUNEL and TB staining.

Nuclear DNA integrity was measured by the TUNEL assay as described previously [21]. The samples were smeared on a silane-coated slide (DAKO, Glostrup, Denmark) and air-dried. Sperms were fixed with 4% neutral buffered formalin for 1 hour at 15-25°C, and then washed with phosphate-buffered saline. Sperms were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (Sigma, St. Louis, MO, USA). A commercial apoptosis detection kit was used (In Situ Cell Death Detection Kit; Roche Diagnostics GmbH, Mannheim, Germany). The remaining procedures were performed as directed by the instructions in the kits. Counterstaining was performed using a mounting medium with 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). The sperms with fragmented DNA had their nuclei stained in green, whereas the nuclei of the other cells nuclei were blue (Figure 1). Sperm heads with >50% of the area stained green were considered positive. At least 500 sperms were counted per experimental set and the percentage of sperms with fragmented DNA was determined.

Chromatin structure was assessed by TB staining as described before [12]. Thin smears were prepared on silane-coated slides (DAKO) and air-dried. Sperms were fixed with 96% ethanol:acetone (1:1) at 4°C for 30 minutes and air dried. Hydrolysis was performed with 0.1 N HCl at 4°C for 5 minutes, followed by three changes of distilled water, 2 minutes each. TB (0.05% in 50% McIlvain's citrate phosphate buffer at pH 3.5; Gurr-BDH Chemicals Ltd, Poole, UK) was applied for 5 minutes. Slides were then rinsed briefly in distilled water, dehydrated in tertiary butanol at 37°C, cleared in xylene at room temperature (Histoclear RA Lamb Labs, Atlanta, GA, USA) and mounted with DPX (water-insoluble embedding medium for histological preparations). The stained sperms were categorized into the following visual TB colors: dark purple, intermediate, and light blue. Only dark purple sperms were considered as having abnormal chromatin structure (Figure 1). At least 500 sperms were counted per experimental set and the percentage of sperms with dark-purple color was determined.

Data before and after extended incubation were compared using a paired Student's *t*-test. The Spearman correlation test was used to assess associations between different parameters. Statistical analysis was performed using statistical software (MedCalc ver. 10.4; MedCalc Software, Mariakerke, Belgium). A *p*-value of <0.05 (two-tailed) was considered statistically significant.

Results

All of the subjects had normal sperm concentrations (mean±SD, 155.1±117.4×10^6/mL). Sperm motility ranged between 32.6% and 86.4% (mean±SD, 55.5±16.7%). After sperm processing, the basal sperm DNA fragmentation ranged from 0.3% to 15.8% (Table 1). The mean percentage of sperm DNA fragmentation was 4.9±4.7% at baseline; the rate significantly increased to 7.0±6.4% after 18 hours' incubation ($p=0.008$, paired Student's *t*-test). The proportion of dark-TB sperm ranged from 0.5% to 24.1%; the mean value also significantly increased after 18 hours' incubation (8.2±5.6% vs. 10.3±6.5%, $p<0.001$; paired Student's *t*-test).

The baseline sperm DNA fragmentation and the proportion of dark-TB sperm showed a strong positive correlation ($r=0.497$, $p=0.007$), but the post-incubation scores did not ($r=0.227$, $p>0.05$). The initial sperm concentration was negatively correlated with the baseline sperm DNA fragmentation ($r=-0.396$, $p=0.037$), but not with the baseline rate of dark-TB sperm. The initial sperm motility and the age of male partner were not correlated with the baseline scores.

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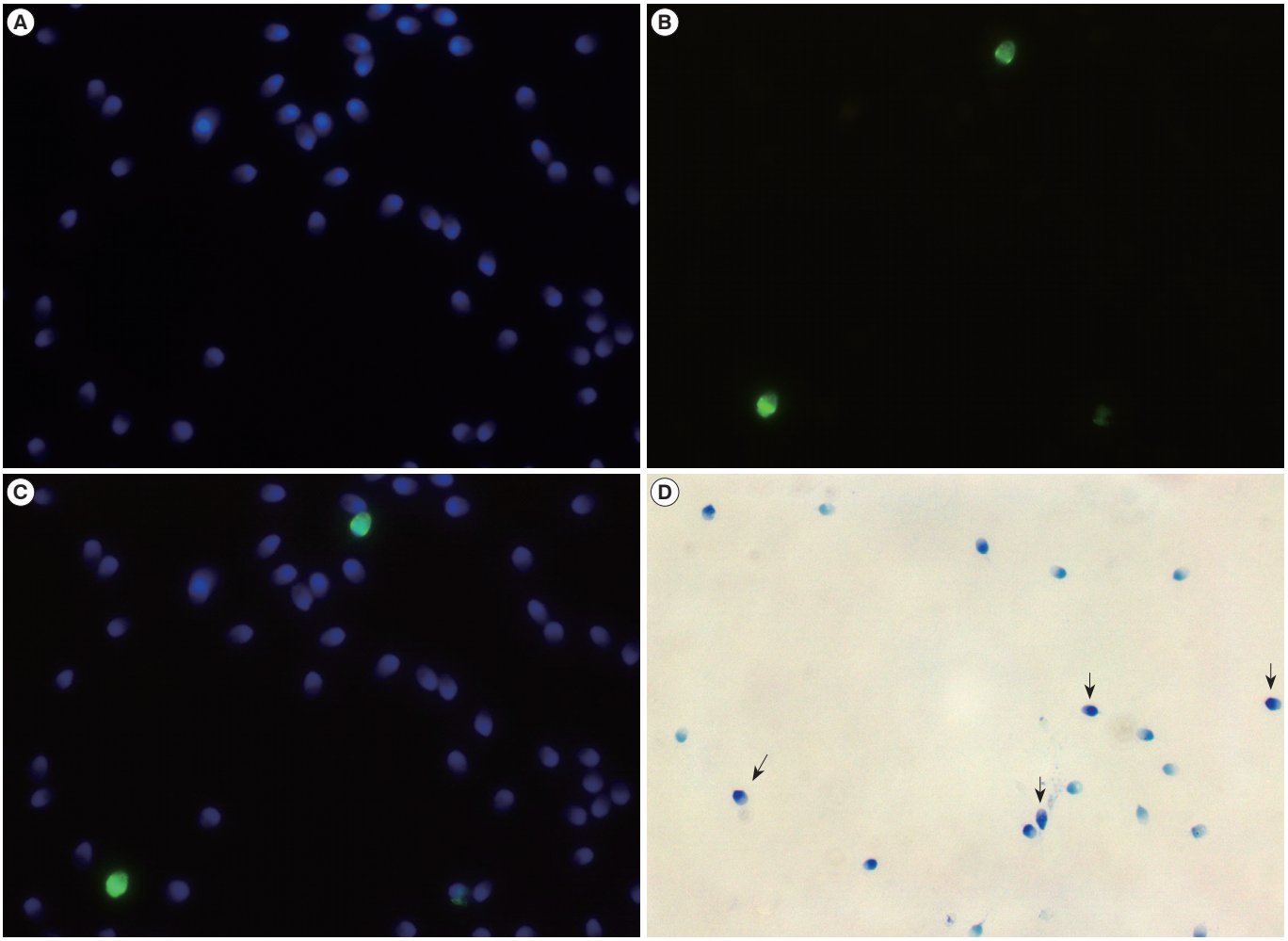

Figure 1. Representative microphotographs showing blue-colored sperm heads stained by 4′, 6-diamidino-2-phenylindole (DAPI) (A, ×1,000); two green-colored sperm heads are clearly seen (B, ×1,000); merged (C, ×1,000). These are stained by TUNEL method and sperm heads with >50% of the area stained green were considered positive. The arrows indicate dark purple sperm head stained by toluidine blue (D, ×1,000); only dark purple sperm heads are considered to have abnormal chromatin structure.

Table 1. Mean percentage and range of sperm DNA fragmentation and abnormal chromatin structure assessed by toluidine blue staining before and after 18 hours incubation

	Before incubation	After incubation for 18 hours	*p*-value
DNA fragmentation (%)	4.9±4.7 (0.3-15.8)	7.0±6.4 (0.48-27.8)	0.008
Abnormal chromatin structure (%)	8.2±5.6 (0.5-24.1)	10.3±6.5 (0.5-26.6)	<0.001

Paired Student's *t*-test.

Discussion

It has been reported that during extended culture of up to 7 days at 24°C, human sperm quality deteriorates severely, especially in motility and viability parameters [22]. It has previously been demonstrated that extended incubation at 37°C for 24 hours significantly decreases sperm motility and viability irrespective of the sperm preparation technique; however, these changes were not accompanied by any increase in the incidence of apoptotic spermatozoa [23]. In frozen-thawed sperms from human donors of proven fertility, the highest increment of sperm DNA fragmentation was observed in the first 4 hour of incubation at 37°C: the velocity of sperm DNA degradation was 8.3% per hour [24]. It has also been reported that DNA fragmentation in testicular sperm from men with obstructive azoospermia is increased by 4 and 24 hours of incubation [25]. Hence extended incubation of sperm *in vitro* in various settings would result in sperm quality deterioration and spontaneous DNA fragmentation.

Muratori et al. [26] suggested the involvement of endogenously produced reactive oxygen species (ROS) as the possible cause of *in*

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vitro sperm DNA fragmentation. Production of ROS has an important role in sperm capacitation and acrosome reaction processes; however, an excessive level of these aggressive compounds may be responsible for cellular and DNA damage [27]. It must be noted that the use of swim-up selected spermatozoa allows us to exclude the presence of contaminating non-sperm cells (immature round germ cells or leukocytes) in the incubation medium; this strongly suggests that ROS derive from the sperm itself.

We demonstrated that sperm chromatin structure was also affected after extended incubation at room temperature. Our finding suggests that sperm chromatin composition can be change in the post-ejaculation period. A previous study reported that impaired chromatin protamination is significantly correlated with DNA fragmentation and oxidative base damage in human spermatozoa [28]. Impaired chromatin remodeling might render the sperm DNA vulnerable to attack by external factors, particularly free radicals. Thus our findings support that impaired protamination during extended culture may be one of the factors causing sperm to be more susceptible to spontaneous DNA fragmentation.

In the present study, the baseline percentage of sperm DNA fragmentation and the proportion of dark-TB sperm showed a strong positive correlation, but the post-incubation score did not. This indicates that extended culture could affect sperm DNA and chromatin status in a different way. The precise mechanism requires further study.

Male germ cells are especially vulnerable to oxidative stress because of the lack of DNA repair systems and antioxidants in spermatozoa [27]. Furthermore, separation from seminal plasma possessing antioxidant ability and extended culture could accelerate this process. Antioxidants supplementation in culture media is therefore needed to reduce sperm DNA damage when extended culture is performed. Although there has been no direct evidence, previous studies support the use of antioxidant supplementation *in vitro* [29-31].

In conclusion, sperm nuclear DNA and chromatin structure were significantly affected after 18 hours' incubation at room temperature. The IVF practitioner should keep this information in mind when performing delayed insemination, especially when dealing with immature oocyte retrieval or early sperm collection prior to oocyte retrieval. Our study also supports the development of improved culture media to protect mature spermatozoa from DNA damage or changes in chromatin during extended culture *in vitro*.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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