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CELL BIOPHYSICS

The Influence of the Storage Temperature and Cryopreservation Conditions on the Extent of Human Sperm DNA Fragmentation

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Abstract—We explored the influence of different storage temperature conditions, including different methods of cryopreservation, on the structure of DNA organization in human sperm using a direct labeling procedure for detecting DNA fragmentation. Nineteen sperm samples that were obtained from healthy men with normozoospermia (according to the criteria of the World Health Organization) were used for the investigation. A significant increase in human sperm DNA fragmentation was observed 8 h after the incubation at +39°C (by 76.7%) and at +37°C (by 68.9%). It was found that cooling the sperm with a cryoprotectant immediately after thawing did not produce a significant difference in the extent of DNA fragmentation; however, the samples that contained cryoprotectants showed a sharp increase in the DNA fragmentation 24 h after the incubation, which could suggest cryoprotectant cytotoxicity.

Keywords: DNA fragmentation, spermatozoa, assisted reproductive technology, cryopreservation, incubation temperature

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The integrity of the genetic material of the both parents is necessary for full-fledged embryo development. The male factor in the problems of biochemical and clinical pregnancy and embryo development is independent in half of the cases. It is known that the fraction of spermatozoa with DNA fragmentation of (for) fertile and infertile men significantly differ [1]. The influence of the extent of DNA fragmentation on fertilization has been proven by the following data:

– the probability of fertilization in vivo is close to zero for DNA fragmentation that exceeds 30% (according to the sperm chromatin-structure assay) [2];

– to achieve a successful pregnancy upon in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), the DNA fragmentation index (DFI) should be < 20%.

At this stage of the development of assisted reproductive technology (ART) unequivocal diagnosis of male infertility requires information on DNA integrity independently of the indices of a routine semen analysis (sperm volume; pH, concentration, motility, and vitality of spermatozoa; morphological analysis), since the methods of bright-field microscopy do not reveal DNA defects of spermatozoa, as well as due to the variability and controversial statistics of these tests [1]. Even spermatozoa that have significant DNA damage preserve the ability to fertilize an oocyte [3].

The physiological reasons for the possible impairments in the DNA structure of a spermatozoid in the form of single- and double-strand breaks are the peculiarities of the DNA packing in germ cells, since in contrast to somatic cells the packing of a primary DNA strand occurs via the nucleoprotamine complex.

As the results of spermatogenesis for the vast majority of mammals are highly homogeneous spermatozoa; for example, the nucleus of a mouse spermatozoid contains more than 95% protamines as the protein component of their nucleoproteins. This makes it possible for the nucleus of a mature spermatozoid to have a volume that is 40 times smaller than that of the nucleus of a somatic cell. The nucleus of a human spermatozoid, however, contains significantly less protamines (approximately 85%), than bovine, horse, hamster, and mouse spermatozoa. The chromatin of a human spermatozoid is thus less regularly packed and often contains DNA breaks [1].

In the beginning of 1990s, a model of the structural DNA organization in a spermatozoid nucleus was proposed by W.S. Ward in [4]. The structural units of the nucleoprotamine chromatin according to this model are toroids, which are circular structures with a thick-

Abbreviations: IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; ART, assisted reproductive technology; TUNEL, terminal deoxynucleotidyl transferases dUTP nick end labeling.

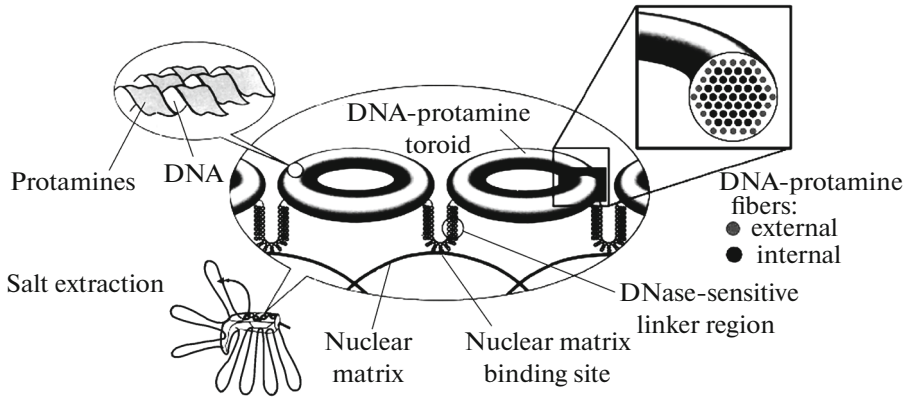

Fig. 1. A toroid model of chromatin structure (according to [4]).

ness of 20 nm, an external diameter of approximately 90 nm, and an internal diameter of approximately 15 nm. The individual toroids are loop DNA domains that are highly condensed by protamines, with their foundation being attached to the nuclear matrix (Fig. 1); these toroids are linked via disulfide bonds that form as a result of oxidation of the sulfhydryl groups of protamine cysteine. Thus, each of the chromosomes represents a garland that is composed of toroids. The centromeres of all the 23 chromosomes are concentrated in the center of the nucleus and form a compact chromocenter; the telomeres are located at the periphery of the nucleus, where they interact with each other, thus forming dimers [4]. This concept is generally accepted, although there are also other hypotheses for the packing of nucleoprotamine complexes. The variability of the chromatin structure in human gametes as compared with other mammals is connected with the preservation of 15% of the histones and the presence of two protamine types (P1, with P2 being added) [1].

The main sources of DNA fragmentation are: defects in the recombination of genetic material during spermatogenesis; abnormal maturation of a spermatid (impairment in protamination); apoptosis of testicular germ cells; and oxidative stress. The biological effect of an abnormal chromatin structure on the whole depends on the damage and ability of the oocyte to restore the damage. If an oocyte is unable to repair the damage before the first division, the embryonic development can be blocked at different stages [3]. The problem of the integrity of male gametes is urgent for the diagnostics and treatment of male infertility, enhancement of the effectiveness of the methods of assisted reproductive technology, and prevention of the transmission of genetic defects using ART, especially upon the injection of a spermatozoid into an oocyte (ICSI) that is able to overcome the natural barrier of a high level of DNA fragmentation and launch a successful pregnancy with negative consequences [5]. The paternal effect can be connected with such genetic factors as genetic mutations, microdeletions, aneuploidy, epigenetic disorders, DNA damage, and impairments in chromatin compaction [6].

The physical factors that are connected with ART, viz., the temperature of the ejaculate, as well as the incubation and storage conditions, could cause DNA fragmentation [7]. It has been noted that prolonged incubation of spermatozoa in vitro under different conditions lead to a loss of motility and viability. These changes could be caused by metabolic changes that are induced by cultivation conditions [8]. Spermatozoa in laboratories are often incubated at physiological temperature (37°C) for 1–2 h before IVF and ICSI. Data that have been obtained in some studies indicate that the incubation temperature significantly affects the level of sperm DNA fragmentation [9]. Earlier, it was shown by the TUNEL (terminal deoxynucleotidyl transferases dUTP nick end labeling) assay that 2 h after incubation at 37°C a decrease in the morphological integrity of a spermatozoid nucleus occurs by a factor of 1.5 as compared with the initial state, while the number of nuclei that contain vacuoles also increases.

ART uses low-temperature storage of sperm (cryopreservation). Cryopreservation is the only way to have children for patients with acquired infertility after previous diseases. It is known that spermatozoa are damaged during cryopreservation due to different causes (cold shock, osmotic shock, and mechanical effects on membranes), which affects their motility and morphological parameters. In this case, the motility and the fertilizing capacity of spermatozoa after cryopreservation decrease within wide limits, on average, by 30–70%. It is important to determine whether freezing affects the DNA integrity of spermatozoa.

We can see that the temperature mode can directly affect the outcome of IVF and ICSI. At a high level of DNA fragmentation of spermatozoa the risk of choosing a spermatozoid that has a hidden defect that could adversely affect the outcome of a pregnancy is particularly high. In this study, the effects of different temperature modes on the structure of DNA organization in spermatozoa were investigated.

MATERIALS AND METHODS

The study was performed using sperm samples of 19 donors and patients that were diagnosed with normozoospermia (according to the criteria of the World Health Organization). This study was approved by the Ethics Committee of IVF Clinic AltraVita and informed consent was obtained from all subjects. To evaluate the parameters of the spermatozoa, routine methods that are used in the AltraVita hospital, concentration and motility assessment using a Makler chamber, and evaluation of morphological characteristics by preliminary stained (with methylene blue and cresyl violet) glass according to Kruger's criteria (the number of normal spermatozoa is 60–150 million/mL with no less than 70% of them being motile (type a + b) and no less than 13% having a normal morphology) were employed. TUNEL was used to assess the integrity of the nuclear DNA. Fluorescein-12-dUTP was the primary fluorochrome; fluorescent counterstaining was carried out using Hoechst 33258. Counting was performed on 500 spermatozoa (using an Olympus BX51 microscope).

Cryopreservation of the samples was carried out according to the standard protocol of slow freezing using the SpermFreeze cryoprotectant (SAGE In-Vitro Fertilization Inc., Trumbull, United States) at the ratio of 1 : 0.5; rapid thawing was conducted at 37°C.

During statistical analysis of the data, the mean values and standard deviations are given for all the series of experiments; the level of statistical significance was $P \le 0.05$.

The modified TUNEL protocol included the following procedures: preparation and drying of smears on glass for 30 min; cell fixation (3.7% paraformaldehyde in phosphate buffer saline) for 20 min at +4°C; washing from the fixing agent by changing the phosphate buffer saline twice for 5 min; permeabilization using a 0.2% triton X-100 in phosphate buffer saline for 5 min; thorough washing by changing phosphate buffer saline three times for 5 min; and the reaction of labeling DNA breaks for 1 h at 37°C. To inhibit the reaction of labeling, the cells were incubated in 2x SCC buffer for 20 min. The preparations were then washed three times for 5 min in phosphate buffer saline, stained with Hoechst, and embedded in glycerol according to the standard method.

A negative control, viz., staining according to the protocol without the addition of terminal deoxynucleotidyl transferase, was performed; thus, the error of the method was calculated (3–5%).

RESULTS AND DISCUSSION

In order to determine the effect of the temperature mode of the storage on the level of DNA fragmentation of spermatozoa, a series of experiments was conducted at temperatures of 21 and 39°C with time intervals of 0, 8, and 24 h. The time interval at the temperature of 37°C that is used in some laboratories consisted of 0, 0.5, 2.5, 5, 8, and 24 h. We have found out a significant increase in the number of spermatozoa with fragmented DNA with time for the all of the selected temperatures. The greatest change was observed for the temperature of 39°C: 24 h after incubation the DNA fragmentation was at approximately the 90% level; the portion of the fragmented DNA increased to 76.7 ± 7.9% at 8 h (Fig. 2).

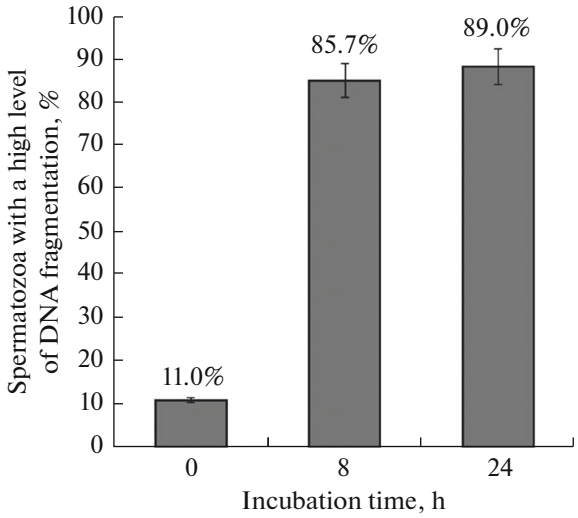

Fig. 2. The time plot of the part of spermatozoa with fragmented DNA at a temperature of 39°C.

Incubation at 21°C for 24 h increased the portion of the fragmented DNA only by a factor of 2.2 ± 0.29.

The experiments at 37°C demonstrated that the major part of the fragmented DNA in the spermatozoa occurs in the first 8 h of incubation (the time dependence of the portion of the fragmented DNA at 37°C is nonlinear). It is likely that the process that leads to DNA fragmentation is initiated in the first hours of incubation (Fig. 3). The nonlinear form of the line of the activation energy makes it possible to suggest some threshold temperature at which other processes, e.g., capacitation begin to evolve in the system. This is proven by the fact that capacitation is not observed during sperm incubation at room temperature, with this inhibition being prevented when the spermatozoa are subjected to the temperature of 37°C. It could be proposed that most spermatozoa have unrepaired DNA breaks that occurred during spermatogenesis and as a result of unfinished apoptosis, which makes them more susceptible to oxidative stress.

To study the effect of cryopreservation on the level of DNA fragmentation, the following experimental scheme was used: the ejaculate was divided into three parts, one for freezing without cryoprotectant, another with cryoprotectant, and a native sample. DNA fragmentation was recorded upon sampling, immediately after freezing and thawing, and 24 h after the thawing. The samples without addition of the cryoprotectant had a level of DNA fragmentation that increased by a factor of 1.9 ± 0.4, while the samples with the cryoprotectant almost did not differ from the native samples (within the limits of the error).

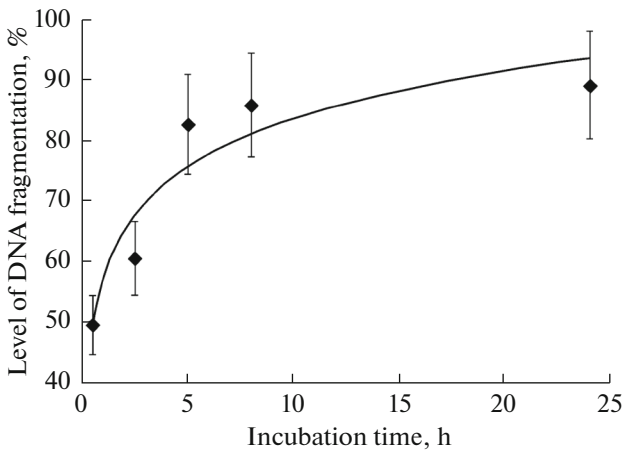

Fig. 3. The kinetics of the change in the number of spermatozoa with fragmented DNA during incubation at 37°C.

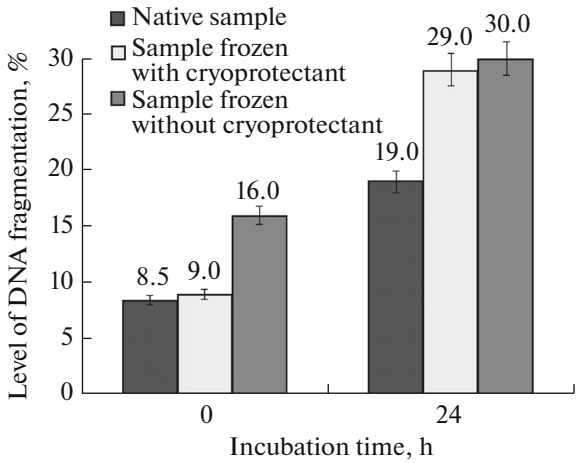

Fig. 4. The effects of cryopreservation conditions on the level of DNA fragmentation in spermatozoa immediately after freezing and 24 h after.

This increase in the fragmented DNA is fatal for the semen of the men that have fragmentation in the native sample that is higher than 7%, since the effectiveness of IVF decreases and the risk of selection of a spermatozoid with damaged genetic material increases, which could lead to abnormal development of the embryo and its loss. After incubation for 24 h at 21°C, the number of spermatozoa with fragmented DNA in the native samples and samples that were frozen without cryoprotectant increased, on average, by a factor of 2 (Fig. 4). The samples that were frozen with the cryoprotectant had a level of DNA fragmentation that increased by more than a factor of 3 (3.1 ± 0.32).

It should be noted that during incubation for 24 h after cryopreservation the cryoprotectant was washed from all of the samples and all of them were under similar conditions. The SpermFreeze cryoprotectant is permeant. The data from the experiment could lead to conclusions of the cytotoxicity of the cryoprotectants and their effect on the level of DNA fragmentation in cells. It is hypothesized that deviations during spermatogenesis could lead to changes in chromatin packing and defects in protamination that make the spermatozoid DNA more susceptible and vulnerable to different exposures. One mechanism of the occurrence of damage in spermatozoid DNA is believed to be a cascade of changes that starts with oxidative stress and the formation of adducts of oxidized DNA bases, which eventually results in DNA fragmentation and cell death [10].

CONCLUSIONS

Thus, we showed that the temperature mode can directly affect the results of IVF and ICSI. This study demonstrated a significant increase in the level of DNA fragmentation of spermatozoa over time for all of the tested temperatures. However, the level of fragmented DNA at 37 and 39°C was higher than at 21°C. There were no significant differences in the level of DNA fragmentation upon cryopreservation with the application of cryoprotectants immediately after thawing; however, 24 h after incubation the samples that had the cryoprotectant experienced a sharp increase in the level of fragmented DNA.

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REFERENCES

1. J. Erenpreiss, M. Spano, J. Erenpreisa, et al., Asian J. Androl. **8** (1), 11 (2006).
2. D. P. Evenson, L. K. Jost, D. Marshall, et al., Hum. Reprod. **14**, 1039 (1999).
3. D. Sakkas and J. G. Alvarez, Fertil. Steril. **93** (4), 1027 (2010).
4. W. S. Ward and D. S. Coffey, Biol. Reprod. **44** (4), 569 (1991).
5. R. J. Aitken and C. Krausz, Reproduction **122**, 497 (2001).
6. E. V. Markova and A. S. Zamay, Probl. Reprod. **4**, 42 (2006).
7. K. L. Larson, C. J. DeJonge, A. M. Barnes, et al., Hum. Reprod. **15**, 1717 (2000).
8. P. N. Schlegel and D. A. Paduch, Fertil. Steril. **84** (4), 854 (2005).
9. L. H. Dalzell, C. M. McVicar, N. McClure, et al., Fertil. Steril. **82**, 1443 (2004).
10. R. J. Aitken and L. Bennetts, in *The Sperm Cell Production, Maturation, Fertilization, Regeneration,* Ed. by Ch. J. De Jonge and Ch. L. R. Barratt (Cambridge Univ. Press, Cambridge, 2006), pp. 170–194.

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