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THE INFLUENCE OF HIGHLY DISPERSED SILICA NANOPARTICLES ON THE FUNCTIONAL ACTIVITY OF MITOCHONDRIA AND CHROMATIN STATE IN NATIVE AND DEVITRIFIED *Bos taurus* OOCYTES

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Abstract

Mitochondria are the only cellular compartments which generate and transform energy in the cell. These organelles are among the first to respond to changes in extra- and intracellular conditions (e.g., ionic homeostasis, dehydration level, temperature). Exposition to ultra-low temperatures, due to lipid peroxidation, causes ATP synthase complex disorders and destruction of genetic material (E.A. Novocherkina et al., 2016). Highly dispersed silica nanoparticles (HDSns) can be proposed as cytoprotectors. An amorphous form of silicon dioxide, or highly dispersed silica, which exhibits its biological activity through high adsorption capacity, reduces the concentration of ions and biopolymers upon cell dehydration during cryopreservation (T.T. Turov et al., 2011). This paper deals with the first report of the increase in cryoresistance and maturation rate of *Bos taurus* oocytes due to exposure to 0,001% HDSns during vitrification and in vitro culture, which ensures higher functional activity of mitochondria and preserves structural properties of chromatin of native and devitrified (DV) oocytes. The study aimed to identify the impact of HDSns on functional activity of mitochondria and chromatin state of cow's native and devitrified oocytes during in vitro culture. In the experiments, we used cumulus—oocyte complexes (COCs) of Holsteinized cows. The oocytes with homogeneous cytoplasm surrounded by five or more layers of cumulus cells were subjected to vitrification. COCs intended for vitrification were exposed in three solutions of cryoprotective agents (CPAs) based on T-199 medium with 10 % fetal bovine serum (FBS, HyClone, UK), the CPA-1 containing 0.7 M dimethyl sulfoxide (DMSO) + 0.9 M ethylene glycol (EG) (for 30 s), CPA-2 (1.4 M DMSO + 1.8 M EG (for 30 s), and CPA-3 (2.8 M DMSO + 3.6 M EG + 0.65 M trehalose) (for 20 s). The straws with oocytes were directly plunged into liquid nitrogen. During thawing, the oocytes were washed with T-199 medium containing 0.25 M (for 3 min), 0.19 M (for 3 min), and 0.125 M (for 3 min) trehalose, and finally with T-199 medium. In the test group of oocytes, highly dispersed silica nanoparticles (HDSns, 4-17 nm, mass concentration 0.001 %) synthesized by high-temperature hydrolysis were added to the CPAs, devitrification solutions and washing solutions. In the control group, native and devitrified oocytes were cultured for 24 hours in maturation medium consisted of the T-199 with 10^6 granulosa cells/ml supplemented with 10 % fetal bovine serum and 50 ng/ml bovine prolactin (38.5 °C, 90 % humidity, and 5 % CO_2 atmosphere). In the test group, the maturation medium for native and devitrified oocytes was supplemented with HDSns at a final concentration of 0.001 %. Mitochondrial activity was measured by fluorescence intensity (FI) of MitoTracker Orange CMTMRos (Thermo Fisher Scientific, UK) in μA. In studding HDSns effects on the nuclear maturation, oocytes were exposed for 5-10 min to 0.9 % sodium citrate solution and mechanically denuded from cumulus cells with a needle. Then the cells were placed on a glass slide and fixed with methanol/acetic acid solution (3:1). The dry-air samples were stained with azure-eosin by Romanowsky-Giemsa method. In DV

oocytes exposed to HDSns, the FI of MitoTracker Orange CMTMRos probe increased from 77±6.3 μA to 169±12.8 μA ($p < 0.05$). The functional activity of mitochondria in DV oocytes which were treated with HDSns increased from 169±12.8 μA to 181±7.7 μA ($p < 0.05$) during the period from diplotene to the metaphase I stages, and subsequently decreased to 141±11.2 μA at metaphase II stage, what is probably associated with the completion of nuclear and cytoplasmic maturation of oocytes. When assessing the chromatin state of oocytes in the HDSns-treated group, we revealed a decrease in the number of oocytes with chromatin degeneration at diplotene and metaphase II stages compared to those in the HDSns -untreated DV oocytes (40 % vs. 21 %, and 59 % vs. 38 %, $p < 0.01$), that is probably associated with the DNA reparation processes. In general, our findings reveal a positive effect of HDSns on the functional activity of mitochondria and the chromatin state in DV female gametes of *Bos taurus*. The obtained results expand views and available information on the functioning of cell compartments at ultra-low temperatures and the mechanisms of HDSns action on female gametes.

Keywords: oocyte, vitrification, highly dispersed silica nanoparticles, functional activity of mitochondria, MitoTracker Orange CMTMRos, chromatin, *Bos taurus*

Improvement of cryoprotective media for vitrification of oocytes through the introduction of various compounds necessary to maintain viability after the thawing procedure continues to be an urgent problem in cryopreservation technology. Recently, biologically active substances with a nanoscale structure synthesized from various compounds of trace elements and minerals, for example, hydroxyapatite (HA), silicon dioxide (SiO_2), aluminum oxide (Al_2O_3), and titanium dioxide (TiO_2) [1, 2] have been widely used for modernization of combined cryoprotective media. Cryoprotective agents containing nanoparticles improve freezing by increasing the thermal conductivity of vitrification solutions and reducing the consequences of recrystallization processes during thawing compared to that when the standard cryoprotective agents such as ethylene glycol and dimethyl sulfoxide [1, 2] are used.

Highly dispersed (pyrogenic) silica (HDS) is an amorphous form of silicon dioxide with a range of spherical particle size 4-17 nm (90% of the range) [3]. Due to the pronounced adsorption activity resulted from a large specific surface area ($S_{sp.} = 200$ m^2/g), HDS nanoparticles are able to reduce the concentration of polymers and ions during cell dehydration [3, 4], and can bind extracellular water [5]. The large size of the finely dispersed silica aggregates and the low density of particles in these agglomerates ensure the binding of a significant amount of extracellular water, which is not subject to changes in interactions with cells during freezing/thawing [5].

Under oxidative stress conditions during freezing, there are a denaturing effect of free radicals on ion-transporting proteins, impaired DNA synthesis and thermotropic mitochondrial defects, which ultimately leads to the cell death [6]. Mitochondria provide cell with the ATP necessary for the completion of meiotic maturation. The features of their functioning determine the gamete quality [7, 8]. Anomalies in the chromatin transformation of the cell nucleus under temperature-dependent oxidative stress are mainly caused by the disruption of the biosynthesis of nuclear proteins and their connection with DNA, the destruction of nucleotides, and single- and double-stranded DNA breaks [9].

This paper for the first time shows that when nanoparticles of highly dispersed silica at a concentration of 0.001% are used in the technology of vitrification and extracorporeal maturation of devitrified (DV) bovine oocytes, the mitochondrial potential of DV oocytes increases and the level of degenerated cells drops.

The work aimed to identify the nature of the effect of highly dispersed silica nanoparticles on the functional activity of mitochondria and chromatin status in native and devitrified bovine oocytes during extracorporeal maturation.

Materials and methods. The ovaries of Holsteinized cows (*Bos taurus*) after ovariectomy were delivered from the slaughterhouse to the laboratory in a 0.9%

NaCl solution with penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 ng/ml) at 30-35 °C. Cumulus—oocyte complexes (COCs) were aspirated from follicles with a diameter of 3-8 mm.

Oocytes with homogeneous cytoplasm surrounded by five or more layers of cumulus cells were subjected to vitrification. COCs intended for vitrification were treated with three solutions of cryoprotective agents (CPA) based on T-199 medium supplemented with 10% fetal bovine serum (FBS, HyClone, UK). The CPA solutions for vitrification of oocytes in control (without treatment with HDSns) contained 0.7 M dimethyl sulfoxide (DMSO) and 0.9 M ethylene glycol (EG) for CPA-1, 1.4 M DMSO and 1.8 M EG for CPA-2, and 2.8 M DMSO, 3.6 M EG and 0.65 M trehalose (Trehalose) for CPA-3. All reagents, with the exception of those specifically indicated in the text, are manufactured by Sigma-Aldrich (USA), plastic laboratory glassware is manufactured by BD Falcon™ (Becton Dickinson and Co., BD Biosciences, USA). HDSns (4-17 nm, mass concentration 0.001%) were synthesized at the Chuiko Institute of Surface Chemistry of NAS of Ukraine by high-temperature hydrolysis. HDSns were used in the form of a stable suspension consisting mainly of submicron aggregates. The choice of concentration was based on the data provided by the developers [10].

COCs were exposed to CPA-1 for 30 s, then to CPA-2 for 30 s, and to CPA-3 for 20 s. Thereafter, the straws with oocytes were plunged into Dewar flask with liquid nitrogen. COCs were removed from the liquid nitrogen not earlier than after 1 h and thawed by placing sequentially into a 0.25 M trehalose solution prepared in T-199 medium supplemented with 10% FBS for 3 min at 37 °C, then into 0.19 M trehalose solution for 3 min at 37 °C, and finally into 0.125 M trehalose solution for 3 min at 37 °C, after which they were washed three times in T-199 medium with 10% FBS. In the control, all the solutions did not contain HDSns, in the experimental groups all media were supplemented with HDSns to a final concentration of 0.001%.

Native and devitrified oocytes were cultured for 24 h at 38.5 °C and 90% humidity in a 5% CO_2 atmosphere using culture medium containing T-199 + 10% FBS + 10^6 granulosa cells/ml + 50 ng bovine prolactin/ml. During culturing native and devitrified oocytes in the control group, this medium did not contain HDSns; in the testing groups, HDSns were added to a final concentration of 0.001% [11].

MitoTracker Orange CMTMRos probe (Thermo Fisher Scientific, UK) was used to assess the functional state of mitochondria in native and DV oocytes at different stages of meiosis. COCs, 15-20 per drop, were placed into 500 μl drops of 500 nM MitoTracker Orange CMTMRos solution and incubated for 30 min in the dark at 37 °C. Then oocytes were washed from the dye in phosphate buffered saline (PBS) with 0.3% bovine serum albumin. The washed oocytes were denuded from cumulus cells by treatment with a 0.1% trypsin solution at 37 °C for 5-10 min, transferred into Hanks solution containing 3.7% paraformaldehyde, and then fixed for 15 min at 37 °C. After fixation, oocytes were washed from paraformaldehyde in PBS and placed on Super frost glasses into drops of Hoechst 33258 solution (2.5 μg/ml, Thermo Fisher Scientific, UK).

Fluorescence intensity (IF) of MitoTracker Orange CMTMRos were measured and nuclear maturation were assessed using the Hoechst 33258 dye and a ZEISS Axio Lab.A1 fluorescence microscope with a photometric attachment (Karl Zeiss, Germany). The excitation wavelength for MitoTracker Orange CMTMRos was 554 nm, the emission wavelength - 576 nm; for Hoechst 33258 these were 352 nm and 461 nm, respectively. IF of MitoTracker Orange CMTM-Ros was measured in μA.

To identify the HDSns effect on the nuclear maturation of female gametes, oocytes were exposed for 5-10 min to 0.9% sodium citrate solution and mechanically denuded from cumulus using a needle. Then the cells were transferred to dry defatted glass and fixed with a mixture of methanol/acetic acid 3:1. Dry-air preparations were stained with azure-eosin according to Romanowsky-Giemsa method.

The results were processed by the method of two-way analysis of variance ANOVA using the SigmaStat statistical program package (Jandel Scientific Software, USA). Data are presented as means (*M*) and standard errors of means (±SEM). The Student's t-test and Pearson's χ^2 test adjusted for likelihood were used to assess the significance of differences between the compared mean value. The significance of the differences was assessed at $p < 0.05$, $p < 0.01$ and $p < 0.001$ for 3-5 independent experiments.

Results. In previous works, we found a positive effect of 0.001% HDSns on the chromatin status of somatic cells of cow ovarian follicles [12], on oocyte-cumulus interactions during maturation of female gametes [13], and on the development of pre-implantation embryos [11]. In the present study, it is found that the introduction HDSns in cryoprotective media contributes to the increase of MitoTracker Orange CMTMRos probe fluorescence in DV oocytes compared to gametes not exposed to HDSns before vitrification (169±12.8 vs. 77±6.3 μA, $p < 0.05$) (Table, Fig. 1).

Intensity of fluorescence (FI, μA) **of MitoTracker Orange CMTMRos probe** (Thermo Fisher Scientific, UK) **in native and devitrified oocytes of Holsteinized cows (*Bos taurus*) during meiosis, treated by highly dispersed silica nanoparticles** (HDSns) (*M*±SEM, number of experiments $N = 5$, total number of oocytes $n = 529$, in vitro culture)

Oocytes	HDSns	*n*	FI of MitoTracker Orange CMTMRos		
			diplotene	metaphase I	metaphase II
Native	–	133	331±16.2[a]	188±11.3[e]	143±10.1[i]
	+	139	309±15.6[b]	203±11.8[f]	149±14.7[j]
Devitrified	–	128	77±6.3[c]	139±11.7[g]	101±9.7[k]
	+	129	169±12.8[d]	181±7.7[h]	141±11.2[l]

a:c; c:d; b:d; g:h; e:g; i:k; k:l; a:d; b:c; f:g; j:k Differences are statistically significant at $p < 0.05$ (Student's *t*-test).

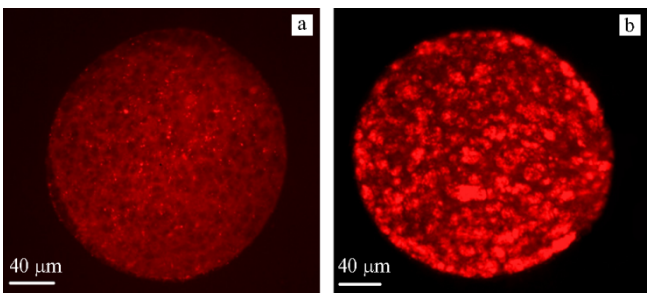

Fig. 1. Representative image of mitochondria in devitrified oocytes of Holsteinized cows at diplotene stage with low (a) **and high** (b) **fluorescence intensities of the MitoTracker Orange MTMRos probe (Thermo Fisher Scientific, UK), treated with highly dispersed silica nanoparticles** (HDSns)**:** a — oocytes not treated with HDSns), b — oocytes treated with 0.001% HDSns (ZEISS Axio Lab.A1, Karl Zeiss, Germany, in vitro culture).

In 14 h, that is, after the devitrified oocytes reached the stage of metaphase I, we detected statistically significant differences in the IF of MitoTracker Orange CMTMRos probe between cells treated and not exposed to HDSns (181±7.7 vs. 139±11.7 μA, $p < 0.05$). By the time the oocytes reached metaphase I, the IF of native oocytes did not differ from the degree of DV oocytes treated with HDSns (188±11.3 and 181±7.7 μA, respectively). In DV oocytes, an increase in mitochondrial activity occurred from diplotene to metaphase I ($p < 0.05$) (see Table). At the final stages of maturation, a general drop in the IF of MitoTracker Orange CMTMRos was observed in all experimental groups, and the indicators of mitochondrial activity were minimal.

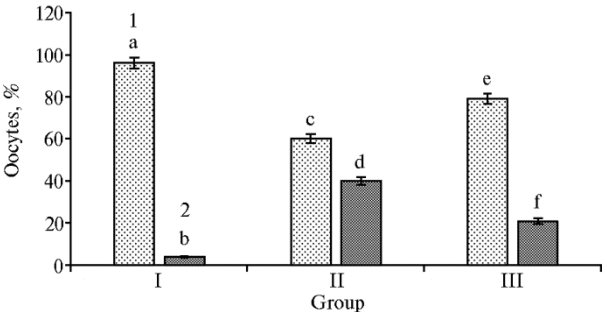

Fig. 2. Oocytes of Holsteinized cows with normal (1) **and degenerated** (2) **chromatin at the diplotene stage, treated with highly dispersed silica nanoparticles** (HDSns)**:** I — native oocytes (control), II — devitrified oocytes (control), III — devitrified oocytes treated with 0.001% HDSns. (0 h of culture, number of oocytes $n = 293$, number of experiments $N = 4$; in vitro culture). Differences are statistically significant: for a:b; a:c; e:f; b:d; b:f; a:d; a:f; c:b; c:f; e:b; d:e at $p < 0.001$; for c:d; c:e; d:f at $p < 0.01$ (Pearson's χ^2 test).

In assessing the chromatin state of native and DV oocytes at diplotene stage, there was a higher level of untreated DV gametes with signs of chromosome degeneration compared to the cells treated with HDSns (40 vs. 21%, $p < 0.01$) (Fig. 2). During culture to the metaphase I stage, we did not find significant differences in the number of oocytes with normal or degenerated chromatin between control and HDSns-treated native and DV gametes (Fig. 3, A). However, 24 h after DV oocytes reached the metaphase II stage (Fig. 4), there was an increase in the number of cells with signs of degeneration of nuclear material among oocytes that were not treated with HDSns (59% vs. 38%, $p < 0.01$, see Fig. 3, B).

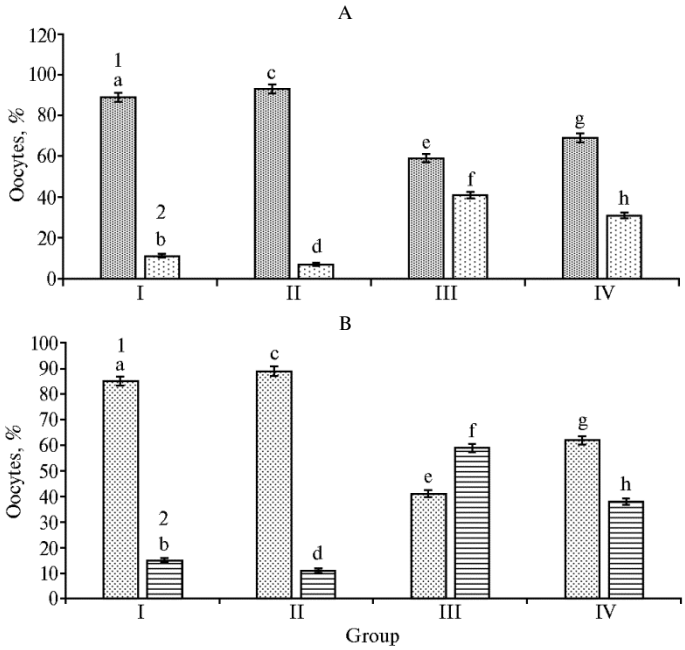

Fig. 3. Oocytes of Holsteinized cows with normal (1) **and degenerated** (2) **chromatin at the metaphase I** (A) **and metaphase II** (B) **stages, treated with highly dispersed silica nanoparticles** (HDSns): I — native oocytes (control), II — native oocytes treated with 0.001% HDSns, III — devitrified oocytes (control), IV — devitrified oocytes treated with 0.001% HDSns. (24 h of culture, number of oocytes $n = 419$, number of trials $N = 4$; in vitro culture).

A: a:b; c:d; g:h; a:e; a:g; c:e; c:g; b:f; b:h; d:f; d:h; a:d; a:f; a:h; c:b; c:f; c:h; e:b; e:d; e:h; g:b; g:d; g:f — differences are statistically significant at $p < 0.001$; differences for e:f are statistically significant at $p < 0.01$.

B: a:b; c:d; g:h; a:e; a:g; c:e; c:g; b:f; b:h; d:f; d:h; a:d; a:f; a:h; c:b; c:f; c:h; e:b; e:d; g:b; g:d — differences for the pairs are statistically significant at $p < 0.001$; e:g; f:h — differences are statistically significant at $p < 0.01$; differences for e:f are statistically significant at $p < 0.05$ (Pearson's χ^2 test).

During freezing functional activity of mitochondria decreases, that is caused

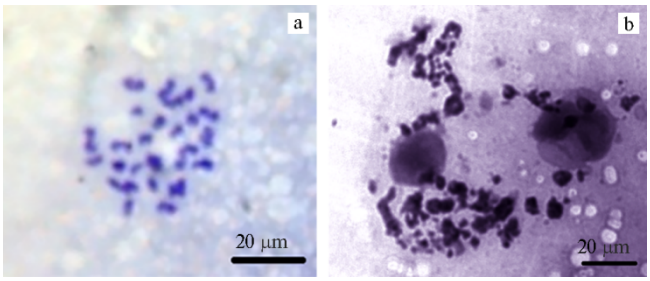

Fig. 4. Representative image of normal (a) **and degenerated** (b) **chromatin in devitrified oocytes of Holsteinized cows at methaphase II stage, treated with highly dispersed silica nanoparticles** (0,001%) (cytogenetic analysis, staining with azure-eosin according to Romanowsky-Giemsa method, ZEISS Axio Lab.A1, Karl Zeiss, Germany, in vitro culture).

not only by the disruption of the antioxidant system, but also by the formation of non-selective mitochondrial pores (MP) [7, 14]. Vitrification changes calcium receptors on the membrane of the endoplasmic reticulum, leading to atypical fluctuations in the Ca^{2+} level, which causes unregulated opening of the MP [15]. The spontaneous membrane permeabilization triggers a cascade of apoptosis events [7]. Our results suggest that HDSns optimize processes associated with calcium regulation of MP functioning and increased ion exchange between the cell cytoplasm [3, 6]. The oxidative stress of mitochondria during cryopreservation is associated with the oxidative modification of proteins [16] and the formation of protein fibrillar complexes [17, 18]. These processes provoke oxidation of the cell membrane, disrupt ionic homeostasis, the functioning of the nuclear apparatus, and inter- and intracellular signal transmission [18]. It was shown that nanoparticles ranging in size from 3 to 9 nm exhibit the properties of quantum dots and are capable to inhibit the formation of protein oxidation products [16] and aggregate protein structures [17, 19]. Ultra-small particles of silica supposedly have antioxidant and antiaggregatory effects, due to which the preservation of cellular functions, including nuclear apparatus, occurs. It should also be noted that the effect of HDSns on cell mitochondria may be due to the concentration of particles in solution [20, 21]. An increase in the functional activity of mitochondria in oocytes treated with HDSns and control DV oocytes during the transition period from the diplotene to the metaphase I stages is possibly explained by the restoration of the electrochemical gradient and increased ATP production [22], which is necessary for the completion of the nuclear-cytoplasmic maturation of the DV oocytes.

HDSns have no genotoxic effect [23] and are able to initiate an anti-stress response of cells to ultra-low temperatures through the activation of DNA repair proteins [24, 25]. Importantly, the HDSns genotoxicity, as cytotoxicity, have a size- and dose-dependent effect [25, 26]. In our experiments, HDSns had a positive effect on the nuclear maturation of DV oocytes, which was probably associated with the induction of an anti-stress response and a weakening of the effects of oxidative stress caused by the ultra-small size of HDS particles and their low concentration in maturation medium.

The observed effect of a decrease in mitochondrial activity in all experimental groups during culture may be a consequence of the completion of the nuclear-cytoplasmic maturation of the oocyte with a further block of meiosis at the metaphase II stage before the activation of the oocyte by the sperm. However, the decrease in the IF of MitoTracker Orange CMTMRos and the rise in the proportion of cells with degenerated chromatin in the DV group of oocytes at the metaphase II stage as compared to other experimental groups, apparently, also indicates a significant damage to mitochondria and chromatin caused by cryopreservation procedure.

Thus, the evidence from this study suggests the increase in the mitochondrial potential and the decrease in the number of degenerated cells because of the use of highly dispersed silica nanoparticles (HDSns) in extracorporeal maturation

procedure of devitrified (DV) bovine oocytes, which may indicate the increase in ion exchange and repair processes. An increase in mitochondrial activity when DV oocytes reach the metaphase I stage indicates the increase in the energy supply of cells during this period. The general decrease in the transmembrane potential in native and DV oocytes at the metaphase II stage can be associated either with the completion of nuclear-cytoplasmic maturation, or with a large number of cryo-injuries in the structure of mitochondria (membranes) and chromatin. Consequently, 0.001% HDSns have a positive effect on the indices of nuclear-cytoplasmic maturation of DV oocytes during vitrification of *Bos taurus* female gametes. Creating a cryobank of oocytes is of particular relevance in the context of large-scale selection, a decrease in the reproductive qualities of highly productive cows, and necessity to preserve the gene pool of elite individuals for constructing new genotypes, including by the CRISPR-Cas9 technology. The obtained data can be also used to improve technique of extra- and intra-ovarian vitrification of oocytes of other animal species, and to address problems of human infertility.

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