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Biopolymer gels as a basis of cryoprotective medium for testicular tissue of rats

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Abstract Cryopreservation of testis tissue is a promising approach to save fertility in prepubertal boys under going gonadotoxic cancer therapies. The using biopolymers as a basis of cryoprotective medium can be effective for the optimization of cryopreservation protocols of immature testicular tissue. The research purpose was to determine morphological parameters and metabolic activity of seminiferous tubules of immature rat testes under exposure to cryoprotective solution (DMSO) based on collagen or fibrin gels (CG or FG) as one of the first stages of developing the cryopreservation protocol. It was found that 30-min exposure of tissue samples to CG and FG with 0.6 M DMSO did not impair the spermatogenic epithelium and metabolic activity of the cells (MTT test and total lactate dehydrogenase activity). The use of FG at the time of exposure of 45 min did not lead to significant changes in the metabolic activity in contrast to other groups. The findings could be used to substantiate and develop the effective techniques for cryopreservation of immature seminiferous tubules.

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Introduction

In prepubertal boys affected by cancer, cryopreservation of testicular fragments prior to gonadotoxic treatment is an ethically accepted strategy to save fertility (Abrishami et al. 2010; Michele et al. 2017). Frozen immature testicular tissue can later be used for completion of spermatogenesis after in vitro maturation, germ cell transplantation or testicular tissue grafting (Travers et al. 2011). Despite the fact that the low temperature preservation of testis is associated with some difficulties (different cell size, post thawing ischemia), it can allow to preserve the different testicular cells in their "niche" with respect interactions between germ cells and Sertoli cells (Milazzo et al. 2010). Nevertheless, the optimization of cryopreservation protocols for improving recovery has not been practically investigated. In our opinion, the various biotechnological methods using natural biopolymers can be effective for this purpose, but they require additional studies.

Tissue fragment encapsulation is based on cell scaffold technology (Nicodemus and Bryant 2008). The latter relies on immobilization of cells in a

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biomaterial which allows bidirectional diffusion of nutrients, oxygen, and waste, thus promoting cell interactions. Substances for encapsulation should also be biocompatible and favor (subject to application) vascular invasion and tissue integration in the host (Dhandayuthapani et al. 2011; Jafari et al. 2017).

Polymers are very often used as scaffolds. They can be synthetic or biological origin, and degradable or non-degradable. The two main classes of natural biopolymer are proteins and polysaccharides (Guarino and Ambrosio 2014; Jafari et al. 2017).

Collagens are most abundant family of the complex enzymatically degradable proteins that has unique biological properties. They are involved in forming of membranes, fibrillar system and other components of the extracellular matrix, determining structural integrity and physiological functions in cells (Gelse et al. 2003). They have been oftentimes applicated for biomedical investigation (Nair and Laurencin 2007; Bret et al. 2011). The studies in field of cryobiology, conducted by Allenspach and Kraemer (1989) showed that the distribution of water and structure of ice crystals in collagen gels depend on the composition of the gel and the program of freezing. Thus, the presence of an extracellular matrix may affect the structure of ice formation during freezing-warming, and hence the final result of cryopreservation.

Now, using of fibrin gel is widespread as an alternative to collagens. The products derived from autologous blood are used in the regenerative medicine for the recovery of damaged tissues and organs. One of the important properties of fibrin gel is ability to degrade in a controllable method and self-organizations into a polymer system that imitates of blood coagulation. At the same time, fibrin gel and other derivatives of blood (serum, plasma) contain a large number of different bioactive substances that used as protectors from the damages during the cryopreservation of the cells (Nair and Laurencin 2007; Li et al. 2015).

The apply of gels as a matrix for fragments of the tissue of testicles will allow to minimize tissue damage during cryopreservation by reducing the amount of free water and increasing of tissue resistance to overcooling. In addition, it is known that biopolymer gels have high viscosity which makes them capable to influence the processes of ice crystal formation inhibiting their growth in volume and thus reduce the degree of mechanical action on the tissues (Koebe et al. 1990; Takahashi et al. 1988).

In our preliminary study, it has been shown that a 30-min incubation of samples of tubules of immature rat testes in Hanks medium with add of 5% BSA and 0.6 M DMSO did not cause change to the morphological characteristics of tissue and metabolic parameters of cells (Volkova et al. 2017). The aim of this research was to determine morphological and functional parameters of seminiferous tubules of immature rat testes under exposure to cryoprotective solution (DMSO) based on collagen and fibrin gels as one of the stages in optimizing of the cryopreservation protocol.

Materials and methods

Animals

Outbreed white sexually immature male rats (50 ± 15 g weight, aged 7–8 weeks, $n = 50$) were used in the study. All the manipulations were carried out in accordance to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 18.III.1986). The protocols were approved by the Bioethics Committee of Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine (Permit No 2016-05).

Preperation of biopolymer gels

Collagen gel (CG) was prepared using collagen type I, which was obtained from rat tendons as described by Chandrakasan et al. (1976). The pH was brought to neutral using 0.34 N NaOH solution.

Fibrin gel (FG) was received from the fresh autologous blood of animals, which was obtained from a cardiac vein and centrifuged for 12 min at a rate of 1000 g. After centrifugation, three fractions of blood were received: the lower fraction was erythrocyte mass; the upper fraction was platelet-poor plasma, and the medium one was platelet-rich fibrin gel. Only blood samples without signs of hemolysis were used in the work.

Cryoprotective media

We used DMSO (PanEco, Russia) as the widespread cryoprotectant for testicular tissue freezing at final concentrations of 0.6 M (Keros et al. 2007), but cryoprotective media differed in the basis. The first experimental medium (CG + DMSO) consisted of collagen gel with 0.6 M DMSO, and the second one (FG + DMSO) consisted of fibrin gel with 0.6 M DMSO. The controls were corresponding gels without cryoprotectant (CG and FG respectively) and intact samples of testicular tissue.

Exposure of testicular tissue

Samples of testicular tissue weighing 75 ± 5 mg were isolated mechanically immediately before the study. They were immersed in the cryoprotective media and exposed during 15, 30, 45 and 60 min at 4 °C (Keros et al. 2007; Milazzo et al. 2010; Travers et al. 2011). The samples after exposure were (washed out by a three-step change of Hanks solution and used for morphological and biochemical studies (Fig. 1).

MTT test

Quantification of metabolic activity was executed at 15, 30, 45 and 60 min of term expositions on testicular tissue samples. MTT test is based on the ability of dehydrogenases to restore [3-(-4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] in living cells to blue crystals of formazan, which are insoluble in water. A final concentration of 0.5 mg/mL MTT (Fluka, Germany) was added to the samples and incubated for 3 h at 37 °C. Then the medium was deleted and 100% DMSO was added to each samples of tissue to solubilize the precipitate (Mossman 1983). Absorbance was read at 570 nm.

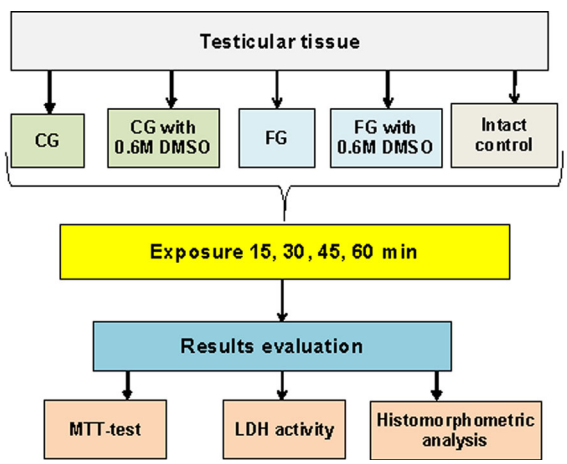

Fig. 1 Experimental scheme. *CG* collagen gel, *FG* fibrin gel, *DMSO* dimethyl sulfoxide, *MTT* 3-(4,5-dimethylthi azol-2-yl)-2,5-diphenyltetrazolium bromide, *LDG* lactate dehydrogenase

Analysis of total lactate dehydrogenase (LDH) activity

The total LDH activity was estimated quantitatively by the method of UV spectrophotometry was using test kits (Felicit, Ukraine). The samples of tissues of all experimental groups were homogenized and filtered, with following centrifugation (1000 g for 10 min). Reaction was started by the addition of enzyme sample to 20 µL supernatant of tissue homogenat with following spectrophotometrical measuring of a decrease in absorbance of NADH at 365 nm.

Histomorphometry

Histomorphometry was carried out maintaining blinding by involving the third person who did not take part in the experiment. Paraffin blocks were prepared and slices of 7 µm thickness were stained by hematoxylin and eosin. Histological preparations were studied using Axio Observer Z1 inverted microscope (Carl Zeiss, Germany). Obtained image files were processed using the Axiovision v. 4.8 (Carl Zeiss).

The following qualitative criteria were studied: cell retraction, nuclei condensation, cell detachment and formation of gaps (Fig. 2). Additionally, a cell density

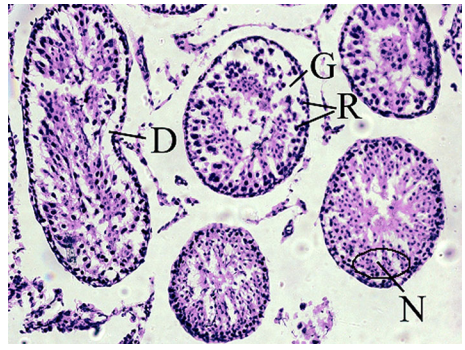

Fig. 2 Histological evaluation of spermatogenic epithelium damages. *R* cell retraction, *N* nuclei condensation, *D* cell detachment, *G* formation of gaps

of spermatogenic epithelium was detected as the average of the number of nuclei in the section area (0.102 mm^2) with following conversion per 1 mm^2.

Statistical analysis

The Mann–Whitney U-criterion was used to determine the statistical significance of the differences in continuous variables when comparing between the groups with multiple (more than two) comparisons Kruskal–Wallis ANOVA tests using Excel (Microsoft, USA) and Statistics 8 (StatSoft, USA) software.

Results

Metabolic activity

The obtained experimental dates of the MTT test indicate that the 15- or 30-min exposures in all studied media did not lead to a significant change in metabolic activity of the immature testis tissue if compared to the intact control and between the investigated biopolymers (Fig. 3). In addition, the metabolic activity was decreased in groups with CG by 19.5% and CG + DMSO by 21.9% compared to intact control at the 45 min. In the media based on FG this parameter remained at the level of intact control. After 60 min of exposure the investigated index in all cases was lower if compared to intact control: for CG and CG + DMSO by 37.5 and 32.7%, for FG and FG + DMSO by 17.3 and 19.2% respectively.

The results of the study of the influence of cryoprotective media on the total LDH activity are shown in Fig. 4. The time of exposure of 15 and 30 min in all tested biopolymers media did not lead to strong changes in LDH activity if compared to the intact control as well as between the investigated media. At the 45-min exposure there was a tendency to decrease the investigated index in the CG and CG + DMSO media by 17.2 and 18.3% respectively compared to the intact control. At 60-min exposure of tissue in media based on CG, we observed a decrease in LDH activity index by more than 1.5 times if compared to intact control. Thus, the increase in the tissue exposure time in the media on base of CG more than 30 min resulted in significant changes in LDH activity, which probably indicated the violation of metabolic processes in the cells. The use of FG at the timing of exposure of 15–45 min did not lead to significant changes in this parameter. The data of the LDH activity on the 60th minute of observation are consistent with the results of MTT test at the same time, which indicates the inhibition of metabolic processes in experimental samples due to the exposure duration.

Histomorphometry

Dates of the biochemical study showed no influence of investigated biopolymers on seminiferous tubules of testes under the 30-min term of exposure. Significant changes were noted starting from 45 min. Therefore the next step was histomorphometric analysis to obtain

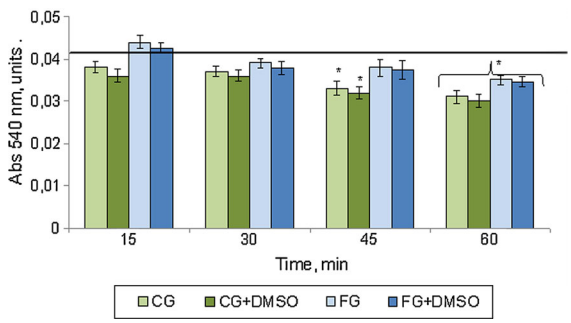

Fig. 3 Dynamic of metabolic activity (MTT test) of seminiferous tubules of immature rat testes under exposure to investigated media. Solid line is an index of intact control. *The difference is statistically significant relative to intact control (M ± m; n = 5; $p < 0.05$)

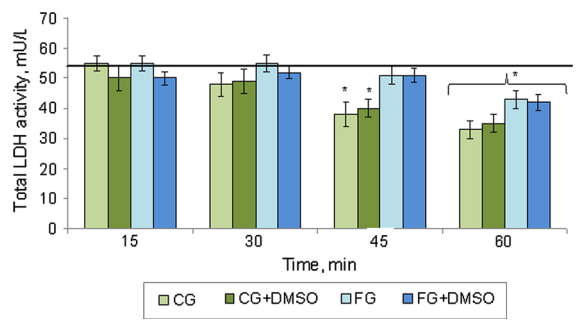

Fig. 4 Dynamics of total LDH activity of seminiferous tubules of immature rat testes under exposure to investigated media. Solid line is an index of intact control. *The difference is statistically significant relative to intact control (M ± m; n = 5; $p < 0.05$)

more information about structural characteristics at this term of exposure (Fig. 5).

It was found that seminiferous tubules of the intact group had a form of the rounded formations of the basal membrane inside of which there was a multilayer germinative epithelium (Fig. 5 Intact control). The population of spermatogenic epithelium was represented by all types of spermatogenic cells excepted of spermatozoa. Spermatogonia were located on the basal membrane, above them, spermatocytes were placed in 2–3 rows. In some tubules, the cells reached a developmental level of early spermatids.

After 30-min exposure in CG (Fig. 5 CG), in the most cases, there was slight desquamation of epithelium into the lumen of seminiferous tubules. The detachment of spermatogenic epithelium from the basal membrane was partial. In addition, there was a chaotic location of the germinative cells with formation of gaps and ruptures inside the spermatogenic epithelium due to the obvious cell retraction. Nuclei condensation was slightly present.

In the case of CG + DMSO combination, germinative cells in most cases saved their connection with the basal membrane and orientation within the spermatogenic epithelium despite the obvious retraction and gap formation. However, there were extensive regions covered by karyolysis (the cell nuclei are swollen, slightly colored with hematoxylin, without distinct contours) in the center of separate seminiferous tubules. At the same time, the tubules were detected in which spermatocytes had a hyperchromic nucleus and a sharp eosinophilic cytoplasm (Fig. 5 CG + DMSO).

When FG was used, cell nuclei of the primarily spermatocytes had a swollen appearance and were sharply hyperchromic. The contours of seminiferous tubules were fuzzy and deformed but desquamation phenomena were absent. The cytoplasm of germinative cells was slightly retracted (Fig. 5 FG).

After exposure in FG + DMSO, as well as in the previous case, cell nuclei of the spermatogenic epithelium in some seminiferous tubules had a swollen appearance and were sharply hyperchromic. But it should be noted that the FG, in contrast to the CG, had a more pronounced protective effect in combination with DMSO, preventing the development of necrosis in germinative cells (nuclei condensation was slightly present). Cell retraction and formation of gaps in most cases were slightly present too and cell detachment was absent (Fig. 5 FG + DMSO).

The morphometric study showed that significant differences in the average density of spermatogenic

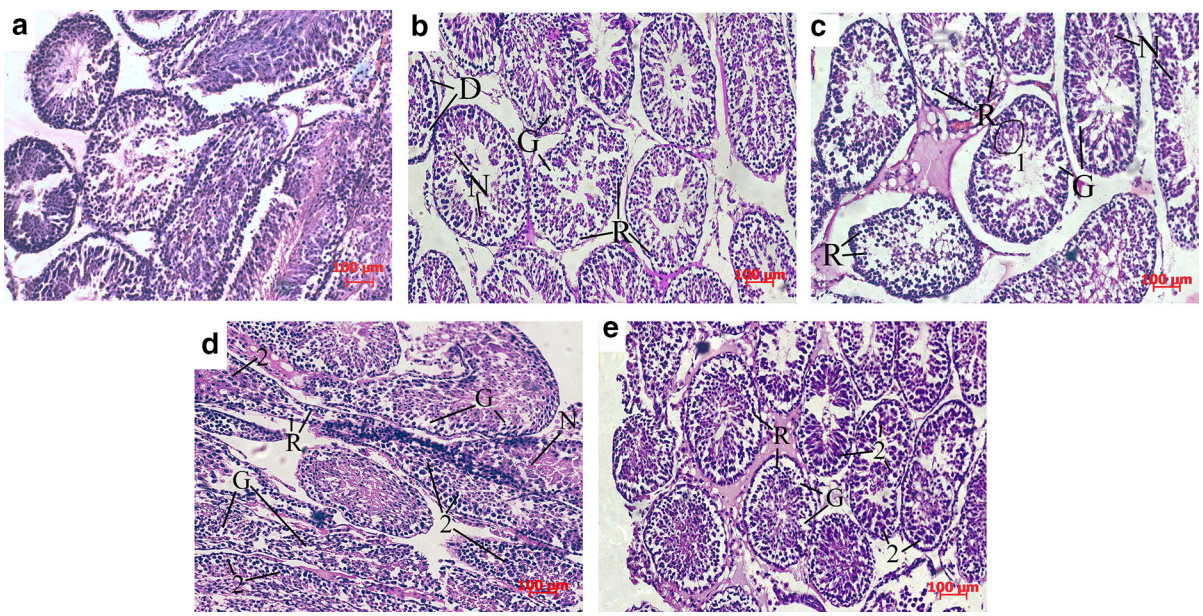

Fig. 5 Seminiferous tubules of intact immature rat testes (**a**) and after 30-min exposure in the CG (**b**), CG + DMSO (**c**), FG (**d**), FG + DMSO (**e**). Hematoxylin and eosin staining. *R* cell retraction, *N* nuclei condensation, *D* cell detachment, *G* formation of gaps, *1* regions covered by karyolysis, *2* swollen and hyperchromic nuclei

cells were absent in both investigated media without cryoprotectant compared to the intact control (Fig. 6). In the case of exposure of testicular tissue to DMSO based on CG this parameter was in 1.3 times lower than in the intact. However, this statement should be treated with caution since according to the data mentioned above some of the cells could have signs of karyopicnosis/kariolysis. The use of FG as a base of medium for cryopreservation was more effective in contrast to CG. In this case (FG + DMSO), the average cell density did not differ significantly from to the intact control.

Discussion

Now it is absolutely clear that progress in the field of biotechnology is inextricably linked with the development of cryobiological approaches because low temperature preservation allows storing biomaterial for future medical procedures. Furthermore, cryopreservation makes the use of cell and tissue products easy in time and place. Therefore, materials for such purposes should be acceptable and even effective in the process of freezing–thawing. This work is the first stage in the study of the possibility to use the biopolymer gels for improving of the efficiency of immature testicular tissue cryopreservation that is so necessary for prepubertal boys undergoing gonadotoxic therapy.

Modern development of innovative biomaterials has made a significant contribution to the development of reproductive medicine, especially in the aspect of maintaining fertility. At the moment hydrogels are considered as a unique solution for stem cell storage

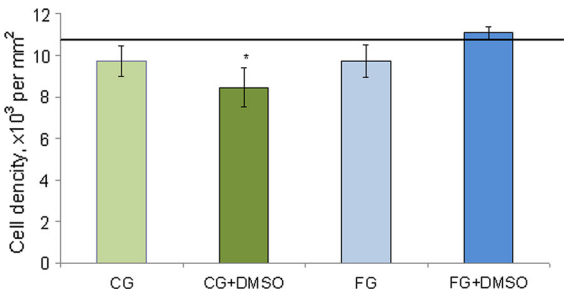

Fig. 6 The average density of spermatogenic epithelium cells after 30-min exposure in the investigated media. The solid line is an index of intact control. *The difference is statistically significant relative to intact control (M ± m; n = 5; $p < 0.05$)

and transport. Their effectiveness has been studied in relation to embryonic stem cells (Ji et al. 2004), multipotent stem cells from bone marrow (Chen et al. 2013), adipose tissue (Swioklo et al. 2016) etc. Nevertheless, the using of gels for tissue cryopreservation is insufficiently studied. Some recent studies (Hatte et al. 2016) have shown that DMSO with polysaccharide-based hydrogel can be recommended as a new effective tool for improving procedure of cell or tissue low temperature preservation. The authors note that this combination allow to ensure safety discarding serum and to reduce DMSO concentration and its cytotoxicity or clinical side-effects. Other results (Itoh et al. 2001) indicate that covering with CG improved the recovery and viability of the small preantral follicles during vitrification steps. Furthermore, the vitrified follicles embedded in CG could be maintained in culture during 1 week. Microencapsulation in ultrahigh viscous alginate allows maintaining of neurosphere morphology, membrane integrity of cells, their metabolic activity, and cell-specific interactions, which are major requirements for their applications after thawing. The use of gels in cryopreservation has also been studied in relation to hepatocyte spheroids (Lee et al. 2004) and multicellular tumor spheroids (Sakai et al. 2012) and optimistic results were received. In the same way FG provides optimal support for adhesion, proliferation, differentiation and biochemical signaling of cells. It is used to encapsulate fragments of integral tissue or isolated cells to support their 3D structure, as well as to study biological phenomena that would be impossible in 2D systems (Chiti et al. 2017). The studies, conducted by Murdock et al. (2018) showed that extracellular matrix can promotes mitogenesis, migration, and/or differentiation of various stem/progenitor cells and contribute to the survival of human spermatogonial stem cells in culture.

So encapsulation is considered at this point as a promising approach for low temperature preservation of 3D cell systems, as this approach protected cells against mechanical damages during ice crystal formation and reduce the risk of cell–cell disruption through immobilization within the hydrogel (Malpique et al. 2010).

In this research, we examined the potency of the using of collagen and fibrin gels as the basis of the cryoprotective medium at the exposure stage. Collagen and fibrin gels are commonly used in tissue

engineering as the foundational matrix due to their availability, scaffolding function, and bioactive qualities. Undoubted advantage of them is an ability to closely mimic native extracellular matrix of tissues. Scaffolds on basis of this substance in various forms have been studied and employed for different tissue regeneration purposes (Geckil et al. 2010).

It was found that FG led to the prolongation of the exposure time of the seminiferous tubules to 45 min according to the results of metabolic tests as well as to less histological changes in the tubular structure with preservation of the average cell density of the spermatogenic epithelium. More pronounced protective effect of FG in our opinion is due to the presence of different biologically active substances in its composition in contrast to CG, whose composition is poorer. It is known that various blood products contain a number of growth factors (hepatocyte growth factor (HGF), transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), insulin like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), nerve growth factor (NGF) etc.) but in different ratio (Nishiyama et al. 2016). Growth factors play a huge role in the cell functioning and can affect cell metabolic processes including on the stages of low temperature preservation. Thus a significant improvement was observed in viability, motility and apoptosis level of human asthenozoospermic sperm after addition of NGF to the cryoprotective media (Saeednia et al. 2016). The using of IGF-I reduced the ratio of sperm with disrupted membranes and the number of Annexin V+ sperm after hypothermic storage (Makarevich et al. 2014). VEGF treatment can prevent germ cell death in bovine testis tissue explants due to stimulation of an intracellular response (Caires et al. 2009). Moreover in the case of application of cryopreserved testicular tissue in vivo VEGF can support engraftment of the transplant promoting angiogenesis in the tissue (Tian et al. 2016; Del Vento et al. 2018).

Thus the use of FG as an integral component of freezing medium is effective and contributes to the preservation of the average density of germinative cell and their metabolic activity during the exposure stage of cryopreservation. We recommend an optimal exposure time of 30-min in the case of cryoprotective media on the bases of CG or FG. However, 45-min exposure of testicular tissue to FG with 0.6 M DMSO did not impair the spermatogenic epithelium and the metabolic activity of the cells (MTT test and LDH activity) and therefore time interval can be increased for this gel. In the future, we plan to study the morphological and functional parameters of immature testicular tissue after freezing- thawing and evaluate the contribution of biopolymer gels to the protection of the spermatogenic epithelium during low-temperature storage.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All the manipulations with animals were carried out in accordance to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 18.III.1986). The protocols were approved by the Bioethics Committee of Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (Permit No 2016-05).

Human and animal rights This article does not contain any studies with human participants performed by any of the authors.

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