EXPERIMENTAL ARTICLES

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APPLICATION EFFICIENCY OF BOVINE SERUM ALBUMIN FOR RECOVERY OF SEMINIFEROUS TUBULES OF TESTES AFTER CRYOPRESERVATION

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Background. An optimal approach to the recovery of testicular tissue fragments after cryopreservation is critical for their further use in order to successful fertility restoration.

Aim. The purpose of this study was to investigate the effect of bovine serum albumin (BSA) addition to the rehabilitation medium on the morphofunctional characteristics of fragments of seminiferous tubules of testes (FSTT) of immature rats after cryopreservation.

Methods. The object of the study was cryopreserved by slow cooling and vitrified FSTT. Warmed samples were incubated for 30 min in Leibovitz medium supplemented with BSA at concentrations of 0, 2, 5 or 10%. After that, morphological characteristics, the activity of the metabolic and antioxidant systems were evaluated.

Results. It was found that in the samples cryopreserved by slow cooling, the use of 5% BSA contributed to the increase in the safety of spermatogenic epithelium cells, in the levels of metabolic and antioxidant activities. In case of vitrified FSTT samples, it was observed that the addition of 5% BSA to the medium promoted the repair of minor tissue damage and increased metabolic activity level, but did not affect the state of the antioxidant defense system.

Conclusions. The obtained data can be used to develop an effective rehabilitation medium for cryopreserved fragments of the seminiferous tubules of testes using BSA.

Key words: bovine serum albumin, rehabilitation, cryopreservation, fragments of the seminiferous tubules of testes.

Today, there is an active developing of the direction of cryopreserved immature testicle fragments transplantation for fertility restoring in the field of reproductive biotechnology. According to the WHO, most people who have experienced childhood cancer are prone to infertility. High doses of chemo- and radiation therapy lead to the destruction or dysfunction of spermatogonial stem cells [1, 2]. Therefore, cryopreservation of fragments of testicular tissue containing a large number of germ cells, especially spermatogonia, which can be a source of male gametes if properly stored and cultured, is now widely recommended for patients with prepubertal cancer [3]. This procedure is currently available in many reproductive centers around the world. Wherein, both slow freezing and vitrification have shown their effectiveness in immature testicular tissue preservation [4, 5].

A search for new and an improvement of existing methodological approaches to cryopreservation of fragments of convoluted seminiferous tubules is important for their further application. It is known that low-temperature storage leads to a decrease in the activity of the protective systems of biological objects, as a result of which damage to the integrity of cell membranes and initiation of apoptosis processes occur [6]. According to the authors [7], free radicals, such as superoxide

and hydroxyl radicals, are damaging factors for tissue. The contact of OH radicals with the polyunsaturated fatty acids leads to increased permeability of cell membranes, disruption of mitochondrial function, decrease in the activities of glyceraldehyde-3-phosphate dehydrogenase, ATPase, etc. [8, 9].

Male gametes are very sensitive to the harmful effects of reactive oxygen species (ROS) due to the large number of unsaturated fatty acids found in their cell membranes [10]. In the work of authors [11] it has been shown that increasing of ROS content leads to reduced sperm motility, DNA fragmentation and activation of apoptosis. Therefore, the attention of scientists has long been attracted by the study of various additional components in the composition of the medium for low-temperature preservation of reproductive cells. As such additive, it is effective to use blood serum, as well as one of its constituents — albumin. Serum albumin is known for its ability to regulate oncotic pressure, to normalize acid-base balance, to bind different ligands, and to exhibit powerful antioxidant properties. Based on the literature, it was found that in the process of sperm storage bovine serum albumin (BSA) (5 mg/ml) and fetal bovine serum (FBS) (10%) have a protective effect on various parameters of sperm: morphological characteristics, plasma membrane and acrosome integrity, motility, DNA structure, fertilization potential [12]. In addition, it was shown that the addition of both BSA and FBS plays a protective role in maintaining the antioxidant activity of glutathione peroxidase and in reducing the level of malonic dialdehyde during sperm storage. Based on the above, both of these impurities are alternative protective agents, but FBS gives in to BSA in widespread use, because its application is burdened by ethical standards, and obtaining is more expensive. Previously, we have shown the effectiveness of BSA addition to cryopreservation solution for fragments of seminiferous tubules of the testes (FSTT) of immature rats [13]. However, the effectiveness of the use of protein impurities in the stages of deconservation and subsequent rehabilitation for FSTT of immature rats has not been studied so far, although it is of interest to develop modern biotechnological approaches for potentiating the functional state of tissues after low temperature storage.

The aim of the study was to investigate the effect of bovine serum albumin (BSA) addition to the rehabilitation medium on the morphofunctional characteristics of fragments of seminiferous tubules of testes (FSTT) of immature rats after cryopreservation.

Materials and Methods

Immature outbred rats aged 7–8 weeks (n = 15) were used in the study. Experimental scheme is shown in Fig. 1.

The testes were isolated from animals after humane euthanasia (CO_2 asphyxia) and were washed in sterile Leibovitz L-15 medium (BioWest, France). Tunica albuginea was removed with sterile medical instruments, and the testicles were then washed in Leibovitz medium again. Samples weighing 75±5 mg and 25±3 mg were made from the obtained material and used respectively for slow-cooling cryopreservation [14] and for vitrification [15]. In the case of slow cooling cryopreservation, rat FSTT were exposed to a medium based on fibrin gel (FG) and 6% glycerol for 30 min at 4 °C, then they were frozen in nitrogen vapor to −70 °C at an uncontrolled rate (40 min) with following transfer to liquid nitrogen (−196 °C). Heating of FSTT samples was carried out in a water bath at 40 °C with preliminary exposure to liquid nitrogen vapor. Removal of the cryoprotectant from the samples was performed by a three-step change of the cryopreservation medium to Hanks' solution. In the case FSTT vitrification, a combination of cryoprotectants was used: medium 1 (FG + 5% Me2SO + 6% glycerol + 0.1 M sucrose) and medium 2 FG + 15% Me2SO + 18% glycerol + 0.5 M sucrose). Sequential exposure of FSTT to each medium 1 and 2 was performed for 5 min at a temperature of 4 °C, followed by rapid immersion of the fragments in liquid nitrogen. Heating was performed in 1 M sucrose solution at 50 °C and then samples were transfered to sucrose solutions of decreasing concentrations (0.5 M, 0.25 M, 0 M) at temperature of 20 °C. The exposition to each sucrose solution lasted for 5 min.

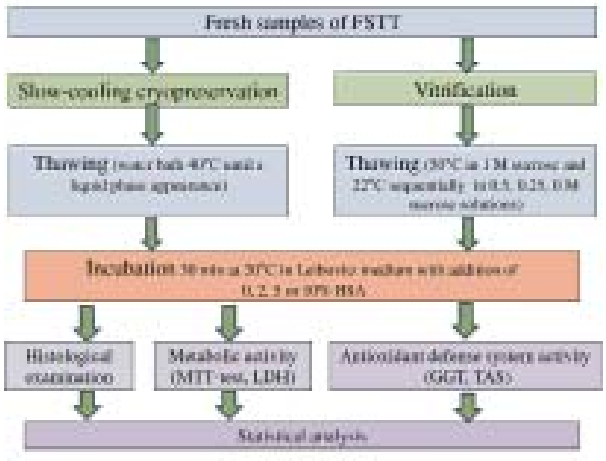

Fig. 1. **Experimental scheme**

FSTT samples after heating and removal of cryoprotectants were incubated for 30 min in Leibovitz medium with the addition of BSA (PAA, Austria) at concentrations of 2, 5 or 10%. The control samples were incubated in Leibovitz medium without BSA addition.

Five fragments from each experimental group were taken for histological examination and fixed in 10% formalin solution (Sigma-Aldrich, USA). Sections of 7 μm thickness from paraffin blocks were stained with hematoxylin&eosin. A general assessment of histological structure of the seminiferous tubules, the relative number of cells with intact and pathological nuclei and the average density of spermatogenic epithelial cells per 1 mm^2 were determined. A Carl Zeiss LSM 510 Meta light microscope (Oberkochen, Germany) and ZEISS ZEN 2 (blue edition) software were used.

The total metabolic activity of rat FSTT samples was investigated using a MTT test (Sigma-Aldrich, USA). To do this, the samples (5 fragments from each group) were transferred into 1 ml of Hanks' solution, where 0.5 ml of 5 mg/ml MTT was added. After three hour incubation (5% CO_2, 37 °C), the medium was removed and the formed formazan was dissolved with Me_2SO (1 ml/sample). Precipitate of proteins was released by centrifugation for 10 min at 1000 *g*. Measurements of the optical density of the formazan solution in the supernatant were performed on a biochemical analyzer CHEM 7 ("ERBA", Czech Republic) at a wavelength of 540 nm. Cell-free medium was used as a blank.

Randox test kits (UK) were used to measure the activity of lactate dehydrogenase (LDH), gamma-glutathione transferase (GGT) and total antioxidant status (TAS) in rat FSTT samples. To test the samples, a homogenate from 5 fragments was prepared, filtered through a nylon filter and centrifuged (5 min, 840 *g*). Measurements of optical density were performed in the supernatant on the biochemical analyzer CHEM 7. The calculation of activity was performed according to the manufacturer's instructions and normalized to mg of protein. The latter was determined by the biuret method (Randox, UK).

Significance of the differences between groups was assessed using the Mann-Whitney test. The statistical parameters are presented below as means and their errors ($M \pm m$). The critical value of the significance level (P) was taken to be 0.05. Data analysis was performed using software packages "Microsoft Excel" and "Statistica 8".

Results and Discussion

The results of histological examination showed that in control samples after cryopreservation using slow cooling, the structure of the seminiferous tubules of the testes was characterized by moderate damage, namely: unexpressed retraction of cells, insignificant spherical lacunae in the epithelial layer, partial desquamation, karyopyknosis in germinogenic cells (Fig. 2). In addition, the basement membrane in the areas of desquamation looked thickened and swollen.

The BSA addition to the rehabilitation media of slow frozen FSTT of immature rats had a positive result (Fig. 2). The number of tubules with the phenomena of spermatogenic epithelium desquamation and swelling of the basement membrane decreased in all experimental groups compared to the control. The number of cells with karyopyknosis underwent pronounced changes, which under the influence of rehabilitation decreased with the maximum effect in the medium with 5% BSA (Fig. 3, *A*).

Histological structure of seminiferous testicular tubules in control samples after vitrification was characterized by moderate cell retraction (Fig. 4). Desquamation of the spermatogenic epithelium was pronounced, often becoming total. The nuclei of most cells looked pyknotic, and in some cells they were vacuolated.

The BSA addition to the rehabilitation medium of vitrified FSTT samples reduced the degree of cell retraction, the frequency of karyopyknosis and spermatogenic epithelium desquamation compared to the control (Fig. 3, *A;* 4). These changes occurred in all groups with the use of BSA, but the best result was found at a concentration of 5%.

The BSA addition to the rehabilitation medium also helped to increase the average density of spermatogenic epithelial cells in the seminiferous tubules, both cryopreserved using slow cooling and vitrified: in the first case this index increased by 1.47 times ($P < 0.05$) when BSA was used at concentration of 5%, and in the second one it did by 1.43 times ($P < 0.05$) relative to the control. In other experimental groups, the average density of spermatogenic epithelial cells remained at the control level (Fig. 3, *B*).

The next step was to determine the metabolic activity in deconserved rat FSTT after rehabilitation in the medium with BSA addition. The obtained results, that

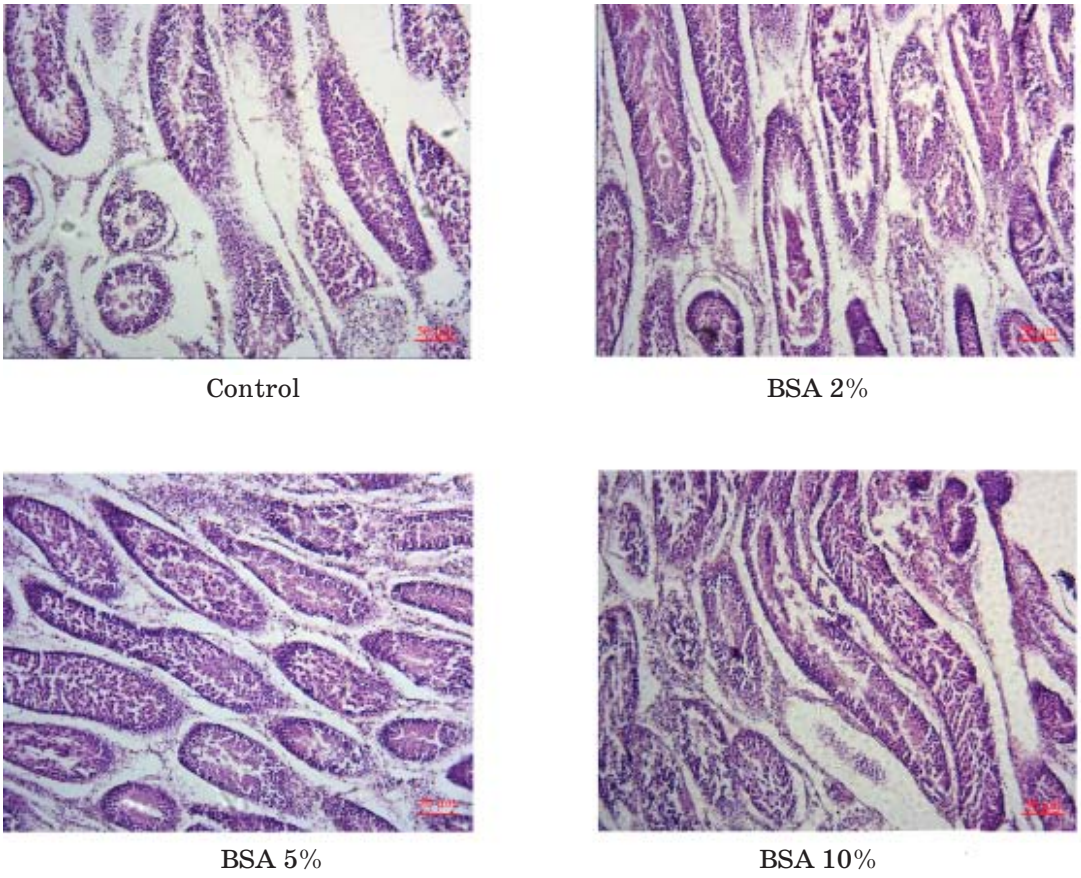

Fig. 2. **Histological structure of FSTT of immature rats after cryopreservation using slow cooling and subsequent rehabilitation in the medium with BSA addition: desquamation frequency reducing in spermatogenic epithelium and basement membrane normalization after incubation in medium with 5% BSA addition**
Light microscopy; staining with hematoxylin and eosin

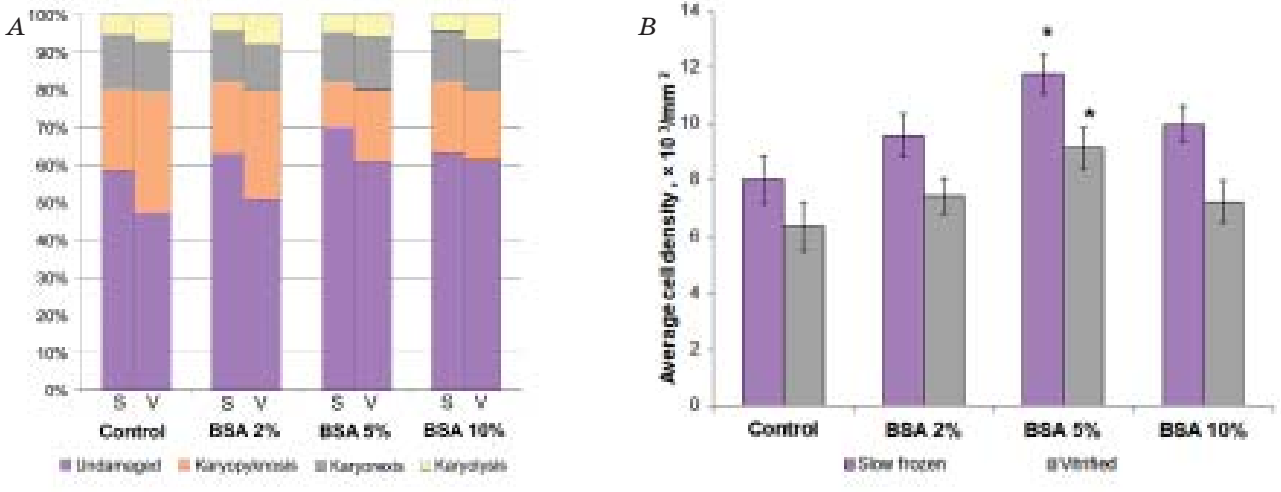

Fig. 3. **Nucleus condition (*A*) and average density of spermatogenic epithelial cells (*B*) in cryopreserved FSTT of immature rats after rehabilitation in the medium with BSA addition:**
S — slow frozen; V — vitrified; * — the difference is significant compared to the control ($P < 0.05$; $n = 5$)

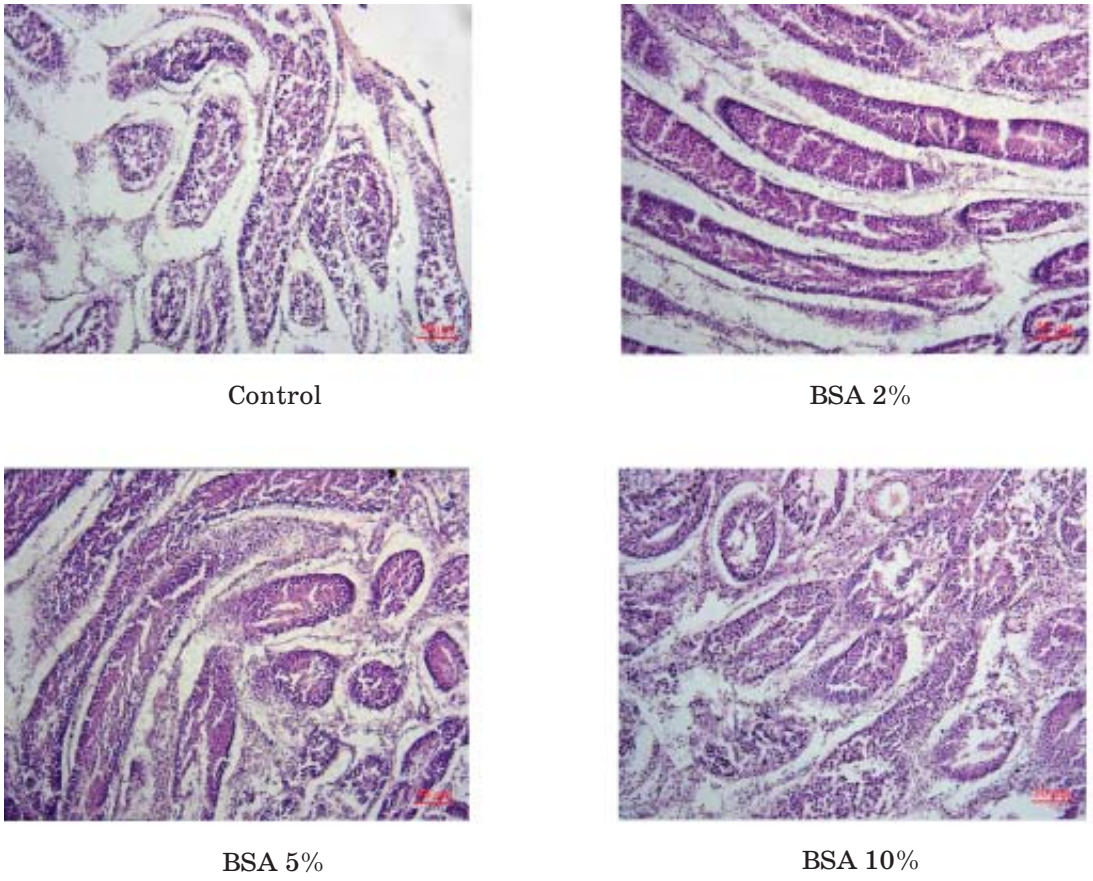

Fig. 4. **Histological structure of FSTT of immature rats after vitrification and subsequent rehabilitation in the medium with BSA addition: cell retraction and desquamation frequency reducing after incubation in medium with a BSA concentration of 5% after incubation in medium with 5% BSA addition**
Light microscopy; staining with hematoxylin and eosin

are presented in Fig. 5, *A*, indicate a 1.5-, 1.8- and 1.6-fold increase ($P < 0.05$) of total metabolic activity in experimental FSTT samples, cryopreserved using slow cooling and rehabilitated in the medium with 2, 5 and 10% BSA addition respectively compared to the control. Metabolic activity in vitrified FSTT samples with following rehabilitation in the medium with BSA addition at concentrations of 5 and 10% was increased by 1.9 and 1.7 times ($P < 0.05$) respectively compared to the control. The rate of total metabolic activity in samples cryopreserved by slow cooling exceeded that in vitrified FSTT.

In experimental samples cryopreserved using both slow cooling and vitrification and then rehabilitated in the medium with BSA addition in the studied concentrations, no significant changes in LDH activity were observed relative to the control (Fig. 5, *B*).

Determination of GGT activity and TAS level in rat FSTT samples after freezing-thawing and subsequent rehabilitation in the medium with BSA addition are shown in Fig. 6 (*A, B*).

The obtained data showed a 1.2- and 1.3-fold increase ($P < 0.05$) relative to the control in the GGT activity and TAS level respectively in FSTT samples, cryopreserved using slow cooling and rehabilitated in the medium with BSA addition at concentration of 5%. The use of BSA at concentrations of 2 and 10% did not lead to significant changes in these indexes in rat FSTT samples, cryopreserved using both slow cooling and vitrification, compared to the control.

GGT is known to be an important enzyme involved in glutathione metabolism. In the seminiferous tubules, this enzyme is localized in Sertoli cells [16] and stimulated by the corresponding hormones. Meroni et al. [17] showed that GGT was present in epididymal epithelial cells and fluid of the lumen. It has been assumed that it plays a role in

sperm protection from oxidative stress in the epididymal duct and/or in extracellular cysteine reduction for protein synthesis [18].

The use of BSA at concentration of 5% had the positive effect on the histological structure, reduced the frequency of karyopyknosis in spermatogenic epithelial cells, increased levels of metabolic and antioxidant activities in samples cryopreserved by slow cooling. During the rehabilitation of vitrified FSTT, it was observed that the addition of 5% BSA contributed to the repair of minor histological damages and increased the level of total metabolic activity without significant changes in the state of the antioxidant defense system.

The authors of the paper [19] described a possible mechanism of the protective effect of BSA on cell membranes. It was shown that protein components are needed to preserve the solubilized form, reduce diffusion and stabilize connection of phospholipids with the cell membrane. In a study by the authors [20], it was shown that BSA can exhibit a membrane stabilizing effect (by absorption on the lipid bilayer and prevention of vesicle aggregation). Assumptions about the possible interaction of BSA with cell membranes are confirmed by data [21, 23] on the absence of concentration

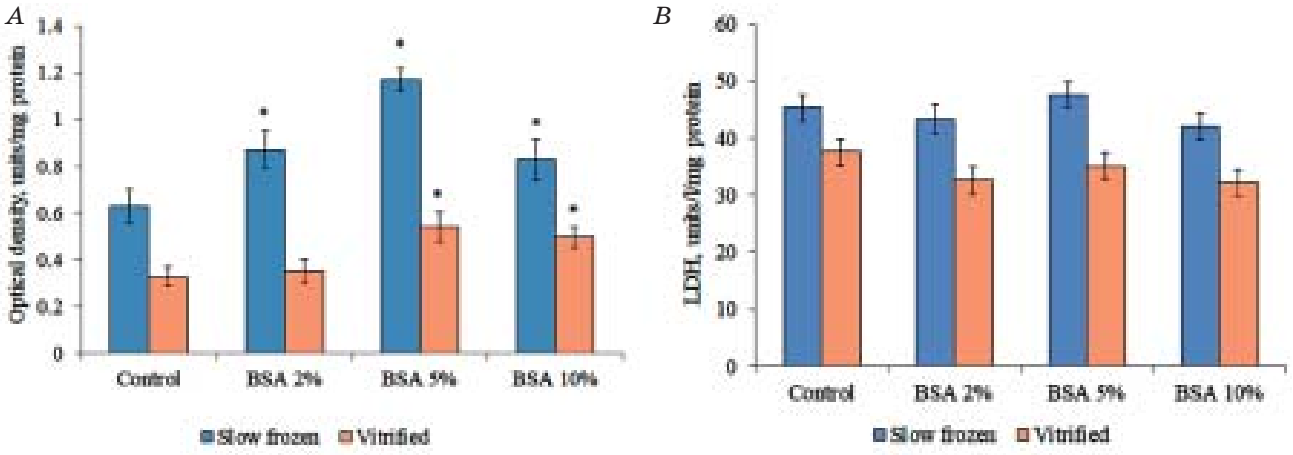

Fig. 5. **Rates (A) of total metabolic activity (MTT-test) and (B) LDH activity in cryopreserved rat FSTT after rehabilitation in the medium with BSA addition**
* — the difference is significant compared to the control ($P < 0.05$; $n = 5$)

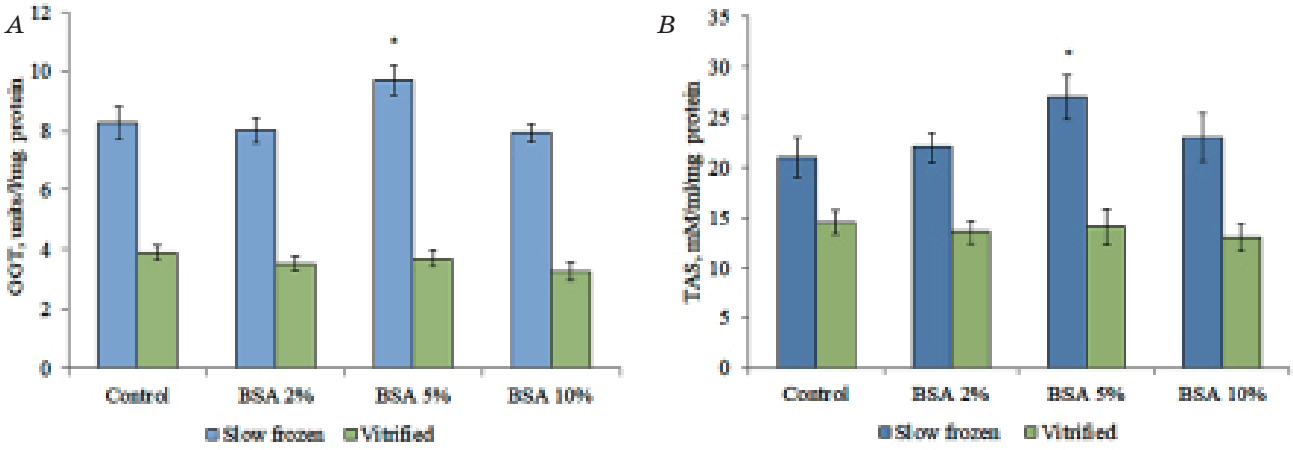

Fig. 6. **GGT activity (*A*) and TAS level (*B*) in cryopreserved rat FSTT after rehabilitation in the medium with BSA addition**
* — the difference is significant compared to the control ($P < 0.05$; $n = 5$)

dependence (1 and 4.6%) and its positive effect on the motility and integrity of sperm membranes during incubation *in vitro*.

Serum albumin is a large protein molecule and is found in the fluids of reproductive tract. Today, serum albumin, including BSA, is one of the available egg yolk replacement proteins, which is often used for sperm cryopreservation. Matsuoka et al. [23] reported that BSA can replace egg yolk in sheep sperm dilution solutions, enhancing sperm motility and viability after cryopreservation. Yokuchi and others [24] suggested that the adsorption of BSA on liposomes is likely due to their hydrophobic interaction. It has also been shown that the microfluidity of liposomal bilayer membranes near the central bilayer decreases, while their permeability increases due to the adsorption of BSA on liposomes.

Conclusions

Thus, BSA addition at concentration of 5% to the rehabilitation medium helped to increase the preservation of spermatogenic epithelial cells, levels of metabolic and antioxidant activities in samples of FSTT, cryopreserved by slow cooling. During the rehabilitation of vitrified FSTT, it was observed that the addition of 5% BSA led to the repair of minor tissue damage and increasing the level of metabolic activity, but did not affect the antioxidant defense system state. The obtained data can be used to develop an effective rehabilitation medium for cryopreserved fragments of the seminiferous tubules of testes using BSA.

Statement on animal protection. All animal manipulations were performed in accordance with international bioethical norms, legislative documents of Ukraine, materials of the IV European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the protocol of the Bioethics Committee of the Institute of Cryobiology and Cryomedicine of the NAS of Ukraine (No. 2014-02).

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Conflict of interests. The authors declare that they have no competing interests.

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ЕФЕКТИВНІСТЬ ЗАСТОСУВАННЯ БИЧАЧОГО СИРОВАТКОВОГО АЛЬБУМІНУ ДЛЯ ВІДНОВЛЕННЯ ЗВИТИХ КАНАЛЬЦІВ СІМ'ЯНИКІВ ПІСЛЯ КРІОКОНСЕРВУВАННЯ

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Вступ. Оптимальний підхід до відновлення фрагментів тканини яєчка після кріоконсервування має вирішальне значення для їх подальшого успішного використання у лікуванні безпліддя.

Мета роботи — дослідити вплив додавання бичачого сироваткового альбуміну (БСА) до середовища реабілітації на морфофункціональні характеристики фрагментів звивистих канальців сім'яників (ФЗКС) статевонезрілих щурів після кріоконсервування.

Методи. Об'єктом дослідження були кріоконсервовані з повільною швидкістю охолодження та вітрифіковані ФЗКС. Відігріті зразки інкубували впродовж 30 хв у середовищі Лейбовіца з додаванням БСА у концентраціях 0, 2, 5 або 10%, після чого оцінювали морфологічні характеристики, активність метаболічної та антиоксидантної систем.

Результати. Встановлено, що у зразках, кріоконсервованих шляхом повільного охолодження, застосування 5% БСА сприяло підвищенню збереженості клітин сперматогенного епітелію, рівнів метаболічної та антиоксидантної активностей. У разі вітрифікованих зразків ФЗКС додавання до середовища 5% БСА сприяло репарації незначних ушкоджень у тканині та підвищувало рівень метаболічної активності, однак не впливало на стан системи антиоксидантного захисту.

Висновок. Отримані дані можуть бути використані для розроблення ефективних реабілітаційних середовищ для кріоконсервованих фрагментів звитих канальців сім'яників з використанням БСА.

Ключові слова: бичачий сироватковий альбумін, реабілітація, кріоконсервування, фрагменти звитих канальців сім'яників.

ЭФФЕКТИВНОСТЬ ПРИМЕНЕНИЯ БЫЧЬЕГО СЫВОРОТОЧНОГО АЛЬБУМИНА ДЛЯ ВОССТАНОВЛЕНИЯ ИЗВИТЫХ КАНАЛЬЦЕВ СЕМЕННИКОВ ПОСЛЕ КРИОКОНСЕРВИРОВАНИЯ

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Вступление. Оптимальный подход к восстановлению фрагментов ткани яичка после криоконсервирования имеет решающее значение для их дальнейшего успешного использования в лечении бесплодия.

Цель работы — исследовать влияние добавления бычьего сывороточного альбумина (БСА) к среде реабилитации на морфофункциональные характеристики фрагментов извитых канальцев семенников (ФИКС) неполовозрелых крыс после криоконсервирования.

Методы. Объектом исследования были криоконсервированные с медленной скоростью охлаждения и витрифицированные ФИКС. После отогрева образцы инкубировали в течение 30 мин в среде Лейбовица с добавлением БСА в концентрациях 0, 2, 5 или 10%, после чего оценивали морфологические характеристики, активность метаболической и антиоксидантной систем.

Результаты. Установлено, что в образцах, криоконсервированных путем медленного охлаждения, применение 5% БСА способствовало повышению сохранности клеток сперматогенного эпителия, уровней метаболической и антиоксидантной активностей. В случае витрифицированных образцов ФИКС добавление к среде 5% БСА способствовало репарации незначительных повреждений в ткани и повышало уровень метаболической активности, однако не влияло на состояние системы антиоксидантной защиты.

Выводы. Полученные данные могут быть использованы для разработки эффективных реабилитационных сред для криоконсервированных фрагментов извитых канальцев семенников с использованием БСА.

Ключевые слова: бычий сывороточный альбумин, реабилитация, криоконсервирование, фрагменты извитых канальцев семенников.