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Biopolymer gels as components of protective medium for cryopreservation of spermatogonial stem cells

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Abstract

Biopolymer gels attract a lot of attention in a field of biothechnology due to their excellent compatibility and degradation. Their application is also promising for cryopreservation of spermatogonial stem cells (SSCs) which is so necessary to preserve the fertility of young patients. The aim of the study was to determine the effectiveness of biopolymer gels as a component of cryopreservation medium for SSCs of immature rats at the stage of exposure to cryoprotectants. It was found that 30-min exposure to cryopreservation media based on collagen or fibrin gel with an addition of 5% Me_2SO or 6% glycerol did not lead to significant changes in membrane integrity, cytochrome C content, metabolic, mitochondrial and antioxidant activities in SSCs compared to the control (Leibovitz-based cryomedium). But fibrin gel more than collagen reduced the toxic effects of Me_2SO and glycerol on SSCs increasing exposure time up to 45 min without significant changes in cell viability. The same cryoprotectants in Leibovitz-based media showed significant toxicity starting from the 15th minute of exposure. Necrosis was the main cause of cell death at this stage of cryopreservation in all experimental groups. The obtained results can be used to optimize SSC cryopreservation protocols for further cell autotransplantation for spermatogenesis initiation in boys who undergo gonadotoxic therapy in prepubertal age.

Keywords

Spermatogonial stem cells, fibrin gel, collagen gel, cryopreservation medium, exposure, apoptosis, necrosis

Introduction

Fertility restoration in men has great potential in basic and applied science.[1] A development of biotechnological methods can give hope for the preservation of reproductive function in patients who are shown gonadotoxic therapy.[2] Many clinics have already implemented cryobanks of testicular cells and tissues from prepubertal boys with cancer. As a result, advances in biotechnology remain inextricably linked to the development of cryobiological approaches, as low temperatures allow bio-objects to be stored for future medical procedures. In addition, cryopreservation facilitates a practical use of cellular and tissue products in clinics.[3]

However, despite the high rates of research in this area, a methodology for isolation and cryopreservation of spermatogonial stem cells (SSCs) of testes has not yet been developed and standardized; their biological properties and methods of fertility restoration have not been fully described. Therefore, there is no doubt that the search for optimal conditions for SSCs cryopreservation and methods for isolation of certain pools of stem cells is an extremely important task for both scientific purposes and practical application.[4] Now several protocols using of cryoprotectants of endo- and extracellular types or their combinations can be applicable.[5,6] The studies of authors[7] have shown that Me_2SO, glycerol, ethylene glycol, polyethylene glycol, trehalose are effective protectors for the cryopreservation of cells and tissues, but their effectiveness is variable for different testis cell populations. It has been previously demonstrated that Me_2SO, being a low molecular weight substance and rapidly penetrating into cells, minimizes a cryodamage of human SSCs to a greater extent.[8] The analysis of studies has shown that the use of classic

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combination of 10% Me_2SO and fetal bovine serum in concentration from 5 to 90% allows to preserve the viability of about 55–65% of cells after freezing.[9,10] Thus, the development of approaches aimed at reducing concentrations of Me_2SO and the search for an alternative to fetal serum are actual tasks of biotechnology.

It is known that natural polymers based on proteins such as collagen, gelatin, silk fibroin and fibrin are characterized by high biocompatibility and bioactivity.[9–11] Therefore, they, and especially extracellular matrix proteins, collagen and fibrin, are promising for use in the biotechnology due to their ability to mimic key biochemical and biophysical factors that are important for cell viability.[12,13]

There is a parallel interest in these gels in the field of cryobiology. It is known that the main factor that leads to cell death at the stages of cryopreservation[5,6] is a violation of the pro- and antioxidant balance, which increases oxidative stress and induces apoptosis.[14,15] The activation of these processes depends on the composition of the cryoprotective medium. It is believed that biopolymer gels in the composition of the cryoprotective medium contribute to the stabilization of membranes, thereby ensuring high safety and metabolic activity of cells.[16] Thus, the use of biopolymer gels makes it possible to minimize oxidative stress and damage to macromolecules during cryopreservation.[17] However, application of collagen and fibrin gels for cryopreservation of SSCs was scarcely studied. Because of their large molecular mass, biopolymers are not able to penetrate into cells. However, their use as a basis for cryoprotective media will be reduce the amount of 'free' water in an extracellular space that participates in the formation of ice crystals, which in turn contributes to an increased cells resistance to cooling. One of the important properties of the fibrin gel is also the ability to control the disintegration and self-organization into a polymer system, and, like other blood derivatives (serum, plasma), it contains a large number of various substances that can have a protective effect during cryopreservation.

It should also be noted that the efficiency of cryopreservation of cells, including SSCs, using gels is determined not only by their membrane integrity, but also by level of viability and functional activity, which is significantly influenced by changes in physical and mechanical properties of the carrier during freezing-thawing.[18] Therefore, the use of gels as matrices for cryostorage of SSCs is promising, but insufficiently studied. SSC cryopreservation protocols using biopolymer gels require careful optimization, as their effectiveness depends on many parameters, including heterogeneity and cryosensitivity of this pool of cells, nature and structure of gels used, individual initial characteristics of cells before freezing, cryopreservation process parameters, etc.[19]

Therefore, the aim of the study was to determine the effectiveness of biopolymer gels as a component of cryoprotective medium for spermatogonial stem cells of the testes of immature rats at the stage of exposition to cryoprotectants.

Materials and methods

The study was performed on 20 sexually immature male outbreed white rats aged 7–8 weeks. All manipulations with animals were carried out in accordance with international bioethical norms (IV European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes) and agreed with the Bioethics Committee of the Institute of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (protocol № 2014–02).

Fragments of seminiferous tubules (100 ± 5 mg) were obtained from both testes of rats mechanically. Cell disaggregation was performed by enzymatic treatment using a mixture of type IV collagenase (1 mg/ml, Gibco, UK) and DNase (500 μg/ml, Sigma, USA).[20] After enzymatic disaggregation cell suspension was filtered (nylon filter with a pore diameter of 100 μm), centrifuged at 250xg, resuspended in nutrient medium and plated on culture vials. An initial cell density was 10^3 cells per cm^2 of a culture vial that has an area of 25 cm^2 (PAA, Austria). A culture medium contained: αMEM medium (PAA, Austria), 10% fetal bovine serum (HyClone, USA), gentamicin (150 μg/ml) (Farmak, Ukraine) and amphotericin B (10 μg/ml). (PAA, Austria). The culture medium was changed every 3 days. A standard culture conditions at 37°C in an atmosphere of 5% CO_2 using an incubator (Biosan, Lithuania) were used. SSCs were detached with 0.25% trypsin-EDTA (Hyclone) at 90% confluence and were replanted in other flasks with 1:2 ratios. The third passage of SSCs was used in all experiments.

Collagen gel (CG) (type I, Sigma-Aldrich, USA) and fibrin gel (FG) were used. FG was derived from fresh rat blood that was obtained from the heart vein in a volume of 5–7 ml and was centrifuged for 12 min at 1000xg. After centrifugation, three blood fractions were received: the lower fraction was erythrocyte mass, the upper fraction was platelet-poor plasma, and the middle fraction was platelet-rich FG. Blood samples with hemolysis were not used in the experiment.

Cryopreservation solutions based on Leibovitz medium (PAA, Austria), fibrin or collagen gels supplementing with cryoprotectants Me_2SO (Sigma-Aldrich, USA) or glycerol (Dow Chemical, Germany) in final concentrations of 5 and 6%, respectively, were used in the study (Figure 1). They were made *ex tempore*. Leibovitz medium without cryoprotectants served as a control.

After detachment rat testicular SSCs were resuspended at a concentration of 10^6 in the studied solutions and thoroughly mixed by pipetting. Exposure was performed

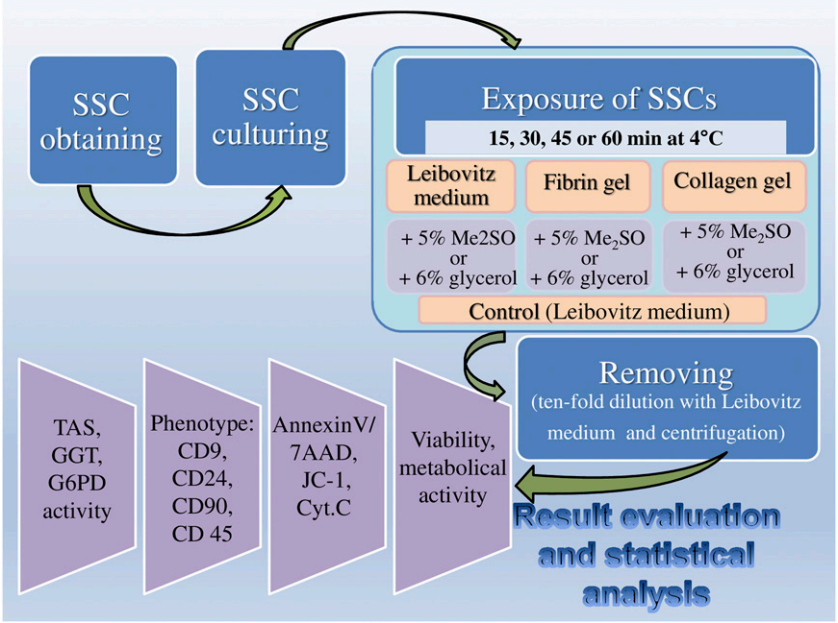

Figure 1. Experimental scheme.

depending on the experiment design for 15, 30, 45 and 60 min at a temperature of 4^0C (n = 5). At the end of exposition, cryoprotective media was removed by adding ten-fold volume of Leibovitz medium and centrifugated. Then the sediment was transferred into 1 ml Leibovitz medium at 25°C for 10 min.

Cell viability was determined by supravital trypan blue (0.05%) staining (Fluka, India), and the relative number of unstained cells was counted.

Metabolic activity of SSCs was studied using MTT test (Sigma-Aldrich, USA). MTT solution at a concentration of 5 mg/ml (0.5 ml) was added to the cell suspensions ($1x10^6$ cells/ml) and they were incubated for 3 h at 37°C. Then the solution was completely removed, and Me_2SO (1 ml/sample) was added to dissolve the formazan crystals with following centrifugation for 10 min at 1000xg. An optical density of formazan solution in supernatant was measured on a CHEM 7 biochemical analyzer (Erba Lachema, Czech Republic) at a wavelength of 540 nm. Cell-free medium was used as a reference solution.

Necrosis-apoptosis studies were performed using Annexin-V-FITC (Annexin V) (BD) and 7-Amino-Actinomycin D (7AAD) dyes (BD, USA). Mitochondrial activity was assessed using JC-1 test system (BD, USA). Determination of the number of positively stained to cytochrome C cells was performed using the test system Cyt. C (BD, USA). For phenotypic analysis cells were stained with anti-CD9 primary monoclonal antibody followed by FITC labeled secondary goat anti-mouse antibody, anti-CD24-PE, anti-CD45-FITC, anti-CD90-FITC according to the manufacturer's instructions (BD Biosciences, USA). The results were analyzed on a flow cytofluorimeter FACS Calibur (Becton-Dickinson, USA) using the program Win MDI v.2.8.

For biochemical tests the samples of SSCs were lysed in RIPA buffer (Millipore, Germany), were filtered and were centrifugated (1000xg for 10 min). A total protein level, γGGT, G6PD activity and total antioxidant status (TAS) was quantitatively estimated in supernatant by UV spectrophotometry (CHEM 7, ERBA, Czech Republic) using test kits (Randox, UK) according to the instructions and calculated per 1 mg of protein (Randox, UK). To clarify the changes in experimental samples, we took the values of TAS activity of control as 100%.

$$\mathrm{TAS}_{\text{experimental group}} \mathrm{x}\ 100\%/\mathrm{TAS}_{\text{control}} = \text{TAS activity}_{\text{experimental group}}\ (\%)$$

Significance of the differences was assessed using a Kruskal-Wallis one-way analysis of variance on ranks followed by Student-Newman-Keuls multiple comparison test. Statistical processing was carried out in Statistika 8 program (StatSoft Inc., USA). The parameters given below in the tables and diagrams are presented as means and their standard deviations (M±SD). The significance level was set at $p < 0.05$.

Results

Under the influence of various physiologically active substances, cells may undergo morphological and

functional changes, so it is necessary to assess cytotoxicity for each potential agent of impact on biological objects.[21]

Thus, at the first stage of our study we assessed the toxic effect of cryopreservation media based on biopolymers on SSCs for selecting an optimal exposure time. This was determined by a decrease in the number of the viability cells (trypan blue exclusion test) after 15-, 30-, 45- and 60-min exposure at 4°C to the studied solutions. The obtained results are shown in Table 1.

Exposure of SSCs for 15 and 30 min in FG or CG based media with 5% Me_2SO or 6% glycerol did not alter the viability compare to the control. It should be noted that no significant difference was found between the gels. A decrease in viability for samples exposure to Leibovitz based cryopreservation media with 5% Me_2SO and 6% glycerol was respectively 12% and 13% ($p < 0.05$) at the 15th minute as well as 17% and 16% ($p < 0.05$) at the 30th minute compared to the control.

After 45-min exposure to FG based media with 5% Me_2SO or 6% glycerol, the viability of SSCs kept at the level of index in the control group at this observation time. The use of Leibovitz and CG based cryopreservation media led to a decrease in SSC viability: by 23% and 20% ($p < 0.05$) in the first case as well as by 17% and 14% ($p < 0.05$) in the second one respectively for 5% Me_2SO and 6% glycerol.

At the 60th minute of exposure, a significant decrease in the index of viability was observed in all studied cryomedia, namely: the use of Leibovitz medium in combination with 5% Me_2SO or 6% glycerol resulted respectively in 39% and 33% ($p < 0.05$) reduction of viability of SSCs; the use of FG in combination with 5% Me_2SO or 6% glycerol led respectively to 14% and 10% ($p < 0.05$) decreasing; the use of CG in combination with 5% Me_2SO or 6% glycerol resulted respectively in a decrease in SSC viability by 28% and 25% ($p < 0.05$).

Thus, Me_2SO and glycerol have less toxic effect on SSCs at the stage of exposure when biopolymer used compared to Leibovitz medium. Thus, the use of FG reduced the toxic effects of Me_2SO and glycerol on rat SSCs and increased the exposure time to 45 min without changes in the viability relative to control. The taking into account the existing trend towards a decrease in viability with increasing exposure time regardless of the basis of cryopreservation media, the suitable contact time of cells with cryoprotectant should be considered 30 min. So, our results showed that cell viability is inversely related to of exposition time. These findings are consistent with the work[22] that shows a decrease of cell viability after exposition in cryoprotective media. In parallel, alteration of cellular functions has been reported after exposure with cryoprotectants,[23] while the viability of cells decreased. That is why the 30-min exposure was used by us in further experiments to determine the effectiveness of collagen and fibrin gels as a basis of cryopreservation media for SSCs.

The next step was to study the effect of exposure to the cryopreservation media on the overall metabolic activity of SSCs. Obtained data are presented in Figure 2.

To assess the metabolic activity of cells we used a colorimetric MTT test based on the ability of NADPH-dependent oxidoreductase enzymes to display the number of viable cells. There was no significant difference in metabolic activity between the control and the groups using FG or CG based cryopreservation medium in combination with both studied cryoprotectants. Absence of significant differences in the metabolic activity indicates a good preservation of cells in cryopreserved media basis on fibrin and collagen gels which is also consistent with the results of the viability at the same time of exposure. A 1.33- and 1.26-fold ($p < 0.05$) decrease of the studied indicator compared to the control was observed using Leibovitz based media with 5% Me_2SO or 6% glycerol, respectively. An inhibition of

Table 1. Viability of rat SCCs after exposure to cryopreservation mediam (x ± SD; $n = 5$).

	Exposure time,min			
Group	15	30	45	60
Control(Leibovitz)	94.4 ± 5.9	86.5 ± 4.3	78.6 ± 3.8[3]	74.6 ± 4.3[3]
Leibovitz+5% Me_2SO	83.2 ± 4.3[1]	71.4 ± 3.8[1,3]	60.5 ± 3.9[1,3,4]	45.7 ± 3.3[1,3,4]
Leibovitz+6% glycerol	82.4 ± 3.1[1]	72.8 ± 3.5[1,3]	62.8 ± 3.3[1,3,4]	50.2 ± 3.9[1,3,4]
FG + 5% Me_2SO	92.5 ± 3.3[2]	78.6 ± 3.3[2,3]	72.1 ± 3.7[2,3]	64.4 ± 3.8[1,2,3,4]
FG + 6% glycerol	94.6 ± 3.6[2]	80.5 ± 4.1[2,3]	75.5 ± 4.5[2,3]	67.1 ± 3.5[1,2,3,4]
CG + 5% Me_2SO	93.6 ± 3.3[2]	78.2 ± 4.8[2,3]	65.2 ± 3.4[1,3,4]	53.5 ± 4.4[1,2,3,4]
CG + 6% glycerol	90.8 ± 3.7[2]	79.6 ± 3.9[2,3]	67.5 ± 5.7[1,3,4]	56.3 ±4.2[1,2,3,4]

Notes: [1]Indicates values that significantly differed from control at the same time of exposition ($p < 0.05$); [2]Indicates values that significantly differed from Leibovitz based medium supplemented with corresponding cryprotector at the same time of exposition ($p < 0.05$); [3]Indicates values that significantly differed from 15-minute exposition in the corresponding group ($p < 0.05$); [4]Indicates values that significantly differed from previous time of exposition in the corresponding group ($p < 0.05$).

metabolic processes in cells from these groups can pointed to the probable toxic effect of the cryoprotectants.

Studies have also been performed to determine the effect of exposure to the investigated cryopreservation media on apoptosis/necrosis processes in the SSCs. It is known that Annexin binds to phosphatidylserine, the presence of which in the outer layer of the plasma membrane of cells indicates the initiation of the process of apoptosis. 7AAD+/− is used to determine viable/necrotic cells. The results of flow cytometry are presented in Table 2.

After exposure to Leibovitz based cryomedia with supplementing of 5% Me_2SO or 6% glycerol, a 1.21- and 1.15-fold decrease in the number of viable SSCs (Annexin $V^-/7AAD^-$) was respectively observed relative to the control. It turned out that most damaged cells underwent to necrosis (Annexin $V^+/7AAD^{++}$+Annexin $V^-/7AAD^+$), while the number of SSCs with preserved order of membrane phospholipids (Annexin $V^+/7AAD^-$) did not changed. That is, the main cell losses occurred as a result of their rapid death. After SSC exposure to FG based cryomedia with 5% Me_2SO or 6% glycerol, number of viable cells (Annexin $V^-/7AAD^-$) was maintained at the appropriate control level without apoptosis/necrosis initiation. In SSC samples after exposure to CG based cryopreservation media in combination with 5% Me_2SO or 6% glycerol, a slight but significant reduction (1.13- and 1.11-fold respectively) in viable cell content (Annexin $V^-/7AAD^-$) was observed, as well as there was 1.5- and 1.52-fold increase in the number of necrotic cells (Annexin $V^+/7AAD^{++}$+Annexin $V^-/7AAD^+$) relative to the control.

It is known that the fluorescent probe JC-1, depending on the energy activity of mitochondria, glows in different regions of the spectrum: its luminescence changes from green to orange with a potential increase.[24] In addition, mitochondria are associated with the release of cytochrome C and the activation of caspases, i.e. the launch of the main links of apoptosis process in the cell.[25,26]

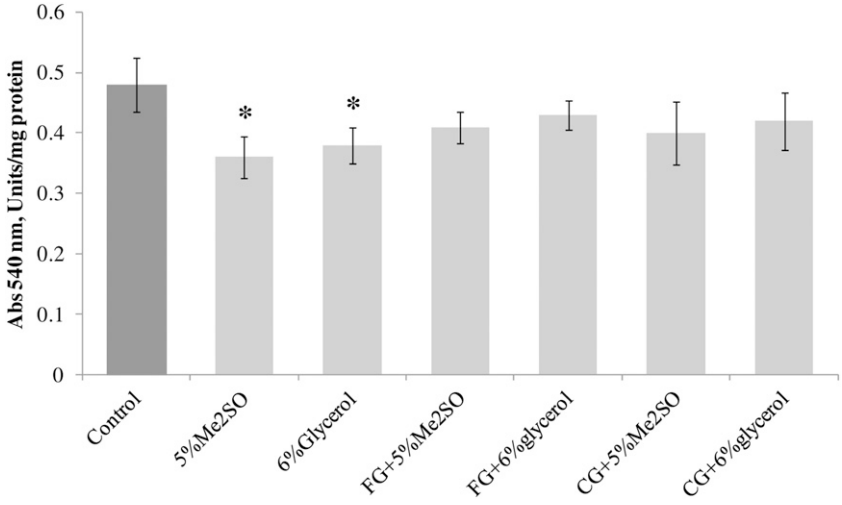

Figure 2. Metabolic activity of SSCs after 30 min of exposure to cryopreservation media. Note: *indicates values that significantly differed from control ($p < 0.05$).

Table 2. Influence of exposure to cryopreservation media on the appoptosis/necrosis processes in the SSCs, % of cells.

Group	Viable cells (Annexin $V^-/7AAD^-$)	Apoptosis (Annexin $V^+/7AAD^-$)	Necrosis (Annexin $V^+/7AAD^+$+Annexin $V^-/7AAD^+$)
Control(Leibovitz)	85.49 ± 2.93	3.63 ± 0.52	10.88 ± 1.27
Leibovitz+5% Me_2SO	70.92 ± 1.56[1]	3.73 ± 0.21	25.35 ± 1.34[1]
Leibovitz+6% glycerol	74.64 ± 1.03[1]	3.03 ± 0.42	22.33 ± 1.68[1]
FG + 5% Me_2SO	83.5 ± 2.05[2]	3.27 ± 0.11	12.80 ± 1.37[2,3]
FG + 6% glycerol	85.33 ± 2.17[2]	3.15 ± 0.33	11.52 ± 1.59[2,3]
CG + 5% Me_2SO	79.42 ± 2.92[1,2]	3.98 ± 0.29	16.60 ± 1.88[1,3,4]
CG + 6% glycerol	80.19 ± 1.69[1,2]	3.27 ± 0.92	16.54 ± 0.93[1,3,4]

Notes: [1]Indicates values that significantly differed from control at the same time of exposition ($p < 0.05$); [2]Indicates values that significantly differed from Leibovitz based medium supplemented with corresponding cryprotector at the same time of exposition ($p < 0.05$).

Therefore, in this study we investigated the impact of exposure to the cryopreservation media on the activity of mitochondria and content of cytochrome C in SSCs (Figure 3). Analysis of obtained results has shown that SSCs after 30 min of exposure to Leibowitz based media in combination with 5% Me_2SO or 6% glycerol had respectively 1.36- and 1.27-fold reduced mitochondrial activity, and 2.17- and 1.95-fold increased cytochrome C content compared to the control. Exposure of SSCs to cryopreservation media based on FG and CG with the supplementing of 5% Me_2SO or 6% glycerol did not lead to changes in mitochondrial activity (number of JC-1^+ cells) and cytochrome C content relative to the corresponding indicators in the control group.

The effect of exposure to the studied cryopreservation media on the phenotypic characteristics of SSCs of immature rats was also determined. The studied cells showed a phenotype typical for spermatogonial cells with high (≥70%) expression level of markers CD9 (≥40%), CD24 (≥70%), CD90 (≥70%) and low one for CD45 (≤1%). It should be noted that there was no significant difference in the expression of these surface cell markers, regardless of the exposure medium.

TAS is often used to assess the antioxidant defense system of biological samples evaluating their response against the free radicals produced under the influence of various factors, including stages of cryopreservation. Gamma glutamyltransferase (GGT) involves in oxidant protection of sperm and/or in restoring extracellular cysteine for protein synthesis.[27] Glucose-6-phosphate dehydrogenase (G6PD) is the first pentose phosphate pathway enzyme that provides the cell with energy. Its main function is to reduce NADP to NADPH, which is necessary for the transition of oxidized glutathione to the reduced form.[28] Thus, some authors indicated GGT and G6PD as impotent enzymes involved in the antioxidant defense system of testicular cells.[29,30]

The 30-min exposure of SSCs to Leibovitz based cryopreservation media inhibited the activity of some enzymes (GGT, G6PD) (Table 3). And their decrease for 5% Me_2SO and 6% glycerol was respectively: 1.34- and 1.27-fold for GGT, 1.12- and 1.11-fold for G6PD relative to the corresponding indicators in the control. No significant differences in the activity of GGT and G6PD were found in the SSC samples after exposure to FG and CG based media in combination with both studied cryoprotectants versus control.

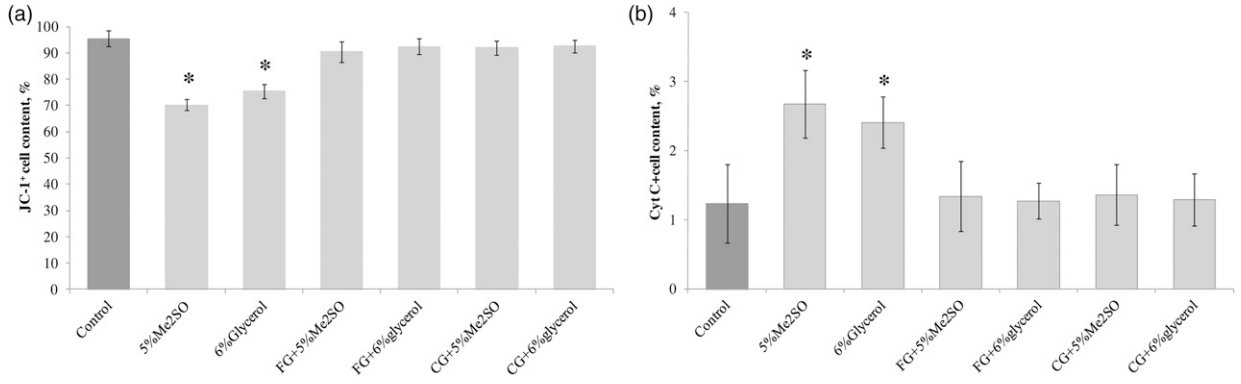

Figure 3. Effect of 30 min exposure in cryopreservation media on mitochondrial activity (a) and cytochrome C content (b) in the SSCs. Note: *indicates values that significantly differed from control ($p < 0.05$).

Table 3. Activity of GGT and G6PD in the SSCs of immature rats after exposure.

Group	GGT UI/mg protein	G6PD UI/mg protein
Control(Leibovitz)	23.75 ± 1.24	891.52 ± 65.87
Leibovitz+5% Me_2SO	17.71 ± 1.34[1]	705.11 ± 51.78[1]
Leibovitz+6% glycerol	18.75 ± 1.39[1]	711.53 ± 1.39[1]
FG + 5% Me_2SO	21.85 ± 1.26[2]	850.34 ± 55.38[2]
FG + 6% glycerol	21.73 ± 1.14[2]	870.95 ± 43.82[2]
CG + 5% Me_2SO	19.08 ± 2.06	825.93 ± 64.78[2]
CG + 6% glycerol	19 68 ± 1.62	838.47 ± 49.32[2]

Notes: [1]Indicates values that significantly differed from control at the same time of exposition ($p < 0.05$); [2]Indicates values that significantly differed from Leibovitz based medium supplemented with corresponding cryprotector at the same time of exposition ($p < 0.05$).

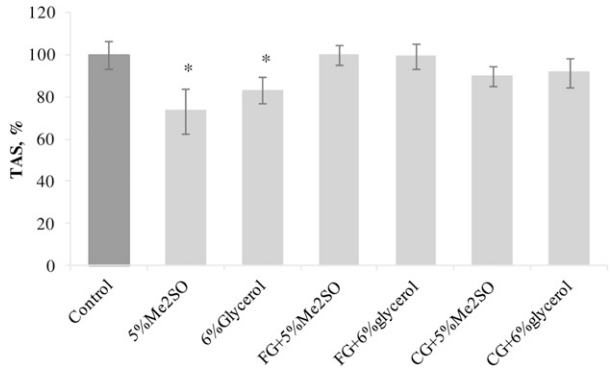

Figure 4. Effect of 30 min exposure in cryopreservation media on activity of total antioxidant system in the SSCs. Note: * indicates values that significantly differed with control ($p < 0.05$).

The results of the study of total antioxidant system activity in SSCs are represented in Figure 4. Thus, there was a 1.36- and 1.21-fold decrease of its value respectively for 5% Me_2SO and 6% glycerol Leibowitz based media relative to the control. There were no significant differences in TAS activity of SSC samples after exposure to media based on FG or CG in combination with both studied cryoprotectants compared to the control.

Discussion

It is known that the process of low-temperature preservation of biological objects consists of several stages: isolation, exposure to cryopreservation medium, freezing, storage at low temperatures and thawing. At all these stages, the biological object is affected by adverse factors. So, during exposure to cryopreservation media, the stage that is investigated in this study, the most important damaging factor is a cytotoxicity of cryoprotectants, which depends on the following parameters: temperature, treatment duration, type of active substance, its concentration, etc.[31,32]

The cytotoxic effect of cryoprotectants on a biological object can be reduced by using protein components in cryopreservation media.[33,34] Currently, in the development of cryopreservation technologies, much attention is paid to biopolymer gels due to their unique properties, such as excellent biocompatibility and biodegradation, so they have already found wide application in tissue engineering, clinical medicine and drug delivery.[35,36]

In the presented study the comparative assessment of changes in the state of SSCs of immature rats during exposure to cryopreservation media of different composition based on nutrient medium or biopolymers was carried out. Obtained results have shown that the use of collagen and fibrin gels reduced the toxic effects of the studied cryoprotectants, Me_2SO and glycerol, on SSCs. It should be noted that FG as a component of cryopreservation medium at the stage of exposure was more effective than CG. It was found that the use of FG can extend the exposure time for SSCs up to 45 min.

The greater efficiency of FG is probably due to its physical properties and/or due to the presence of a rich spectrum of biologically active substances and 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.) compared to CG, the composition of which is poorer. It is known that biologically active substances and growth factors play a huge role in cell function and can affect metabolic processes in them, including when exposed to cryoprotectants.[37–39] The authors were researched physical properties of gels[40,41] and showed that fibrin can be completely decomposed within a few hours after polymerization in vitro by reason of proteases located in the cellular medium and secreted by cells. Fibrin in gel form has ability to be formulated at much higher concentrations than collagen (up to ~100 mg/ml compared to ~10 mg/ml), which affects their mechanical properties.

Gels have a unique three-dimensional network structure that allows small molecules (such as nutrients or metabolites) to pass through them without affecting normal cell function, and provides immune protection against foreign substances. In addition, studies have shown that gels are useful in minimizing cell damage during the cryopreservation process: they can limit the growth of ice crystals and provide a lower rate of osmotic changes during exposure of cells to penetrating cryoprotectants such as Me_2SO or glycerol. In addition, they are able to mimic the native extracellular tissue matrix, which may be also important for cells.[42] The results obtained in our work are consistent with the results of the authors that applied a cryopreservation method to increase the degree of cell viability in the cooling and thawing processes using gel systems.[43] The researchers showed that gel system under cryopreservation can increase the neural cell viability by means of: confine the ice crystal growth in the porous of gel system; decrease the amount of ice crystallization and cryopreservation system's freezing point; reduce the change rates of cell volumes and osmotic shock. In experiments[44] there was shown that fibrin gel and slow freezing led to greater post-thawed follicle quality than in the control group. This methodical approach allowed the researchers to storage the high percentage of follicle viability, morphology, protein phosphorylation at stages of tissue encapsulation and cryopreservation.

Thus, the use of FG as a component of cryopreservation medium is effective and helps to preserve the integrity of cell membrane, metabolic, mitochondrial and antioxidant activities of SSCs, which is also confirmed by the absence of

apoptosis/necrosis activation and changes in cytochrome C content after 30 min of exposure as shown in this study.

Consistent data were obtained by authors.[45,46] Thus, one of the causes of apoptosis is oxidative stress that associated not only with increased generation of reactive oxygen species (ROS), but also associated with decreased mitochondrial membrane potential,[45] which was also shown for SSCs in our work using JC-1 fluorescent probe. In mitochondrial pathway of apoptosis activation, the key link is a change in the state of the mitochondrial membrane that lead to the release and activation of a number of proteins: cytochrome C in combination with Apaf-1 and procaspase 9 forms caspase 9, which activates caspase 3.[46] In addition, there is also a non-caspase pathway of apoptosis initiation in mitochondria.

Cytotoxic effect is inherent to both studied cryoprotectants, and its degree depends on the type, concentration, exposure time and temperature, as well as the features of biological objects. From the published reports it is known that Me_2SO toxicity is associated with protein denaturation and destabilization of phospholipid bilayer,[47] as well as with the possible caspase activation and apoptosis.[48] Toxic effects of glycerol depend on equilibration period and are manifested in a change of permeability of cell membranes, concentration of osmiophilic granules in nucleus, pinocytosis, appearance of cisterns and vacuoles in cytoplasm, damage of mitochondria and endoplasmic reticulum dehydration.[49]

In general, in the case of cryopreservation media based on CG or FG, in our opinion, the optimal exposure time is 30 min. However, 45-min exposure to 5% Me_2SO or 6% glycerol in FG does not impair SSC viability, and therefore, if necessary, the time interval for this gel can be increased. Our further research will focus on the investigation parameters of SSCs after freezing-thawing and evaluation of the contribution of biopolymer gels to the protection of cells during low-temperature storage.

The obtained results can be used to optimize SSC cryopreservation protocols for further cell autotransplantation initiation of spermatogenesis in boys who undergo gonadotoxic therapy in prepubertal age.

Conclusions

A comparative evaluation of the effect of exposure to cryopreservation media based on biopolymer gels on the state of spermatogonial stem cells (SSCs) of rat testes was made. It was found that 30-min exposure to cryopreservation media based on collagen or fibrin gels with the supplementing of 5% Me_2SO or 6% glycerol did not lead to significant changes in viability, metabolic and total antioxidant activity, mitochondrial potential as well as in cytochrome C content in SSCs. The use of fibrin gel as a basis for cryopreservation media reduced the toxic effects of 5% Me_2SO and 6% glycerol on SSCs and increased exposure time up to 45 min without significant changes in cell viability. Obtained results can be used to develop effective cryopreservation protocols for the creation of SSC cryobanks to restore male fertility.

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