INFLUENCE OF CONDITIONED MEDIA FROM GLIAL CELL CULTURES ON CONTRACTILITY OF UTERINE IN RATS OF DIFFERENT AGES

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INTRODUCTION

The rate of delivery by cesarean section has increased dramatically in most developed countries, which is potentially a negative trend due to possible risks to the maternal and newborn health [1]. The physiological regulation of the contractile activity of the uterine myometrium changes with age. It has been established that the duration of labor and the number of prolonged labors increase with the mother's age [2]. Considering that in the modern world the trend of having a child at a later age is spreading [3], it is relevant to study the mechanisms of changes in the contractile activity of the uterus and ways to correct it.

As shown earlier, age-associated rearrangements of structural elements involved in the development of contraction occur in smooth muscle cells. For example, in the smooth muscle cells of blood vessels the expression of smooth muscle α-actin is reduced [4] as well as there is a loss of smooth muscle caveolae in the bladder [5]. The expression of genes encoding ion transport, metabolism of steroid hormones and the presence of receptors for uterotonics changes in the uterine myometrium [6].

The family of neurotrophic factors (NF), which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophins 3 (NT-3), 4/5 (NT- 4/5) and 6 (NT-6) are involved in the regulation of proliferation, migration and contractility of smooth muscle cells in various organs [7, 8]. At the same time, NF plays an important role in maintaining the contractile function of the uterus throughout the entire reproductive lifespan and particularly during pregnancy [9–11].

In order to correct the function of the female reproductive system cell therapy is often used [12, 13]. The experience accumulated at present indicates that its main effects in the patient's body are being realized due to biologically active substances that are synthesized by transplanted cells [14]. With this in mind, the modern approach is to use conditioned media (CM) from cell cultures, which minimizes the risks of immune rejection of transplanted stem cells or tumorigenicity due to their inadequate engraftment and differentiation [15–17].

CM (otherwise "cell secretome") is a nutrient medium obtained during the cultivation of cells *in vitro* and containing their metabolic products. CM contains signal peptides that are processed along the classical secretion pathway; proteins exfoliating from the cell surface; intracellular proteins released by non-classical secretion, exosomes. It includes enzymes, growth factors, cytokines, hormones, mediators [14, 15, 17, 18]. Clinical trials of CM obtained from different sources have been reported, which show the effectiveness of this approach as a safe alternative to cell therapy [19–22].

It has been established that CM of glial cell cultures contains the majority of neurotrophic factors, as well as basic fibroblast growth factor (bFGF), antioxidants, and other regulatory proteins [23, 24]. Consequently, they can be used as biologically active compositions to maintain reproductive function and prevent age-associated structural and functional changes of uterus. Therefore, experimental studies in this direction are relevant.

Obtaining CM is a technology based on the use of cell culture methods, cryopreservation and long-term storage of cell cultures in the cryobank. However, the effect of cryopreservation on the composition and biological properties of CM has been barely studied.

Objective of this study was to investigate the effect of CM obtained from intact and cryopreserved cultures of glial cells on the contractile activity of the uterus in rats of different reproductive ages.

MATERIALS AND METHODS

The experiments were carried out on outbred female rats aged 6 and 14 months, which corresponds to the reproductive age (RA) and late reproductive age (LRA) [25]. All experiments on animals were done in accordance with the Law of Ukraine "On the protection of animals from cruelty" (No. 3447-IV of February 21, 2006) in compliance with the requirements of the Bioethics Committee of the Institute, agreed with the provisions of the "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes" (1986).

Cells were obtained from the dorsal root ganglia of neonatal piglets by the enzymatic

method [26]. The cells were seeded at a concentration of 5 x 10^5 cells/ml into plastic Petri dishes (SPL Life Sciences, Korea) and cultured at 37 °C in an atmosphere with 5% CO_2 and 95% O_2 using a basal medium (BM) α-MEM (Biowest, France) supplemented with antibiotics and 10% fetal calf serum (FCS, BioSera, France). As shown earlier, such a culture consists of 80% satellite glial cells [26].

Cryopreservation was carried out using a cryomedium based on α-MEM with the addition of 25% FCS and 7.5% cryoprotectant dimethyl sulfoxide (DMSO, AppliChem, Germany), as described in [26]. After thawing, the cryoprotectant was removed from the samples by centrifugation; the cells were placed in BM and cultured during 28 days as described above.

CM from intact (ICM) and cryopreserved CM (CCM) cultures were collected (every three days), frozen, and stored at -18 °C. The collected media were thawed, combined, and fractionated by ultrafiltration using a polyethersulfone membrane (Millipore, Germany). Fractions with a molecular weight of up to 30 kDa were obtained from the ICM, CCM as well as BM, which was used as a control. The resulting fractions were administered to rats (the start of administration was in the estrus phase) at a dose of 0.2 ml intraperitoneally for 9 days. Animals were slaughtered on the 30th – 32nd day after the end of the administration. Fragments of the uterus were taken for histological and immunohistochemical (IHC) analysis, as well as to study the uterine contractile activity.

Fragments of the uterus were fixed in 10% neutral formalin, histological sections were made, and stained with hematoxylin/eosin according to the standard method. For IHC, the Ready-to-use kit with monoclonal antibodies against smooth muscle actin (Smooth Muscle Actin 1A4, DAKO, Denmark) was used. Staining with the first antibodies was visualized using UltraVision Quanto Detection Systems HRP (Thermo scientific, USA). Diaminobenzidine (DAB) was used as a chromogen. Visual assessment and microphotography were performed using a light microscope AmScope XYL-403 (AmScope, China).

Using the AxioVision Rel 4.8 application (CarlZeiss, Germany), the area of the myometrium (Sm) was measured in 8–9 serial sections (field of view x 40) of the uterus of each animal. Sm was calculated as the ratio of the area of the myometrium to the total area of the uterine section x 100%. The density of myocytes in the longitudinal (Dl) and circular (Dc) layers of the myometrium was determined as the mean number of cell nuclei per a field of view of 400 x 400 μm. The relative area of positive IHC-labeling was determined as the ratio of the sum of the labeled myometrium areas to the area of the uterine section x 100% (field of view x 200).

Contractile activity was studied by the organ bath method on isolated strips (IS) of the uterus [27]. The contractile activity was recorded using a mechano-electric transducer "Grass FT03C" (Grass Instruments, USA) connected to adapter-multimeter "OWON B41T" (Fujian Lilliput Optoelectronics Technology Co., Ltd., China). Uterine strips 8 x 3 mm were mounted vertically in a thermostated chamber (t = 37 °C, atmosphere with 5% CO_2 and 95% O_2) filled with Krebs solution (pH = 7.4) in such a way that one end of the IS was connected to a stationary holder and another to the transducer. Before the experiment, the IS were incubated for 60 min under isometric loading of 1 g (10 mN). After the end of the experiment, the IS was weighed on a torsion balance BT-500. Solutions of oxytocin (OT) and KCl at final concentrations of 4 nM and 70 mM respectively were used to determine a stimulated IS activity.

Frequency of contraction (FC) was calculated as the average number of IS contraction-relaxation cycles, and the amplitude of contractions (AC) as the average peak height on the IS contraction curve expressed in mN for 10 min. As previously described [27], tension of isometric contraction (TIC) was determined as the ratio of the area under the IS contraction curve to the IS cross-sectional area ((g x s)/cm^2). The IS cross-sectional area was calculated by the formula:

S = m / xl, where m – mass of the IS, l – length of the IS, while x was taken as 1.05 g/cm^3.

The following groups of rats were used in the experiment: 1 – intact RA (n = 10); 2 – RA with the administration of BM (n = 9); 3 – RA with the administration of ICM (n = 10); 4 – RA with the administration of CCM (n = 9); 5 – intact LRA (n = 10); 6 – LRA with the administration of BM (n = 9); 7 – LRA with the administration of ICM (n = 11); 8 – LRA with the administration of CCM (n = 10).

The results were presented as Me x IR (Me – median, IR – interquartile range as the difference between the upper and lower quartiles). The statistical significance of differences between groups was assessed using the non-parametric Mann–Whitney U test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Spontaneous activity of uterine IS in rats of all groups was characterized by rare rhythmic contractions of small amplitude (Fig. 1). The addition of KCl led to the appearance of a peak with increasing amplitude. The OT-induced contraction had two distinct components, tonic and phasic, with an increase in both AC and FC.

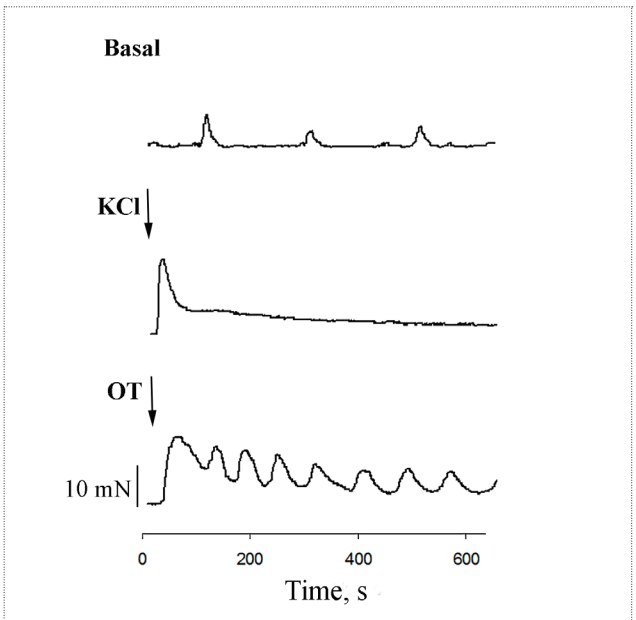

Figure 1. A representative curves of uterine IS spontaneous (top trace), KCl-induced (middle trace) and OT-induced (bottom trace) contractile activity

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Spontaneous, OT-, and KCl-induced TIC significantly decreased in intact LRA rats by 19, 20, and 14%, respectively, compared with intact RA animals while FC and AC did not change (Table 1). The administration of all media did not affect the contractile activity of the uterus in animals of RA. In LRA rats, there was a significant increase in OT-induced TIC after the administration of ICM, as well as spontaneous, OT- and KCl-induced TIC after the administration of CCM compared with the intact control of LRA. Herewith, the indicators of LRA TIC reached the values of the intact RA control rats ($p > 0.05$).

The histological structure of the uterus did not differ in rats of all experimental groups. The endometrium was represented by a simple columnar epithelium and tortuous tubular glands, separated from each other by a connective tissue of lamina propria. In the myometrium, two layers of smooth muscles were distinguished, located longitudinally and circularly, which were separated from each other by layers of loose connective tissue.

Quantitative analysis showed that the area of the myometrium of animals RA did not change depending on the administration of media (Table 2). In LRA animals, a significant increase in the area of the myometrium was observed using ICM and CCM. At the same time, in these groups, the density of myocytes in the longitudinal layer of the myometrium increased significantly.

Immunohistochemical labeling of smooth muscle actin was carried out in the uterus of rats (Fig. 2). Specific labeling on sections of the uterus was observed in animals of all studied groups, mainly in the layers of the myometrium and vascular media. In LRA animals actin expression in the myometrium was dramatically reduced, especially in the circular layer (Fig. 2B). Expansion of intermuscular and intramuscular connective tissue with increased vascularization of the last one was noted. After ICM and CCM administration, an increase in actin expression was visually established in samples of LRA rat uterus (Fig. 2C).

Quantitative analysis of myometrium IHC staining confirmed a decrease in actin positive labeling in intact aged animals (Fig. 3). When using ICM and CCM in animals of both ages, the area of positive actin labeling respectively increased: by 1.3 and 1.1 times in RA animals ($p > 0.05$); by 2.7 and 2.6 times in LRA animals ($p < 0.05$).

DISCUSSION

Previous experimental studies have shown that profile of myometrial contractile activity changes with age [2, 6, 28]. The strength of spontaneous contractions of the myometrium in women declines with age, but the likelihood of multiphase contractions increases, which indicates discoordination of the uterine contractile activity [28]. It was found that the amplitude and strength of spontaneous uterine contractions decreased by three times in 24-month-old rats, but its sensitivity to stimulators of contractile activity increased [6]. There was no change in the frequency of spontaneous contractions in rats of 14 month-old compared with rats of 6 month-old in our studies, but their TIC was significantly lower.

The mechanism of smooth muscle contraction is based on a voltage-dependent increase in intracellular Ca^{2+} concentration and the formation of cross-bridges between myosin and actin. The influx of Ca^{2+} can occur both from the extracellular environment and when it is released from intracellular

Table 1. Indicators of uterine IS contractile activity of rats of different ages after administration of BM, ICM and CCM (Me ± IR)

Indicator	RA				LRA			
	Control	BM	ICM	CCM	Control	BM	ICM	CCM
FC_{bas}	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.3	0.5 ± 0.4	0.3 ± 0.1	0.2 ± 0.1
FC_{OT}	0.8 ± 0.4	0.6 ± 0.4	0.6 ± 0.2	0.9 ± 0.3	0.8 ± 0.3	0.8 ± 0.1	1.0 ± 0.2	1.0 ± 0.5
AC_{OT}	12.0 ± 1.4	12.0 ± 0.7	13.5 ± 0.6	12.1 ± 0.6	12.1 ± 1.1	13.1 ± 1.8	13.9 ± 1.0	12.7 ± 1.0
AC_{KCl}	11.7 ± 0.9	11.9 ± 0.7	13.4 ± 0.3	12.5 ± 0.7	12.1 ± 1.9	13.2 ± 3.5	14.8 ± 1.9	14.2 ± 0.7
TIC_{bas}	32.8 ± 14.2	31.1 ± 7.8	28.8 ± 2.1	33.4 ± 12.6	26.7 ± 7.0*	26.6 ± 7.6*	27.7 ± 1.2*	32.7 ± 2.1**
TIC_{OT}	35.9 ± 14.6	31.9 ± 7.7	29.7 ± 2.5	38.1 ± 14.8	28.5 ± 7.7*	28.4 ± 8.4*	32.3 ± 2.7**	34.8 ± 3.4**
TIC_{KCl}	34.2 ± 16.0	32.0 ± 9.1	30.8 ± 2.0	40.0 ± 13.2	29.3 ± 7.1*	28.4 ± 5.4*	30.3 ± 1.9	39.0 ± 4.5**

* indicator is significantly different from the intact RA control, $p < 0.05$
** indicator is significantly different from the intact LRA control, $p < 0.05$

Table 2. Uterine histological parameters of rats of different ages after administration of BM, ICM and CCM (Me ± IR)

Indicator	RA				LRA			
	Control	BM	ICM	CCM	Control	BM	ICM	CCM
SM	51.8 ± 5.9	49.6 ± 6.8	52.3 ± 8.9	48.7 ± 9.9	50.3 ± 10.8	52.5 ± 9.1	62.8 ± 15.5**	58.0 ± 12.8**
Dc	13.5 ± 4.7	14.5 ± 3.0	14.0 ± 4.0	14.1 ± 2.5	13.0 ± 4.5	14.0 ± 5.5	12.5 ± 4.8	12.0 ± 4.5
Dl	24.0 ± 11.5	27.5 ± 10.2	22.9 ± 6.5	30.0 ± 7.1*	20.2 ± 3.5	22.5 ± 16.5	23.1 ± 5.2**	25.0 ± 5.5**

* indicator is significantly different from the intact RA control, $p < 0.05$
** indicator is significantly different from the intact LRA control, $p < 0.05$

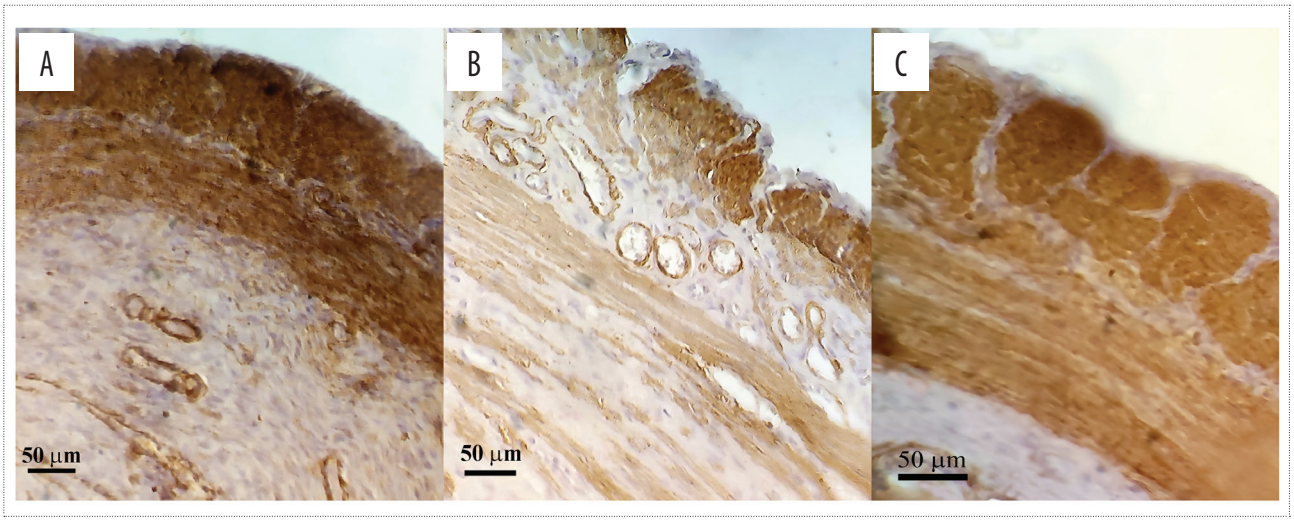

Figure 2. Immunohistochemical labeling of smooth muscle actin in the rat uterus
A – intact RA; B – intact LRA; C – LRA with administration of CCM. Areas of positive labeling are brown, nuclei counterstained in hematoxylin.

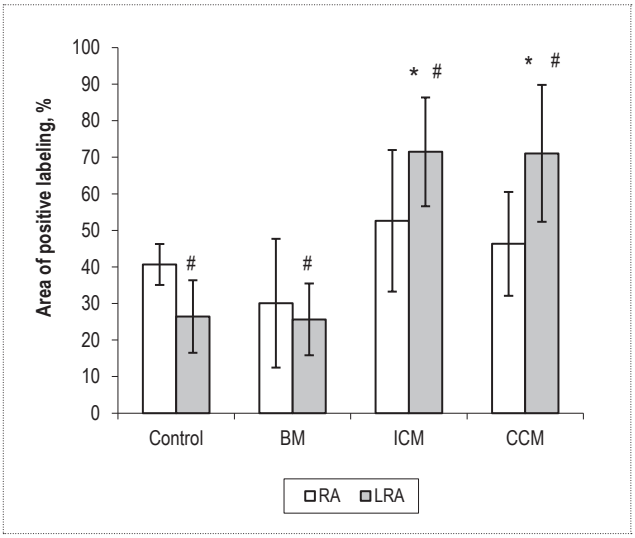

Figure 3. Area of specific labeling of uterine sections with antibodies to smooth muscle actin (Me ± IR)
* indicator is significantly different from the intact control of the corresponding age, $p < 0.05$
# indicator is significantly different from the intact RA control, $p < 0.05$

stores. There are several types of ion channels in the myocyte plasma membrane that stimulate contraction. The main type is voltage-sensitive Ca^{2+} channels, which open in response to membrane depolarization [29]. KCl solution is a non-specific stimulator of smooth muscle contraction by changing the potassium gradient across the cell membrane, which in turn leads to a change in the membrane potential and activation of these channels. Oxytocin, on the other hand, is a specific stimulator of myometrial contraction. It binds to the OXTR (oxytocin receptor gene) receptor, which activates the signal transduction pathway through phospholipase C and inositol-3-phosphate, which causes the release of Ca^{2+} from the sarcoplasmic reticulum inside the cell [30]. In this way KCl-mediated contraction makes it possible to assess the contractile apparatus of the myometrial sample *in toto*, while OT-mediated contraction carries information about the expression of specific receptors in it. Based on the data on KCl- and OT-induced contractile activity obtained in this study, it can be concluded that with age, there is a decrease in both total and receptor-mediated TIC of the myometrium. Interestingly, the introduction of CCM resulted in an increase in both types of contractile activity, while the use of ICM resulted in an increase in OT-induced activity of the myometrium in LRA rats. This may indicate that under the influence of exogenous NF of CM and, probably, other trophic factors, the contractile apparatus of the myometrium is reorganized and its sensitivity to specific stimuli is increased.

These results are consistent with our data on a significant increase in the relative area of the myometrium in animals treated with conditioned media. The effect was realized due to the proliferation of smooth muscle cells, as evidenced by an increase in the average density of myocytes in the longitudinal layer and an increase in the area of specific labeling with anti-actin antibodies in both layers of the myometrium.

It is known that the neuroplasticity of the uterus and the implementation of normal contractile function depend on NF [9–11]. NF, in particular BDNF, induces smooth muscle cell proliferation [8, 31, 32]. Thus, the effect obtained in our studies can be explained by the mitogenic impact of NF contained in the conditioned medium from glial cells. It cannot be overlooked, however, the other biologically active substances that are present in the CM and have a similar effect.

It is well known that in the process of cryopreservation, cells are treated with a cryoprotector and low-temperature cooling, as a result of which a decrease in their viability is observed, as well as a modification of their proliferative and functional activity. As shown by S.G. Ali et al. [26], when using 7.5% dimethyl sulfoxide cryoprotectant and a certain cryopreservation mode, about 90% of viable glial cells are preserved in culture. In our experiments, it was found that the biological effect of CM of intact and cryopreserved glial cell cultures on the contractile activity of the myometrium is similar. This makes it possible to use the technology of cryopreservation of glial cell culture to obtain a conditioned medium.

CONCLUSIONS

Intraperitoneally injected CM from the culture of glial cells increase the contractile activity of the uterus in aged rats. This effect is realized by increasing the relative area of the myometrium, the density of myocytes, and the area of expression of smooth muscle actin. The biological effect of the media from intact and cryopreserved cultures on the contractile activity of the uterus was similar, which makes it possible to use the technology of low-temperature storage of glial cell culture to obtain CM or their components.

Conflict of interest

The authors do not have relevant financial or non-financial interests to disclose.

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INFLUENCE OF CONDITIONED MEDIA FROM GLIAL CELL CULTURES ON CONTRACTILITY OF UTERINE IN RATS OF DIFFERENT AGES

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Background. The physiological regulation of the uterine contractile activity changes with age, which leads to an increased number of prolonged labor and emergency caesarean sections in women giving birth at the age of 35+. One of the modern approaches to correct the function of the reproductive system is the use of from cell cultures. CM from glial cell culture contains neurotrophic factors that play an important role in maintaining the contractile function of the uterus. Current cell culture technologies include cryopreservation.
Objective: to research experimentally the effect of CM obtained from intact and cryopreserved cultures of glial cells on the contractile activity of the uterus in rats of different reproductive ages.
Materials and methods. The monolayer cell culture was obtained from the dorsal root ganglia of neonatal piglets and cryopreserved in the presence of cryoprotectant dimethyl sulfoxide. CM from native and cryopreserved cultures were collected for 28 days, after which fractions with a molecular weight of < 30 kDa were obtained from them by ultrafiltration. Rats at the age of 6 and 14 months, which corresponds to reproductive age and late reproductive age (LRA), were intraperitoneally injected with 0.2 ml of media from intact (ICM) or cryopreserved (CCM) cultures for 9 days. On the 30th – 32nd day after the end of the administration of CM animals were slaughtered and the uterine contractile activity was determined by the organ bath method, the relative area of myometrium and density of myocytes by histological method, the average area of labeling with specific antibodies to smooth muscle actin by immunohistochemical method. The statistical significance of differences was assessed by the Mann–Whitney test.
Results. It was found that spontaneous, OT-, and KCl-induced tension of isometric contraction of the uterus in intact LRA rats decreased by 19, 20, and 14%, respectively, compared with intact reproductive aged animals. After the introduction of ICM and CCM in LRA animals, normalization of isometric contraction parameters was observed. This effect was realized against the background of an increase in the area of the myometrium, the density of myocytes, and actin expression.
Conclusions. Intra-abdominal administration of CM from glial cell culture increases the uterine contractile activity in LRA rats. This effect is realized by increasing the relative area of the myometrium, the density of myocytes, and the area of expression of smooth muscle actin. The effect of media from intact and cryopreserved cultures on the contractile activity of the uterus was similar, which makes it possible to use low-temperature culture storage technologies to obtain CM without losing its biological effect.

Keywords: contractile activity, uterus, conditioned media, neurotrophic factors, glial cell culture, cryopreservation.

ВПЛИВ КОНДИЦІОНОВАНИХ СЕРЕДОВИЩ ВІД КУЛЬТУР ГЛІАЛЬНИХ КЛІТИН НА СКОРОТЛИВУ АКТИВНІСТЬ МАТКИ ЩУРІВ РІЗНОГО ВІКУ

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Обґрунтування. Фізіологічна регуляція скоротливої активності матки змінюється з віком, що призводить до збільшення кількості затяжних пологів та екстрених кесаревих розтинів у жінок, які народжують у віці 35+. Один із сучасних підходів до корекції функції репродуктивної системи полягає в застосуванні кондиціонованих середовищ (КС) від клітинних культур. КС від культури гліальних клітин містять нейротрофічні фактори, які відіграють важливу роль у підтриманні скоротливої функції матки. Сучасні технології отримання клітинних культур включають кріоконсервування.
Мета дослідження: експериментальне вивчення впливу КС, отриманих від інтактної та кріоконсервованої культур гліальних клітин, на скоротливу активність матки щурів різного репродуктивного віку.
Матеріали та методи. Моношарову культуру клітин отримували зі спінальних гангліїв неонатальних поросят і кріоконсервували у присутності кріопротектора диметилсульфоксиду. КС від нативної та кріоконсервованої культур збирали впродовж 28 діб, після чого з них методом ультрафільтрації отримували фракції з молекулярною масою < 30 кДа. Щурам віком 6 і 14 місяців, що відповідає репродуктивному та пізньому репродуктивному віку (ПРВ), внутрішньочеревно вводили по 0,2 мл середовищ від інтактної (ІКС) або кріоконсервованої (ККС) культур упродовж 9 діб. На 30–32-гу добу після закінчення введення КС тварин забивали й визначали спонтанну, окситоцин (ОТ)- та KCl-індуковану скоротливу активність матки методом органної бані, відносну площу міометрію та щільність міоцитів – гістологічним методом, середню площу мічення специфічними антитілами до актину гладеньких м'язів – імуногістохімічним методом. Статистичну значущість відмінностей оцінювали за критерієм Манна–Вітні.
Результати. Встановлено, що спонтанна, ОТ- та KCl-індукована сила ізометричного скорочення матки в інтактних щурів ПРВ знижувалася відповідно на 19, 20 і 14% порівняно з інтактними тваринами репродуктивного віку. Після введення ІКС та ККС у тварин ПРВ спостерігалася нормалізація показників сили ізометричного скорочення. Цей ефект реалізувався на тлі збільшення площі міометрію, щільності міоцитів та експресії актину.
Висновки. Внутрішньочеревне введення КС від культури гліальних клітин підвищує показники скоротливої активності матки в щурів ПРВ. Цей ефект реалізується шляхом збільшення відносної площі міометрію, щільності міоцитів і площі експресії актину гладеньких м'язів. Вплив середовищ від інтактної та кріоконсервованої культур на скоротливу активність матки був схожим, що дозволяє використовувати технології низькотемпературного зберігання культури для отримання КС без втрати її біологічного ефекту.

Ключові слова: скоротлива активність, матка, кондиціоноване середовище, нейротрофічні фактори, культура гліальних клітин, кріоконсервування.