Gliocyte Secretomes as a Source of Growth Factors Affecting the Female Reproductive System

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Abstract. In the context of a growing global trend toward delayed motherhood, which is often accompanied by age-related decline in ovarian reserve and impaired reproductive function, research aimed at developing novel strategies for the assisted reproductive technologies is gaining particular significance. One of the promising approaches is the use of cell-based products in particular biologically active compositions that have a neurotrophin-like effect on the female reproductive system.

This study investigates the effects of biologically active compositions—including cryoextracts and conditioned media of cells isolated from the spinal ganglia of neonatal piglets—on the functional state of the reproductive system in female rats at a late reproductive age. Specifically, the study focuses on changes in the parameters of the estrous cycle following administration of these compositions.

The results revealed a statistically significant prolongation of the estrus phase in treated animals compared to intact controls, indicating potential activation of ovarian function and a favorable modification of reproductive status. These findings suggest that neurotrophic factors contained in the tested biological products may influence the regulation of primordial follicle development and maintenance of the ovarian reserve. This opens new prospects for the development of therapeutic agents aimed at fertility restoration, particularly in women with diminished ovarian reserve or poor response to conventional gonadotropin stimulation protocols in assisted reproductive technology programs.

Keywords: conditioned medium, cell culture, glial cells, spinal ganglia, cryoextract, reproduction, neurotrophins.

Introduction. Declining population growth is a common problem in developed countries. In this regard, strategies for preserving women's reproductive health and assisted reproductive technologies are being developed. In the context of modern social transformations, there is a tendency to postpone motherhood, particularly an increase in the proportion of births over the age of 30. Mature reproductive age is accompanied by a decrease in ovarian reserve and a decrease in the quality of oocytes, which increases the risk of chromosomal abnormalities, miscarriage, and premature birth [1-3].

Given the relevance of the problems of decreasing ovarian reserve and female infertility, research aimed at finding effective preventive and therapeutic approaches, including those based on the use of cell technologies, is of particular importance [4, 5].

Among the newest preventive or therapeutic agents in reproductive medicine, stem cells from amniotic fluid, umbilical cord, menstrual blood, adipose tissue, and bone marrow are used [6].

Experimental data indicate the ability of such cells to influence the ovarian niche and activate follicle development. The therapeutic effect of cell therapy is largely associated with paracrine action - the release of biologically active substances that affect follicular structures [7, 8].

In view of this, there is growing interest in the use of cell secretomes (conditioned media) as an alternative to stem cell transplantation. This approach avoids the risks of immune reactions, as well as uncontrolled cell proliferation or differentiation. Secretomes from cultured stem cells demonstrate potential in tissue repair, stimulation of angiogenesis, neurogenesis, immunomodulation, and wound healing [9, 10].

Modern studies indicate an important role in the regulation of female reproductive function of neurotrophic factors: nerve growth factor (NGF), neurotrophins NT-3 and NT-4/5, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF). They are key regulators of gonadal development, ovarian and uterine function, as well as placenta and embryo formation [11-13].

Previous studies have shown that conditioned medium from the culture of gliocytes obtained from spinal ganglia is a source of neurotrophic factors [14, 15].

Therefore, biological products obtained from spinal ganglia can exhibit neurotrophic effects on the female reproductive system. This can be used in the future to obtain pharmacological drugs based on them.

In view of this, the following series of biologically active compositions from spinal ganglia were used in the work. The total cryoextract was used as a total preparation of all water-soluble substances of the organ. Since the molecular weight of neurotrophic factor dimers is about 30 kDa [16,17]. A cryoextract fraction in this molecular weight range was obtained.

It was previously established that the primary culture from spinal ganglia consists mainly of satellite gliocytes [18]. Therefore, conditioned media from intact and cryopreserved cell cultures from spinal ganglia were used as a product of direct neurotrophic secretion of gliocytes.

The purpose of the study was to study the effect of different biological compositions obtained from spinal ganglia on the estrous cycle of rats of late reproductive age.

Materials and methods. The study was conducted on white outbred female rats aged 14 months, which corresponds to the period of late reproductive age in this species of animals [19].

Total cryoextract (TC) was obtained using saline from the spinal ganglia of neonatal piglets according to the method [20].

Cryoextract fraction (CF) <30 kDa was obtained by ultrafiltration through a polyethersulfone membrane ("Millipore", Germany). To obtain conditioned media, gliocytes were isolated from spinal ganglia and cultured for 22 days according to the method [18]. Cryopreservation of cell culture in the presence of 7.5% cryoprotectant dimethyl sulfoxide was used according to the method [18].

Conditioned medium from the intact cell culture (CMICC) and conditioned media from the cryopreserved cell culture (CMCCC) of gliocyte cultures were collected in the stationary growth phase, after which they were fractionated to obtain a <30 kDa fraction.

Estrous cycle assessment was performed daily at 10:00 by vaginal cytology [21].

Estrous cycle duration was determined as the arithmetic mean for each group. The relative duration of the estrus and diestrus phases was calculated as the ratio of the total duration of the desired phase to the total duration of observation, expressed as a percentage.

Animals were divided into groups: 1 – TC administration (n = 10); 2 – CF administration (n = 10); 3 – CMICC administration (n = 14); 4 – CMCCC administration (n = 15). As controls, groups of intact animals (n = 14) and animals with the administration of carrier solutions (n = 12) were used.

Biologically active compositions and carrier solutions were administered to animals intraperitoneally in 0.2 ml. The experiment was started in the estrus phase to synchronize the start of the administration and continued daily for 9 days. Observation of the estrous cycle was carried out in the next 30 days.

Statistical analysis was performed using the Statistica 10 software (StatSoft, USA).

The results are presented in the format of Median Me (Q1; Q3). To assess intergroup differences, the Mann-Whitney test was used; differences were considered statistically significant at $p<0.05$.

Results and discussion. It is well known that the estrous cycle in rats lasts mostly four, rarely five days and is characterized by cyclic fluctuations in the secretion of the main ovarian hormones - estrogen and progesterone [22].

Changes in the concentration of sex hormones are reflected in vaginal cytology. In cytological vaginal smears of rats, three main cell types are distinguished – lymphocytes, keratinocytes, and leukocytes. The ratio of cell types varies depending on the stage of the cycle [23, 21].

Four stages of the estrous cycle are distinguished: P – proestrus, E – estrus, M – metestrus, D – diestrus.

Proestrus (P), which lasts approximately 14 hours, is easily identified by the presence of nucleated epithelial cells (Fig. 1, P).

In the Estrus (E) stage, which lasts 24-48 hours, vaginal smears are predominantly composed of large, anucleated, keratinized cells (Fig. 1, E).

Metestrus (M) (Fig. 1, M), which lasts 6-8 hours, is characterized by a combination of keratinized epithelial cells and leukocytes.

The longest stage is Diestrus (D) – 48-72 hours.

In Diestrus (D) (Fig. 1, D), leukocytes are the main cell type, but the number, appearance and presence of other cell types and mucus in the smears may vary.

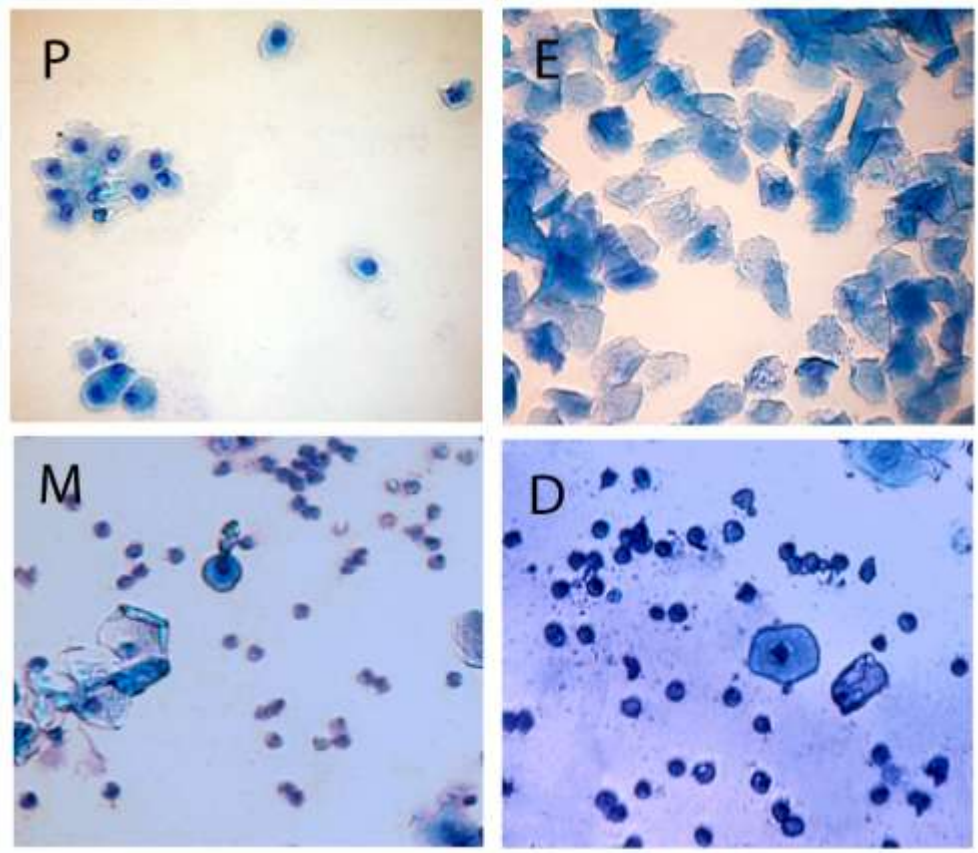

Fig. 1. Typical cytology of vaginal smears of rats: P – Proestrus (P); E – Estrus (E); M – Metestrus (M); D – Diestrus (D).

Regarding our experiments, in intact animals, individuals with both a 4-day and a 5-day estrous cycle were observed. The percentage of animals with a 4-day estrous cycle was 29.8%, while with a 5-day cycle – 70.2%. In animals, the prevalence of the percentage of the Diestrus phase in the structure of the estrous cycle was established in both the 4-day and 5-day cycles (Fig. 2).

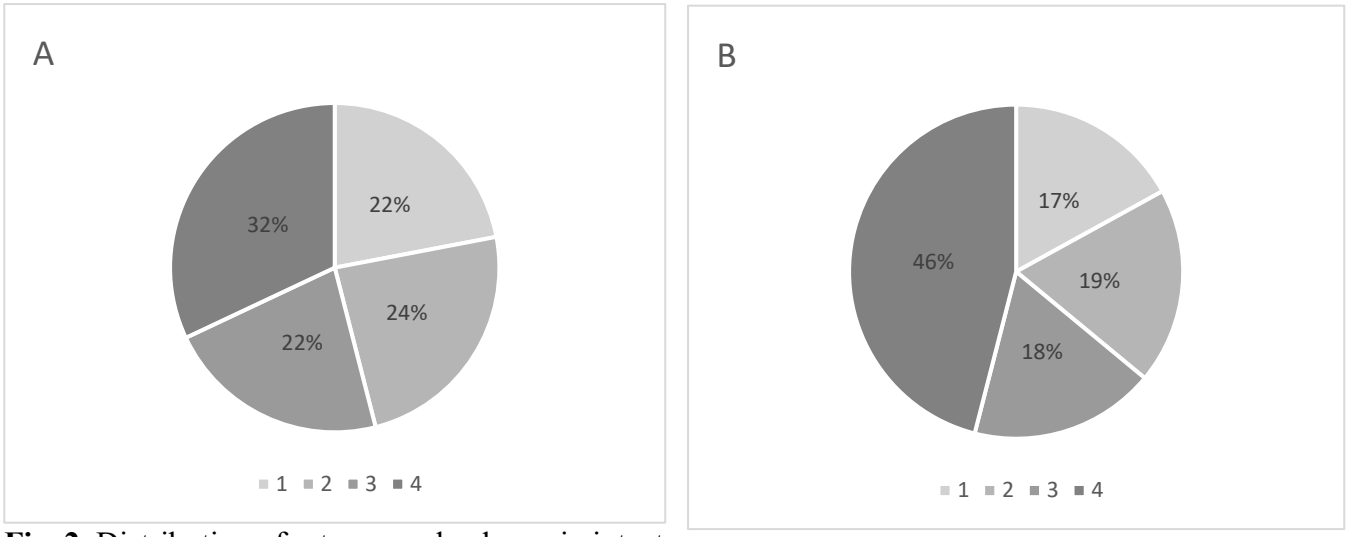

Fig. 2. Distribution of estrous cycle phases in intact rats (average values by group): left – 4-day estrous cycle, right – 5-day estrous cycle. P – Proestrus, E – Estrus, M – Metestrus, D – Diestrus.

Analysis of the estrous cycle of animals of the experimental groups showed that the main effects of the introduction of biologically active compositions from spinal ganglia were manifested in changing the number of Diestrus and Estrus phases.

In animals under the conditions of TC, CMICC and CMCCC administration, there was a statistically significant increase in the percentage of the Estrus phase in the structure of the estrous cycle compared to the intact control (Table 1). At the same time, no statistically significant changes in the number of Diestrus phases were observed.

Table 1. Percentage of Estrus and Diestrus phases in the structure of the 5-day estrous cycle of rats after administration of biologically active compositions from spinal ganglia.

Phases of the estrous cycle	Group of animals			
	TC	CF	CMICC	CMCCC
Estrus	24,2 (22,2; 28,2) *	20,1 (18,0; 20,6)	31,3 (29,1; 32,6) *	30,2 (29,2; 31,3) *
Diestrus	38,5 (33,2; 47,5)	45,4 (33,4; 47,4)	49,9 (32,5; 66,5)	45,8 (44,7; 47,4)

Note: * – the indicator is significantly different compared to the intact control, $p < 0.05$.

It is well known that age-related involutive restructuring of the reproductive system of female rats includes changes in the follicular profile of the ovaries and, as a result, a disorder of the estrous cycle. During reproductive aging, changes progress from a regular estrous cycle to irregular cycles, followed by a persistent Diestrus (anestrus) [24]. At this time, follicle development can occur in the ovaries of rats, but in most cases, it ends with their atresia without ovulation.

In our experiments, characteristic patterns of reproductive aging of rats were also observed, which were expressed in an increase in the average duration of the estrous cycle from 4 to 5 days, mainly due to an increase in the number of days with the Diestrus phase.

However, the introduction of biologically active compositions from spinal ganglia (TC, CMICC, CMCCC) led to an increase in estrus periods and a delay in persistent Diestrus.

This may be explained by the fact that neurotrophic factors present in biologically active compositions from spinal ganglia are participants in many signaling pathways at the level of regulation of the female reproductive system. It is known that neurotrophic factors are involved in

the primary assembly of ovarian follicles and subsequent folliculogenesis, affect oocyte maturation and proliferation of theca and granulosa cells [25, 26].

Previous observations of the authors indicate that the introduction of cryoextract and secretomes from intact and cryopreserved cultures of cells from spinal ganglia activates the development of primordial follicles [27].

Thus, exogenous neurotrophic factors, which are administered to rats as part of biologically active compositions from spinal ganglia, can stimulate the release of follicles of hormone-independent stages of development from the dormant state. After this, their further development and the onset of the Estrus phase are observed.

The established fact is valuable from the point of view of the development of approaches in the field of assisted reproductive technologies. It is generally accepted that ovarian stimulation for in vitro fertilization includes the use of gonadotropins that stimulate the development of follicles of hormone-dependent stages. However, there is a large group of patients who do not respond to such a protocol ("poor responders") [28].

From this point of view, the use of biologically active compositions of neurotrophic factors - a similar effect may be useful in the future for use in protocols of assisted reproductive technologies to activate dormant (hormone-independent) ovarian follicles, to which it is then possible to apply gonadotropin stimulation.

Conclusions. A positive effect of the introduction of biologically active compositions obtained from spinal ganglia on the estrous cycle of rats of late reproductive age has been established. The use of such compositions led to an increase in the number of days with the Estrus phase compared to intact rats of this age group. This can be explained by the regulatory and stimulating effect of neurotrophic factors that are part of the used biologically active compositions (cryoextract and conditioned medium from cultures of gliocytes from spinal ganglia).

Conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The authors also state that they don't have any conflict or potential conflict of interest.

Ethical approval. The Bioethics Committee of the Institute of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine approved the experimental design. All procedures complied with the requirements of the Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447-IV of February 21, 2006, as amended), as well as the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986).

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Data Availability Statement. The data presented in this study is available from corresponding author upon reasonable request.

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