Cultivation of mammals early male germ cells in a semi liquid medium

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Abstract. Spermatogenesis is one of the most complex processes of cell differentiation. The ability to cultivate early male germ cells and subject them to differentiation to produce mature gametes in vitro is an important task for both biology and veterinary and regenerative medicine. In vivo, spermatogenesis is known to occur in a three-dimensional structure, in the convoluted seminiferous tubules of the male reproductive glands, and Sertoli cells and the extracellular matrix have a significant impact on the survival, proliferation and differentiation of early germ cells. At present, methods of creating a microenvironment, similar to the native, are being actively developed to support spermatogenesis in vitro. This paper reflects research on the use of semi-fluid media as a three-dimensional matrix for the cultivation of early male gametes of mammals. These culture systems have been shown to support the differentiation of early germ cells to the stage of elongated spermatids with the subsequent formation of spermatozoa. However, it is necessary to continue research to create a culture system that can induce and maintain complete spermatogenesis.

1. Introduction
Studies of mammalian spermatogenesis have been conducted for a long time and are of interest both from the fundamental side - with the aim of expanding the understanding of the molecular mechanisms underlying the processes of self-renewal and commitment of spermatogonium stem cells, cell proliferation and differentiation, and from the practical side. The creation of new cell models for use in veterinary medicine is one of the promising areas for the development of modern science [1-3]. The ability to cultivate early male germ cells and subject them to differentiation to obtain mature gametes in vitro, relevant for agricultural biotechnology, including genomic and cellular therapy for male infertility, correction of inherited genetic disorders, the creation of transgenic animals, and the preservation of rare and endangered species. Previous studies have led to the understanding that for successful cultivation and differentiation of early germ cells, in addition to growth factors and the presence of somatic cells, it is necessary to create a spatial structure in which the reproduction of a sequence of processes occurring in the seminiferous tubules in vivo is possible [4-7]. At present, three-dimensional (3D) cultivation systems are considered to be the most closely imitating the microenvironment of the seed epithelium. Unlike conventional two-dimensional cell cultures, where the Petri dish is covered with a thin layer of gelatin, collagen, Matrigel, etc., the semi-liquid three-dimensional matrix is represented by a thick (from a few millimeters to several centimeters) layer of gel into which any cell culture can be embedded, including early sex and somatic cells.
Our review describes recent advances in the creation of a 3D method for the cultivation of mammalian early male germ cells.

2. Literature review

J H Lee with the team conducted a series of experiments in which revealed a positive effect of the 3D matrix, represented by collagen gel and collagen + Matrigel complex, on the differentiation and viability of spermatogonia in 18-day rats. A three-dimensional matrix was prepared from a solution of collagen, blood serum of cows (SKPK) and DMEM/F12 concentrated nutrient medium, with or without the addition of Matrigel. As a result, after 22 days of cultivation of the untreated cell population of male reproductive rat cells, the spermatogonia underwent meiosis and differentiated into spermatids. The viability percentage was 42.8%, 70.7% and 76.1% when cultivated in a monolayer, in a 3D matrix of collagen gel without Matrigel and in a complex of collagen + Matrigel, respectively. The authors also noted that shaking has a positive effect on the viability of the culture, very likely due to an increase in oxygen flow [8]. Later, when cultivating human spermatocyte I and II, in a 3D system represented by collagen gel enriched with 5% Matrigel, for 12 days, the team of J H Lee received colonies consisting of 11% and 37% of round and elongated spermatid, respectively. [9].

In 2014, Khajavi and other scientists were able to demonstrate that the joint cultivation of spermatogonial stem cells (SSC) and somatic cells isolated from 7 days of mice in a 3D system represented by collagen gel based on the DMEM/F12 nutrient medium and containing SKPK increases the number of CCK colonies and their size. Studies have shown that the presence of somatic cells, including Sertoli cells (SC), in culture allows an increase in the expression level of SCP3, Crem and TTF1 (spermatid markers) from 6% to 13% [10].

Team Stukenborg J.B. et al. showed that by co-cultivation of spermatogonia with somatic cells obtained from 10 days of mice, in the presence of gonadotropin, a full cycle of spermatogenesis differentiated to spermatozoa can be obtained in vitro [11]. To recreate the testicular microenvironment, scientists used the Soft-Agar-Culture-System (SACS), originally created to study the proliferation and differentiation of bone marrow and hematopoietic cells in vitro [12]. SACS can be used as a bi- or single-phase system. It is a two-layer structure, represented by the top layer - soft 0.37% agar and the bottom layer - solid 0.5% agar. The high-glucose DMEM medium was used as a nutrient medium. Spermatogonia were added to soft 0.37% agar, depending on the experiment, the lower solid layer (0.5% agar) was either empty or with the addition of somatic cells, including Cop. The addition of somatic cells to the lower solid layer of agar increased proliferation and stimulated the differentiation of spermatogonia, which is consistent with the data obtained during classical cultivation [13].

After 30 days of cultivation, about 6% of the cells in the colonies expressed acrosine, which indicates the presence of spermatids, morphological analysis showed that the vast majority of them were round spermatids. Researchers have identified the differentiation of spermatogonia to a spermatozoan with a normal morphology, but in extremely low concentrations - up to 16 cells out of 106, despite the small concentration, this is a big step forward. It is worth noting that researchers observed the formation of sperm cells only when the culture was supplemented with gonadotropins (5 IU/l HCG and 5 IU/l rFSH), which indicates the potentially crucial role of hormones in the development of male germ cells [11,14,15]. The presence of spermatozoa was demonstrated only at the stage of histological evaluation, and therefore fertility was not assessed. In 2017, Navid with colleagues used SACS to assess the effect of melatonin, along with leukemic inhibitory factor (LIF) and neurotrophic factor (GDNF), on the SSC of newborn mice. Adding melatonin to the main nutrient medium significantly increased SSC proliferation [16]. In his further research J B Stukenborg et al. tested a new system - methylcellulose culture systems (MCS), consisting of methylcellulose as an alternative matrix for the three-dimensional cultivation of early germ cells. As a result, it was found that the material of the matrix is not the principal factor for successful three-dimensional cultivation, provided that the material supports the necessary 3D structure [5]. This conclusion is consistent with the work of J H Lee, in which he used collagen gel as a matrix material and also noted proliferation and differentiation [8, 9]. Nevertheless, the use of the matrix based on methylcellulose has the main advantage - the possibility of subsequent purification of cells.
from the matrix material, which opens up opportunities for a more detailed study of the result. Based on the fact that the joint cultivation of spermatogonia and somatic cells lead to more intensive proliferation and differentiation, the researchers in subsequent experiments to enhance the effect, added somatic cells in a single layer with spermatogonia. Spermatocytes and round spermatids were found in SACS after 21 days of cultivation, full maturation of germ cells in spermatozoa was observed after prolonged cultivation for 40 days. In 2015, a message appeared on the three-dimensional cultivation of type A spermatogonia obtained from testicles of 13–33 days of rhesus monkeys (Macaca mulatta); SACS and MCS were used as a matrix based on RPMI medium, with the addition of 25% SKPK. Differentiation was observed after 30 days of cultivation, while in vivo in this species of animals it occurs after 10.5 days. VASA-, SALL4-, GFR-1α-positive cells were observed throughout the entire cultivation period, which indicates the pre-meiotic germ cell preservation. After 1 month of incubation, CREM-1 and acrosin-positive cells appeared, which indicates the development of spermatogenesis to the meiotic and postmeiotic stages. Unlike previously obtained results in mice, in this experiment, elongated spermatids were not identified in the colonies, which may be due to the imperfection of the culture system itself, which does not allow cells to complete the final stage of differentiation, as well as with the possible small number of spermatids formed and difficulty microscopically identify these cells in a thick layer of agar and methylcellulose [17]. These results are consistent with our research. We conducted a series of experiments on the 3D cultivation of boar spermatogonia in a methylcellulose-based matrix [18,19]. For this, two experimental groups were formed: the 1st experimental group is represented by boar spermatogonia, the 2nd experimental group is represented by a mixture of boar spermatogonia and CS. As a result, it was found that spermatogonia in 2.5% methylcellulose gel remain viable up to 21 days. Spermatogonia previously purified from other types of cells multiplied without signs of differentiation and by 21 days their numbers increased 2.5 times. In the second experimental group, represented by a mixture of spermatogonia and CS, we observed a process of differentiation, which was accompanied on the 7th day by the formation of chains of cells, cords, and on the 21st day by the formation of structures similar to the seminiferous tubules. Analyzing the cellular composition of the formed structures, we found that the culture is characterized by heterogeneity and consists of 17% spermatocyte I and II, 33% of the rounded spermatids and single (6%) elongated spermatids. A single cell with a phenotype like sperm was also detected. Histological analysis showed in both groups a uniform distribution of cells in the matrix. As a result, we found that cultivation of boar spermatogonia in the presence of CS leads to their differentiation.

In 2018 Madighem A Abu with colleagues first showed the presence of biologically active spermatogonial cells in the testes of immature mice treated with busulfan. Scientists induced the proliferation and differentiation of spermatogenic cells from immature mice treated with Busulfan (of Busulfan-Treated Immature Mice) using MCS to the meiotic and postmeiotic stages [20].

In 2014, A Reda and colleagues published a paper that analyzed the effects of hormones, nutrient medium, Leydig cells, and CS on 3D cultivation of early germ cells for 7 days. rats in soft 0.35% agar. After testing the culture media: F12, DMEM/F12, DMEM + Glutamax, MEM, DMEM + glutamine and DMEM without glutamine, the researchers concluded that these culture media directly affect the functionality of Leydig cells, but not the differentiation of germ cells in 3D culture. The researchers noted that in the 3D system represented by soft agar, regardless of the nutrient medium, stimulation with gonadotropins contributes to the functionality of Leydig cells after 1, 7 and 14 days, but the DMEM + glutamine nutrient medium was most effective in the direction of testosterone production by Leydig cells in 1 day of cultivation. Nutrient media did not have a significant effect on overall cell survival. At the same time, all colonies contained a mixture of somatic cells (CS and peritubular cells) and early germ cells (including differentiated to pachytenic spermatocyte). In this study, the spermatogonia present in the testes of 7 days of rats differentiated to the stage of pachyten spermatocyte in 21 days, while in vivo this occurs by 25–28 days of age, i.e. for the same period of time [21].

J J Lim et al. conducted an experiment on the 3D cultivation of male germ cells (male germ stem cell) (GSC) -like cells, obtained from human embryonic stem cells [22]. Calcium alginate was used as a matrix. Alginate is a natural polysaccharide derived from brown algae that forms a hydrogel in the
presence of divalent cations, such as calcium. Purified alginate is non-toxic, has a high permeability, soft gel consistency and low immunogenicity. These properties contribute to the active use of alginate in the biomedical field, including as a biomaterial, as well as for the cultivation and transplantation of cells, tissues and embryos. After centrifugation, the concentrate of GSC-like cells was squeezed into a Petri dish with 1 ml of sodium alginate solution (0.01 mg / ml in physiological solution) using a Pasteur pipette. Then they were transferred to a solution of calcium chloride (0.015 mg / ml in physiological solution) to obtain encapsulated cell aggregates. Cells were cultured with and without the addition of somatic cells in DMEM / F12 with the addition of 10% SKPK for six weeks. As a result, scientists concluded that the joint 3D cultivation of GSC-like cells and somatic cells is more effective in differentiation than the 3D cultivation of purified GSC-like cells. After 2 weeks of cultivation, scientists obtained about 3% of spermatid cells differentiated to the stage, but no morphologically normal or functioning spermatozoa was obtained [22]. This result confirms the previously obtained D R Lee et al. data, when, after 35–70 days of 3D cultivation in calcium alginate, from the FCS, spermatids were obtained from 3 days of bulls [23].

T Yokonishi et al. proposed a new combined approach to 3D cultivation, based on the ability of testicular cells to restore structure even after disaggregation into single cells. However, this ability was demonstrated only in vivo: in the subcutaneous [24, 25] and in the renal subcapsular spaces [26, 27]. In his study, T Yokonishi reconstructed the seminiferous tubules in vitro; for this, enzymatically dissociated cells of the seminiferous tubules 0.5-5.5 days of mice were cultured under conditions of suspension, which caused aggregation. On the 2nd day of cultivation, the cell aggregates were transferred to the surface of the agarose gel to continue cultivation by the method of organ culture. Agarose gel aggregates showed a gradual reorganization into a tubular structure that lasted about 2 weeks. The first histological changes were observed for 2 days in the form of single strands, for 5 days the strands continued to form and became apparent. Tubular structures, similar to the seminiferous tubules, were formed on day 14, but the borders of the tubular structure were irregular in shape and resembled a maze. Immunohistochemical analysis showed that Sertoli cells formed tubules, germ cells (germ cells, GS cells) adjoined them, and there were 3β-HSD-positive cells between the tubules and the periphery of the tissues, presumably Leydig cells. Some germ germ cells differentiated to the round spermatid stage. However, the effectiveness of this approach is lower than in experiments with organ cultivation. [28, 29].

3. Conclusion
Spermatogenesis is a complex, multistep process that takes place in the seminiferous tubules of the testes and is controlled by many factors associated with germ cells and other testicular cells, such as Sertoli cells and Leydig cells. The creation of such a vulnerable microenvironment in vitro is one of the main topics of modern biology. Given that in vivo, germ cells are closely interrelated with somatic cells and the extracellular matrix, which are involved in complex proliferation and differentiation processes [30–33], it can be assumed that the imitation of the microenvironment, which resembles 3D organization of the seminiferous tubules, will create ideal conditions for cultivation [4-7]. SACS and MCS are qualitative, three-dimensional systems that provide a microenvironment, similar to the native, and imitate some aspects of the in vivo environment, under the influence of which spermatogenesis occurs. These systems will allow us to broaden our understanding of the interaction between Sertoli cells and early male germ cells, between the germ cells and the extracellular matrix, as well as the effect of these interactions on the process of spermatogenesis. Results demonstrating that 3D culture conditions improve the proliferation of early male germ cells, and also contribute to their differentiation to spermatids, followed by the formation of spermatozoa [11, 14, 15], indicate that 3D cultivation system has great potential for research mechanisms that control the differentiation of male germ cells. However, literary analysis and our own experience shows that to obtain a higher concentration of differentiated cells (including spermatozoa), further optimization of this system is required. In addition, it is necessary to evaluate the functionality of the sperm cells obtained, the state of their DNA, as well as epigenetics.
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