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ISOLATION AND CHARACTERIZATION OF ROOSTER (*Gallus gallus*) SPERMATOGONIA

**N.A. VOLKOVA, S.V. KORZHIKOVA, T.O. KOTOVA, A.N. VETOKH,
L.A. VOLKOVA, N.A. ZINOVIEVA**

L.K. Ernst All-Russian Research Institute of Animal Husbandry, Federal Agency of Scientific Organizations, pos. Dubrovitsy, Podolsk Region, Moscow Province, 142132 Russia, e-mail natavolkova@inbox.ru

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Abstract

Using the male gonad cells of farm animals and poultry in the biotechnological programs to produce chimeric and genetically modified animals is regarded as an alternative to traditional methods of breeding and genome modification and offers great opportunities for obtaining individuals with new desired properties. In transgenesis, this approach provides a targeted genetic modification of male germ cells by introducing the recombinant DNA directly into the testis parenchyma of adults (in vivo), or introducing the transformed donor spermatogonia into testes of a sterile recipient (ex vivo) for further obtaining progeny. In case of introduction of donor spermatogenic cells the key points that determine the effectiveness of the manipulation is to obtain a pure population of donor cells and the elimination of their own spermatogenic cells (spermatogenesis off). In this regard, conducting research related with the development of effective methods for isolating and maintaining the culture of testes stem cells (spermatogonia) is actual. The aim of our study was the optimization of the methodological approaches to the preparation and cultivation of rooster spermatogonia within the development of individual stages for producing transgenic birds. In the experiments we obtained and characterized the culture of rooster spermatogonia. Based on preliminary histological studies of spermatogenesis it was found that in roosters aged under 5 weeks the spermatogenic cells are predominantly presented by one cell type — spermatogonia. In this connection, for obtaining spermatogonia culture the testes from two week old cockerels have been used. Isolation of spermatogenic cells from testicular of rooster was achieved by mechanical and enzymatic treatments of the testis tissue. For enzymatic dissociation of testis tissue we used sequential processing with collagenase solution (at a final concentration of 1 µg/ml) for 20 minutes and 0.25 % trypsin solution for 30 minutes. For obtaining the most pure population of spermatogonia we took into account different capacity of different cell types to adhesion. It was found that after 24 hours of culturing primary culture of rooster testicles the unattached cells were presented mainly by spermatogonia. These cells were pelleted and plated into culture dishes with feeder layers. Continuous cell line STO, transplanted Sertoli cells of swine, a cell line Sc, primary Sertoli cells of rooster were used as feeder layers. Also we carried out the cultivation of spermatogonia on the dishes coated with 0.2 % gelatin. The growth medium for culturing spermatogonial cells was DMEM with high glucose content (4.5 g/l), supplemented with 5 % FCS (Fetal Calf Serum, GE Healthcare Life Sciences HyClone Laboratories, USA), 2 mM α-glutamine (Invitrogen, USA), MEM (Minimum Essential Medium, 10 µl/ml, Invitrogen, USA), antibiotics (100×, Invitrogen, USA), mercaptoethanol (5×10^{-5} M, Invitrogen, USA), albumin (5 mg/ml, Invitrogen, USA), DL-lactic acid (1 µl/ml, Sigma-Aldrich Co., USA), epidermal growth factor EGF (20 ng/ml, Sigma-Aldrich Co., USA), basic fibroblast growth factor bFGF (10 ng/ml, Sigma-Aldrich Co., USA), leukemia inhibitory factor LIF (2 ng/ml, Sigma-Aldrich Co., USA). Spermatogonia colonies have been formed at days 3–4 of cultivation. Best results were obtained by culturing the spermatogonia on primary Sertoli cells of rooster. The presence of spermatogonia colonies was confirmed immunohistochemically in 7-day culture using SSEA-1 (stage-specific embryonic antigen-1) specific antibodies.

Keywords: spermatogonia, spermatogenic cells, roosters, cell culture

Using farm animal and poultry male gonad cells in biotechnological programs for producing chimeric and genetically modified animals is regarded, due to ability of foreign DNA (fDNA) transfer to eggs at fertilization, as a potential

alternative to common breeding and genome modification methods [1-5]. fDNA transgene integrated into host genome can be stably inherited in downstream progenies. Besides, due to adult animals manipulated the time and cost of obtaining transgenic progeny reduce significantly.

Germinal epithelium consists of Sertoli's sustentacular nourishing cells and the spermatogenic cells (spermatogonia A and B, spermatocytes I and II, spermatids, sperm cells) [6]. Genetic modification of type A spermatogonia, the stem cells of the male gonads and the precursors of highly mature sperm cells, are of greatest interest. These cells are small populations of spermatogenic cells located on the basement membrane of the seminiferous tubules, and able to self-renew and differentiate, thus enabling the continuity of spermatogenesis and sperm production [7, 8]. This provides a great opportunity in creating transgenic and chimeric animals with desirable traits.

In transgenesis, a targeted genetic modification of male germ cells can be due to introducing the recombinant DNA directly into the testis parenchyma of adults (in vivo), or introducing the transformed donor spermatogonia into testes of a sterile recipient (ex vivo) for further obtaining progeny. In in vivo manipulation, gene constructs are introduced into testes of adult males by retroviral or lentiviral vectors which can integrate into host cell genome providing a high-frequency transformation of target cells [9, 10]. At ex vivo transgenesis spermatogonia are genetically pre-transformed in vitro [11, 12].

In case of introducing donor spermatogenic cells the key point that determines the manipulation effectiveness is to obtain a pure population of donor cells. Spermatogenic cell culture and in vitro genetic transformation significantly extend capabilities for recombinant DNA delivery into specific target cells using sound systems. At that, the risk of transgenic mosaic individuals with untransformed germ cells which cannot produce transgenic progeny is fully avoided. Positive and negative selection in the modified germ cells ensures single copy transgene integration in a specific locus of fertilized oocyte while integrity of the rest genome remained unaffected. In future, the artificial insemination with the transformed sperm cells may be seen as an approach to mass production of genetically modified poultry.

Isolation and characterization of spermatogonial cells are reported for laboratory and farm animals, such as mice [13, 14] and hamsters [15], pigs [16-20] and goat [21, 22], and in fishes [23]. The spermatogonial cells were also isolated from the gonads of embryos and testes of day-old chicks [24, 25].

In our previous study the spermatogonia were found to prevail among cell populations in the testis of roosters under the age of 5 weeks [26]. In this study, we showed that a preliminary removal of other types of cells, in particular the largest population of Sertoli cells, from cell suspension provides high purity of spermatogonia cell culture. The remaining sporadic somatic cells produce a feeder layer. In this, the own Sertoli cells are most successful to form a feeder layer.

This study is focused on the optimization of methodological approaches to the preparation and cultivation of rooster spermatogonia within the development of individual step in the program for producing transgenic poultry.

Technique. Collected testes of 2-week-old roosters were disinfected with 70 % ethanol, decapsulated, rinsed twice in saline solution containing double dose of antimycotic antibiotic, and then cut into 1-2 mm size pieces and rinsed thrice with DMEM (Dulbecco's modified Eagle's medium) by sedimentation (300 g for 7 min) and pipetting. The resulting cells was subjected to an enzymatic treatment with collagenase (PanEco, Russia) in DMEM concentration of 1 mg/ml for 20 min at 37°C. The cells were centrifuged at 200 g for 5 min, and the supernatant was removed. The sediment was pipetted in 0.25 % trypsin (Invitrogen,

USA) and incubated for 30 min at 37 °C and occasionally shaking. After trypsin neutralization with the medium containing fetal bovine serum (FBS, GE Healthcare Life Sciences HyClone Laboratories, USA) the cells were repeatedly sedimented and rinsed several times in growth medium by pipetting. The resulting cell suspension were inoculated into tissue culture flasks (1×10^6 cells/cm²). The growth medium was DMEM HG (Invitrogen, CIIA) containing 4.5 g/l glucose, 20 % FBS, 2 mM α -glutamine (Invitrogen, USA), MEM (Minimum Essential Medium, 100 $\times$; Invitrogen, USA), and antimycotic antibiotic (100 $\times$; Invitrogen, USA). The cells were removed from the substrate by adding 0.25 % trypsin solution.

When obtaining primary culture of Sertoli cells to be used as a feeder layer, the growth medium containing spermatogenic cells and a very small amount of somatic cells not adhered to the substrate was removed 24 hours after isolation of primary culture of spermatogonial cells. The dishes were rinsed with DMEM, and DMEM HG was added for further incubation of the adhered cells consisting mostly of the Sertoli cells and fibroblasts. Since DMEM HG is not optimal for fibroblasts, they were gradually replaced by Sertoli cells. When feeder layer preparing, the cells after reaching 90 % of the monolayer were treated with 30 μ g/ml mitomycin C (Sigma-Aldrich Co., USA) for 3 hours, then washed three times with Hanks' solution (PanEco, Russia) and used for the spermatogonia cell culture. After methanol fixation the Sertoli cells were stained with Oil red for 15 min for identification.

The growth medium for spermatogonial cells was DMEM HG supplemented with 5 % FBS, 2 mM α -glutamine, 10 μ l/ml MEM, 100 $\times$ antibiotic, 5×10^{-5} M mercaptoethanol (Invitrogen, USA), 5 mg/ml albumin (Invitrogen, USA), 1 μ l/ml DL-lactic acid (Sigma-Aldrich Co., USA), 20 ng/ml EGF (epidermal growth factor, Sigma-Aldrich Co., USA), 10 ng/ml bFGF (basic fibroblast growth factor, Sigma-Aldrich Co., USA) and 2 ng/ml LIF (leukemia inhibitory factor, Sigma-Aldrich Co., USA). The growth medium was refreshed each 3 days.

Freshly-isolated roosters' spermatogenic cells were studied morphologically by phase contrast microscopy (Nikon, Japan). Romanowsky-Giemsa staining was used for permanent preparations. Prior to staining the spermatogenic cells were fixed for 10 min in cold methanol at -20 °C, rinsed twice in distilled water, stained with Giemsa stain (Merck, Germany) solution for 10-15 min and then washed in tap water.

In 7-day culture the spermatogonia were identified by histochemical and immunochemical staining. When histochemical Schiff staining for glycogen according to standard protocol [27] the cells were fixed in 10 % neutral buffered phormalin. Resulted spermatogonial cell colonies were stained crimson. To detect SSEA-1 (stage-specific embryonic antigen-1) expression the cells were fixed in methanol. In immunohistochemical tests the avidin-biotin system (Vector Laboratories, USA) used as described [15] cells were first incubated with primary anti-SSEA-1 antibody, and the peroxidase-labeled Ab-Ag complex was incubated with 3,3-diaminobezidin tetrachloride (DAB, Vector Laboratories, USA) as a substrate to cause detectable colored precipitate.

Results. Figure 1 illustrates spermatogonia isolation (A-D). After mechanical treatment of testes there were small pieces of tissue seen on the preparations (see Fig. 1, A). The enzyme treatment (see Fig. 1, B) led to cell suspension which consists mostly of individual cells. In 24 hours the somatic cells with high adhesiveness to plastic are attached to a substrate and start dividing. Most of the spermatogenic cells were spherical in shape and suspended while some were attached to flattened somatic cells, using them as a feeder layer. On day 2 in the culture (see Fig. 1, D) spermatogenic cells attached to the feeder layer and became spherical in shape.

Fig. 1. Isolation of spermatogonia from testes of a 2 week-old rooster (*Gallus gallus*): A — small fragments after mechanical manipulation (×200); B — individual spermatogenic and somatic cells on enzyme-treated preparation (×400); C — spermatogonia (1), Sertoli cells (2), Leydig cells (3), fibroblasts (4) and myoid cells (5) in a 2-day culture (×400); D — spermatogonia (marked by arrows) in a 2-day culture (Romanowsky-Giemsa staining, ×400). Light (A, B, D) and phase contrast (C) microscopy (Nikon, Japan).

Fig. 2. Primary culture of rooster (*Gallus gallus*) testis somatic cells: A — fibroblasts (1) and Sertoli cells (2) in 4-day culture (×200); B — Sertoli cells (2) in 7-day culture (×200); C — Sertoli cells in 7-day culture (Romanowsky-Giemsa staining, ×100); D — Sertoli cells in 7-day culture detected by lipid inclusions (3) (Oil red staining, ×400). Light microscopy (Nikon, Japan).

There were several cell types on the permanent preparations of which the spermatogonial cells and Sertoli cells were numerous. Spermatogonia were round in shape and had a large spherical nucleus and a narrow rim of cytoplasm on the stained preparations. Sertoli cells had a large nucleus and an indistinct cytoplasm

diffused in shape with lipid inclusions. Additionally, interstitial cells (the fibroblasts and Leydig cells) were found. Leydig cells were irregular-shaped and large in size as compared to other testis-specific cell types. Fibroblasts were spindle-shaped. There were also small spherical myoid cells (see Fig. 1, C).

In view to obtain a homogenous Sertoli cell culture for feeder layer we used a somatic cell culture. On days 3 and 4 (Fig. 2, A) the cells differed morphologically, i.e. Sertoli cells were epithelial-like in shape, while fibroblasts were spindle-shaped. On days 6 to 7 the Sertoli cells displaced other cell types in the culture (see Fig. 2, B). On preparations after Romanowsky-Giemsa staining the cell had morphology similar to that of epithelial cells, and lipid inclusions characteristic of Sertoli cells (see Fig. 2, D).

For the most pure population of spermatogonia we used a method based on unequal adhesive abilities of different cell types. In this, the unattached cells were sedimented by centrifugation of growth media removed from 1-day primary culture of rooster testis. The pelleted cells were mostly spermatogenic, since more adhesive somatic cells remained in the flasks. Cells were pipetted in spermatogonia growth medium and plated onto feeder layers, which were STO cell line, pig Sertoli cells' continuous culture (PSC), Sc cell line and primary Sertoli cells of rooster, or onto dishes with 0.2 % gelatin. The primary Sertoli cells occurred to be the most successful feeder layer (Table).

Effect of different feeder layers on rooster (*Gallus gallus*) spermatogonial cell culture

Parameter	Feeder layer			
	STO cell line	Sc cell line	pig Sertoli cells, continuous culture	rooster Sertoli cells, primary culture
Spermatogonia attached to feeder layer	Days 1-2	Days 1-2	Days 1-2	Days 1-2
Spermatogonia cell colony formation	Days 4-5	Days 4-5	Day 4	Day 3
Spermatogonia count in the colony on day 7 (average for 20 colonies)	6	6	8	10

On days 3 to 4 the spermatogonial cell colonies occurred and grew in size to day 7. In case of gelatin as a feeder layer, the spermatogonial cells attached to the somatic cells a small amount of which was mentioned to be present in the cell suspension when growth media were collected from 1-day culture of the rooster's testis cells (Fig. 3, A, B).

Fig. 3. Colonies (marked by arrows) of rooster (*Gallus gallus*) spermatogonia in cell culture when different feeder layers used: A — own Sertoli cells (Romanowsky-Giemsa staining), B — Petri dishes with 0.2 % gelatin (no staining), B — specific immunohistochemical reaction for SSEA-1 (stage-specific embryonic antigen-1) expression, D — control (no specific immunohistochemical reaction for SSEA-1 in the absence of anti-SSEA-1), $\times 400$; E — colonies after Schiff staining, F — control (feeder layer, no Schiff reaction), $\times 630$. Light microscopy (Nikon, Japan).

Immunohistochemical staining for SSEA-1, a marker of stem cells, including spermatogonial stem cells A (see Fig. 3, C, D), and histochemical Schiff staining for glycogen confirmed the presence of spermatogonia colonies in 7-day cell culture.

Thus, efficient obtaining homogeneous rooster's spermatogonia culture depends on separation of the spermatogonial cells from the rest ones and on the feeder layer used. Different cell types are not the same in ability to adhere to substrate that allows us to purify the spermatogonia cell culture as much as possible from other cells, in particular from the largest population of Sertoli cells. Individual somatic cells, which remain thereafter in the suspension, serve as a feeder layer to which spermatogonia are attached during cell culture growth. Using feeder layers or plates treated with 0.2 % gelatin and an appropriate growth medium allows us to obtain the spermatogonia colonies in 3-4-day culture. Own Sertoli cells are optimal to produce feeder layer for rooster's spermatogonial cells.

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