Study of insulin-like growth factor 1 effects on bovine type A spermatogonia proliferation and viability

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Abstract: Spermatogonial stem cells (SSCs) are the best candidates for exploitation in genetic improvement of cattle herds. In fact, SSCs transmit genetic information to future generations. In vitro improvement in proliferation and frozen-thawed SSCs numbers is therefore needed. In the present study, bovine SSCs were cocultured with Sertoli cells, with or without insulin-like growth factor 1 (IGF-1). During 16 days of incubation, 100 ng/mL IGF-1 showed higher efficacy in increasing the numbers and diameters of SSCs colonies than 40 and 150 ng/mL concentrations. Furthermore, 100 ng/mL IGF-1 increased the frozen/thawed cells' viability rate. Overall, these data demonstrate that usage of 100 ng/mL IGF-1 in vitro improves proliferation and postfreezing viability rate of SSCs.

Key words: Spermatogonial stem cells, proliferation, IGF-1, bovine

1. Introduction
Spermatogonial stem cells (SSCs) are considered an undifferentiated spermatogonial subpopulation that has self-renewal and differentiation potential. SSCs are able to continue spermatogenesis after transplantation into infertile testes (1). Spermatogenesis in mammals is a procedure for transmitting genetic information to future generations, which, after adulthood, continues throughout the male life. Spermatozoa are the final product of this process (2). Cell culture techniques’ development has made an opportunity for germ-line modification and improvement in a broad range of species (3). Transplantation of transfected SSCs into seminiferous tubules of bull calves testes can be used for production of transgenic animals. After transplantation they migrate to the basal layer of tubules and initiate proliferation and spermatogenesis (4). Thus, increasing proliferation and viability rate of SSCs in vitro facilitates great successes in the field of genetic improvement of cattle herds. Type A spermatogonia can be obtained in large numbers in prepubertal bulls (4,5). Isolation of type A spermatogonia is performable by 2-enzyme digestion of calf testes, which gives 65%-87% pure cell populations (6). In the past, researchers used different culture conditions and cryopreservation methods for improvement of proliferation and survival of spermatogonia (6–8).

Insulin-like growth factor 1 (IGF-1) consists of a peptide chain of 70 amino acid residues with 3 disulfide bridges that is homologous to proinsulin (9,10). Leydig cells in normal testes secrete IGF-1, which regulates self-renewal and pluripotency of SSCs via IGF-1 R-mediated PI3K/Akt signaling (11). Synthetic IGF-1 inhibits apoptosis (12,13) and it has mitogenic effects on cells (14,15).

In this context we examined the effects of IGF-1 on proliferation and viability of bovine type A spermatogonia. Appropriate doses of IGF-1 in SSC cultures result in optimal size and numbers of colonies for various experiments in this field.

2. Materials and methods
2.1. Experimental animals
In this survey, 5 male Holstein Friesian calves of 3 months old were used. The research was conducted in accordance with National Research Council guidelines. Calves were sedated with xylazine (0.1 mg/kg of body weight, i.m.) and received a 3-mL injection of 2% lidocaine 20 min before surgery. Respiration rate, heart rate, and rectal temperature were monitored before and after recovery from analgesia. Calves were given flunixin meglumine (2.2 mg/kg of body weight, i.m.) for 3 days after surgery.

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2.2. Germ cell and Sertoli cell collection
Testicular biopsy samples were placed on ice and transferred to the laboratory within 2 h. The testes samples were minced into small pieces and suspended in Dulbecco’s modified Eagle’s Medium (DMEM; Sigma, St Louis, MO, USA), supplemented with 160.7 mM NaHCO₃, single-strength nonessential amino acids, 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, and 40 µg mL⁻¹ gentamicin (all from GIBCO, Carlsbad, CA, USA). SSC isolation was prepared by the method previously described by Van Pelt et al. (16). In brief, testes fragments were rinsed in DMEM containing 1 mg mL⁻¹ collagenase, 1 mg mL⁻¹ trypsin, 1 mg mL⁻¹ hyaluronidase type II, and 5 µg mL⁻¹ DNAse I (all from Sigma) and were incubated at 37 °C for 60 min. Mechanical dissociation was also done by a little pipetting for better testicular tissue digestion. After removal of interstitial cells by 3 washes with DMEM, seminiferous fragments were incubated in DMEM containing collagenase, hyaluronidase, and DNAse for 45 min as described above. By centrifugation at 30 × g for 2 min, the cells were separated from the remaining tubule fragments. Nylon filters of 77 and 55 mm were used, and then isolated cells were cultured.

2.3. Sertoli cell isolation
Following the second enzymatic digestion, Datura stramonium agglutinin (DSA; Sigma) was used for isolation of Sertoli cells. Briefly, 25-cm² flasks were coated with 5 µg mL⁻¹ DSA in phosphate-buffered saline (PBS) at 37 °C for 1 h. After 3 washes with DMEM containing 0.5% bovine serum albumin (BSA; Sigma), the suspensions of cells obtained by enzymatic digestion were placed in coated flasks and incubated for 1 h at 37 °C in a humidified atmosphere of 5% CO₂ in air. After the incubation period, nonadherent cells were collected. After 3 days the Sertoli cells formed a confluent layer.

2.4. Spermatogonial cells’ coculture with Sertoli cells
After achieving appropriate confluency of Sertoli cells, SSCs were cocultured for 2 weeks. DMEM supplemented with 160.7 mM NaHCO₃, 10% FBS, 100 ng mL⁻¹ glial cell line-derived neurotrophic factor (GDNF), single-strength nonessential amino acids, 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and 40 µg mL⁻¹ gentamicin was used. After 1 week of coculturing, colonies of SSCs were seen. The cells were then passaged and cultured in 24-well plates in 4 groups including: the control group 1 (40 ng/mL IGF-1), group 2 (100 ng/mL IGF-1), and group 3 (150 ng/mL IGF-1). Culture medium together with the mentioned doses of IGF-1 were refreshed every 3 days.

2.5. Colony assay
Determination of numbers and diameter of SSC-derived colonies was carried out on day 7 and the following 10th, 13th, and 16th days (8). This assessment was prepared with an inverted microscope (Olympus, Tokyo, Japan) equipped with an ocular grid.

2.6. Identification of spermatogonial and Sertoli cells
For immunocytochemical analysis, 1–2 × 10⁶ cells were expanded in each well of 8-well glass slides (Marienfeld, Lauda-Königshofen, Germany) overnight in a humidified incubator followed by washing with prewarmed medium. After drying for 15 min, cells were fixed and permeabilized in acetone for 2 min at –20 °C and kept at 4 °C for 30 min until slides were completely dried. Slides were then washed 3 times (3 × 3 min) with Tris-buffered saline, pH 7.4, containing 5% BSA (TBS/BSA). Blocking was performed with 5% horse serum in TBS for 10 min and goat anti-OCT-4 polyclonal antibody (Abcam, Cambridge, UK) or mouse antivimentin (Abcam) optimally diluted in TBS/BSA (1/200 and 2 µg/mL, respectively) was applied to slides for 60 min at room temperature. After being washed as above, for Oct-4 identification, fluorescein isothiocyanate-conjugated donkey polyclonal secondary antibody to goat IgG diluted in TBS/BSA in a ratio of 1:200 was used. For vimentin detection in Sertoli cells, Alexa Fluor 568-conjugated sheep antimouse IgG diluted in TBS/BSA in a ratio of 1:50 was added and incubation was further continued for 45 min at room temperature. After washing with TBS/BSA, the nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Calbiochem, Nottingham, UK) or mouse antivimentin (Abcam) optimally diluted in TBS/BSA for 10 min and then the slides were washed, mounted in PBS-glycerol 90%, and examined under a fluorescence microscope (Olympus). Primary antibodies were substituted by the same concentrations of the normal IgG1 in negative control slides and the results were shown to be always negative.

2.7. Statistical analysis
Results are presented as the mean ± SEM, and statistical analysis was performed by one-way analysis of variance and Duncan post hoc test. Differences were considered to be significant at P < 0.05.

3. Results
3.1. Immunocytochemical staining of sorted cells
Onesamplefrom each calf was used in immunocytochemical analysis. Among the markers used for identification of SSCs, anti-Oct-4 was used. As expected, the cells of colonies were positive for these markers as shown in Figures 1a and 1b. The nuclei were counterstained by DAPI, as shown in the mentioned figures. Additionally, vimentin, which is a molecular marker for Sertoli cells, was detected in the feeder monolayer cells, which are shown in Figure 1c.
3.2. Colony assay in spermatogonial cells cocultured with Sertoli cells

The result of this study indicates that after 16 days of coculture, diameters of the colonies in groups 1 and 2 were significantly greater than in the other groups (P < 0.05), as shown in Figure 2. However, as shown in Figure 3, the number of colonies in group 2 (P < 0.05) was greater than in the control and group 1.

3.3. Viability rate after thawing

After 16 days of coculture, viability rates of the cells in the 4 groups were above 91%, but after the freezing process the viability rates between groups were different and the frozen-thawed cells' viability rate in group 2 (P < 0.001) was higher in comparison with other groups, as shown in Figure 4.

4. Discussion

Among the stem cells in the male body, only SSCs have the ability of transmitting genetic information to future generations (2). Thus, in cattle herds, SSCs are exploitable for genetic improvement (4). Large numbers of type A spermatogonia have been isolated and purified from young prepubertal bulls (4,5).

In this research, we isolated the cells of the seminiferous tubules of 3-month-old male calf testes, containing 2 types of cells: Sertoli cells and type A spermatogonia. From morphological aspects, the spermatogonial and Sertoli cells in our cultures were similar to the cells that Bellve et al. (17) expanded.

The size, morphology, and vimentin immunocytochemical staining were regarded as Sertoli...
Our results on expression of vimentin in bovine Sertoli cells were similar to the previous studies by Zimmerman et al. (18) and Tajik et al. (19). In addition to size and morphology, identification of spermatogonial cells was carried out by Oct-4 tracing in the colony cells. Spermatogonial-derived colonies showed Oct-4 expression. This finding is in agreement with reports of Kubota et al. (15), Jeong et al. (20), and Shi et al. (21), who verified Oct-4 expression in the SSCs.

Our method was a coculture of Sertoli and spermatogonia cells in vitro and we investigated the effects of IGF-1 on the colonization and viability of spermatogonia. After day 8 of coculture, the diameters of the colonies in groups 1 and 2 were larger than those of the control group, suggesting the proliferative effect of IGF-1. There were no significant differences between groups 1 and 2. Furthermore, the diameters of the colonies in groups 1 and 2 were larger than those of the group 3 after the 12th day.
The number of the colonies in groups 1 and 2 was higher than of the control and group 3. In the group 3, the number of the colonies did not show significant difference in comparison with the control group at the end of coculture.

Adding 40 or 100 ng/mL IGF-1 to the culture medium containing GDNF increased the number of SSCs at the 16th day of incubation. GDNF maintains proliferation and self-renewal of SSCs for years in vitro (22). The suggested mechanism was described by Leroith et al. (23), where IGF-1 binds to its tyrosine kinase receptors and this triggers the phosphoinositide 3-OH kinase/Akt and mitogen-activated protein kinase pathways, which mediate cell proliferation or cell survival. Kubota et al. (15) also reported that the IGF system and GDNF signaling pathway may cooperate together in supporting self-renewal of SSCs.

In our study, the amount of 150 ng/mL IGF-1 had no positive effect on diameters and numbers of colonies. This is in agreement with Velazquez et al. (24), who stated that higher doses of IGF-1 induce downregulation of IGF-1 receptors.

The viability rate of frozen/thawed cells was lower than that of freshly isolated cells. This is mainly due to diminishing cell recovery following the freeze/thaw procedure. This finding was also reported by Avarbock et al. (25) and Brinster et al. (26). However, in group 2, the viability rate of frozen/thawed cells was higher than in other groups. Apparently, 100 ng/mL IGF-1 has an antiapoptotic effect on cells. Kennedy et al. (27), Khwaja et al. (28), and Kulik et al. (29) described IGF-1 receptors’ antiapoptotic signaling pathway, which plays a role by phosphatidylinositol-3-kinase, the serine/threonine kinase Akt, and the MAP kinase downstream proteins.

In summary, these findings demonstrate that 100 ng/mL IGF-1 has a positive effect on SSC numbers and diameters. Furthermore, 100 ng/mL IGF-1 can increase frozen-thawed cell viability rate.

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