Effect of testosterone on spermatogonial cell colony formation during In vitro co-culture

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Abstract:

BACKGROUND: The complex process of spermatogenesis is regulated by various factors. Studies on spermatogonial stem cells have provided a very important tool to improve herd genetic and different field. 0.2 to 0.3 percent of total cells of seminiferous tubules consist of spermatogonial stem cells. To investigate and biomanipulate these cells, first the proliferation and viability rate of cells should be increased in vitro. **OBJECTIVES:** In the present study, the in vitro effects of testosterone on spermatogonial cell colony formation were investigated. **METHODS:** Sertoli and spermatogonial cells were isolated from 3-5-month-old calves. The identity of the cells was confirmed through analysis of immuno-cytochemistry. Co-cultured Sertoli and spermatogonial cells were treated with testosterone in different doses of $0.2\,\mu mol\,L^{-1}$, $0.4\,\mu mol\,L^{-1}$ and $0.8\,\mu mol\,L^{-1}$, before colony assay. **RESULTS:** testosterone did not decrease the proliferation of spermatogonial stem cells. **CONCLUSIONS:** testosterone can be chosen for in vitro colonization of spermatogonial cells with other factors.

Introduction

Spermatogenesis is a complex developmental process that originates from Spermatogonial Stem Cell (SSC). This process consists of sequential, highly organized steps of cell proliferation and differentiation resulting in the generation of functional spermatozoa (McLean et al., 2002). Many growth factors, hormones and cell interactions of germ cells with Sertoli cells regulate these processes, and the failure of any of the processes can lead to male infertility (Kojima et al., 1997).

In nonprimate mammals, the A single (As) spermatogonia are considered to be the stem cells of spermatogenesis (Huckins, 1971; Oakberg, 1971; de Rooij, 1973). Hence, in bulls, Apr spermatogonia are also thought to have stem cell properties (Wrobel et al., 1995). In the adult mammalian testis, owing to the presence of multiple generations of germinal cells,

purification of spermatogonia is more difficult than it is before puberty. Bellve et al. (1977) obtained a 90% pure fraction of type A spermatogonia from immature mice. Izadyar et al. (2002) concluded that when testis from 5-month-old calves were used, approximately 1×10^6 type A spermatogonia per gram of testis of a purity of about 75% could be obtained routinely.

Sertoli cells have vital roles in the spermatogenic function of the testis for many reasons (Russell and Griswold, 1993). These somatic cells generate and maintain the cytoarchitecture of the germinal epithelium, produce nutrients that provide energy substrates to the germ cells and, in the primate, represent the only cellular component of the blood-testis barrier (Dym and Cavicchia, 1977). The receptor tyrosine kinase c-kit is expressed in germ cells and its ligand, Stem Cell Factor (SCF), is expressed in the Sertoli cells of the testis. The interaction between c-kit and SCF is essential for the

maintenance and/or mitosis of differentiating type-A spermatogonia (Yoshinaga et al., 1991). Co-cultures of gonocytes and Sertoli cells have been used to demonstrate the survival effects of added growth factors on germ cells (Enders, 1994).

Testosterone, directly produced by Leydig cells in response to Luteinising Hormone (LH), indirectly produced in response to FSH or supplied exogenously, has been shown to be a necessary prerequisite for the maintenance of an established spermatogenesis in the adult mammalian testis (Huang and Nieschlag, 1986; Lejeune et al., 1996). Testosterone and FSH have been reported to act synergistically in the adult rat by either stimulating germ-cell development and/or inhibiting germ-cell degeneration (Huang and Nieschlag, 1986). Testosterone to the Sertoli cell culture has been reported to increase the secretion of the mitogenic factor (Holmes et al., 1986). Testosterone and pituitary hormones may be quantitatively required for the maintenance of spermatogenesis in the adult rat (Cochran et al., 1981). To date, attempts to culture spermatogonia in vitro have been unsuccessful owing to the problems in purifying the testicular-cell population and the lack of knowledge about the in vitro regulation of proliferation and differentiation (Hasthorpe, 2003). The difficulty with the co-culture system has been reported to result from the complex interaction between germ cells and somatic cells and the lack of demonstrable cell division in vitro.

In studies on spermatogonial isolation and purification, the availability of markers that can conclusively establish the identity of the spermato-gonia is essential. Oct-4, can be used as a specific marker for spermatogonia in the testis (Kubota et al., 2004).

Consequently, the aim of the present study was to determine the effects of testosterone on spermato-gonial cell colony formation after in vitro co-culture with Sertoli cells.

Materials and Methods

Animals: Male calves (3 to 5 months old, n=5) were chosen from Aboreihan Dairy Farm of the University of Tehran. Calves were maintained with free access to food and water 24 and 12 hours before surgery. Testicular biopsies were obtained from 3 to 5-month-old calves undergoing the TESE procedure.

Germ cell and Sertoli cells collection: Obtained testis pieces were mechanically minced and floated in DMEM containing 1 mg/ml collagenase, 1 mg/ml trypsin, 1 mg/ml hyaluronidase type II and 5 mg/ml DNase I and then incubated at 37°C for 60 min (van Pelt et al., 1996). After washing three times in DMEM and excluding the interstitial cells, for secondary digestion step, seminiferous tubules were incubated in DMEM containing collagenase, hyaluronidase and DNase for 45 min. Finally, the obtained cellular suspension was centrifuged at 30 g for 2 min to achieve favorite cell population.

For Sertoli cell collection, 5 mg/ml Datura stramonium agglutinin (DSA)_lectin (Sigma) in Phosphate Buffered Saline was poured into the sterile flasks. Cells obtained from secondary enzymatic digestion were added to DSA-lectin coated flasks and incubated at 37 °C for 1 hr.

Co-culture: After Sertoli cells confluency, spermatogonial cells were co-cultured in seven groups for 13 days. For co - culture of these cells, DMEM with 10% FBS, 100 ng/ml Glial-Cell Line-Derived Neurotrophic Factor, 100 u/ml penicillin and 100 mg/ml streptomycin were used. Our experimental groups were: Control, group 1 (0.2 μmol L^{-1} Testosterone (Parsdaroo, Iran)), group 2 (0.4 μmol L^{-1} Testosterone), group 3 (0.8 μmol L^{-1} Testosterone). Culture medium plus mentioned doses of testosterone were refreshed every 3 days.

Cell viability: Cell viability was evaluated by means of the dye exclusion test (0.04% trypan blue solution)(Anjamrooz et al., 2006).

Cells identification: Colonies of SSCs were immunocytochemically stained with anti Oct-4 (Sigma) (conjugated with FITC). Oct-4 has been described as a marker for undifferentiated cells (Kubota et al., 2004).

Colony assay: The number and diameters of the spermatogonial cell derived colonies were measured on days 4, 7, 10 and 13. I Inverted microscope (Olympus, Tokyo, Japan) equipped with ocular grid was used for the measurements.

Statistical analysis: Results are expressed as mean±s.d. The statistical significance between mean values was determined by One Way Analysis of Variance (ANOVA) and Duncan post hoc test; p<0.05 was considered significant.

Results

Isolation and identification of spermatogonial Stem Cell: The cell population obtained from the seminiferous tubules of 3-5-month-old calves testis contained mostly two cell types with different immunocytochemical features. The first type proliferated and created a monolayer of cells (Figure 1), whereas the other type created a colony after proliferation, of which Oct-4 is a molecular marker for SCCs identification (Figure 2).

Testosterone effect on colony diameter and number: Diameters of the colonies in the testosterone-supplemented groups were not significantly different compared with the control group ($p \leq 0.05$) (Table 2); Also, the number showed no significant difference with control group ($p \leq 0.05$) (Table 1).

Comparison among experimental groups: The diameter of colonies was similar between the testosterone-supplemented groups ($p \leq 0.05$) (Table 2). There was no significant difference in appearance of colonies in all groups. All groups showed an increase in colony number colony number until day 10 of co-culture and after that a decrease of one, but this was not significant. Colony morphology was similar between all groups. There was no significant difference in viability rate between all groups and these rates were more than eighty five percent in all rates at the end of these at the end of co-culture (Table 3).

Discussion

Suitable populations of Sertoli cells and SSCs can be obtained from 3-5-month-old calves because the seminiferous epithelium of the calves testis contains two distinct cell types: type-A SSCs and Sertoli cells. The most appropriate age of calves from which to isolate type A SSCs appears to be 3-5 months. Most of the tubule cross-sections contained type A SSCs, and this testis proved to be the best source for isolation of this type of SSCs. Highly enriched populations of type A SSCs with final purity of up to 75% could be isolated routinely. Cell recovery was about 1×10^6 type A SSCs per gram of testis and the viability of the isolated SSCs was always >80% (Izadyar et al.,
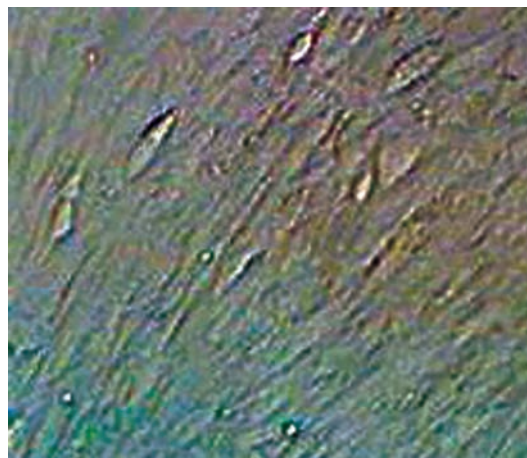

Figure 1. Sertoli cells that created a monolayer of cells.
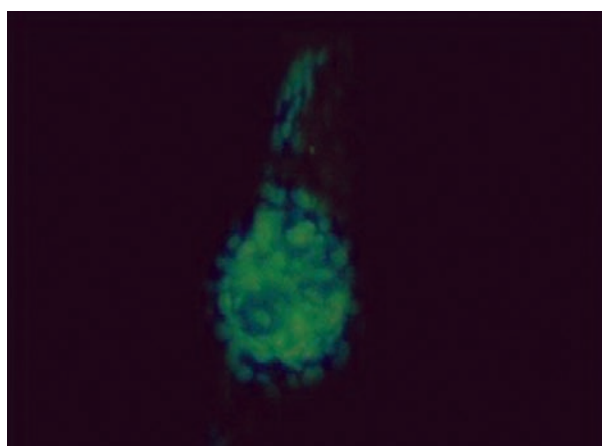

Figure 2. OctT-4 immunocytochemical staining of bovine Spermatogonial Stem Cell.

Table 1. Comparison of colony numbers between control and experimental groups. Values are the mean colony numbers at different times±s.d. There were no significant differences ($p < 0.05$).

Day 13	Day 10	Day 7	Day 4	Group
20.6±0.7	21.2±0.63	19.6±0.66	14±0.37	Control
Testosterone (μ mol L^{-1})				
35.8±1.05	45.8±1.81	37.8±1.64	20.2±0.98	0.2
23±0.71	35.8±1.05	29.4±1.08	18.8±0.61	0.4
25.2±0.91	31.6±0.71	31.2±0.74	18.8±0.9	0.8

2002). Our viability rate results are comparable to those reported for the isolation of type A SSCs from prepubertal mice (Bellve et al., 1977), rats (Morena et al., 1996), pigs (Dirami et al., 1999) and bovine (Izadyar et al., 2002).

About Sertoli cells, as immature ones proliferate, the final number of Sertoli cells is determined before

Table 2. Comparison of colony diameters between control and experimental groups. Values are the mean colony numbers at different times±s.d.

Day 13	Day 10	Day 7	Day 4	Group
163.52±83.28	195.08±184.60	202.88±152.25	135.72±87.04	Control
Testosterone (µ mol L-1)				
85.98±28.18	60.22±23.74	82.86±74.18	58.48±53.98	0.2
96.86±40.82	101.78±91.97	79.54±53.86	70.78±51.9	0.4
145.46±74.42	119.72±126.66	102.3±54.22	84.1±62.46	0.8

adulthood. There appears to be fundamental differences between species as to when Sertoli cells proliferate, but in bull, there is a short gap (weeks) between the neonatal and peripubertal periods (De Franca et al., 2000); nevertheless, recent study has shown that there was proliferation in Sertoli cells after maturation (Tajik et al., 2010).

Therefore, the optimum time for the recovery of Sertoli cells and primitive type-A SSCs is 5 months after birth (Izadyar et al., 2002). Hence 3-5-month-old calves were used as animal sample in our study.

Koruji et al., 2007 concluded that some co-cultures (mean co-culture with Sertoli cells and growth factors) had considerable effect on colonization of SSCs and colony number increased with time. Sertoli cells support SSCs survival and proliferation. Therefore, co-culture of SSCs with Sertoli cells has a supportive effect on proliferation of SSCs. Thus co-culture of SSCs and Sertoli cells were used in this project.

In this research, the isolated cells from the seminiferous tubules of 3-5-month-old calves had two types of cells. This finding is in agreement with that reported by koruji et al., 2007, who demonstrated the immuonocytochemical features of SSCs in mouse.

For confirmation of the presence of SSCs, Oct-4 was traced in the colony cells. Undifferentiated type A SSCs express Oct-4 (Kubota et al., 2004). We therefore assume that the colonies may have been largely derived from the SSCs.

Our approach was to co-culture Sertoli cells and SSCs in vitro and to determine the effects of testosterone on the colonization of SSCs. Then the best factor was chosen for proliferation increscent of co-cultured SSCs.

In the testosterone-supplemented groups, the number of colonies did not show any significant difference in comparison with the control group at the end of co-culture. Like the control group, all the spermatogonial cells that entered colony formation may have chosen the proliferative pathway. This pathway could have been induced by Sertolicell-secreted growth factor. Also, testosterone seemed to have stimulated colony formation, echoing previous findings which indicate that testosterone produced by Leydig cells stimulates Sertoli-cell differentiation and that Sertoli cells in turn, secrete a potent mitogenic factor that initiates spermatogenesis (Holmes et al., 1986; Singh and Handelsman, 1996).

In conclusion our in vitro co-culture system for SSC colony formation proved that testosterone can be used as a hormone with other factors in the in vitro co-culture for SSCs proliferation and colonization with no negative effects.

Acknowledgments

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تاثیر تستسترون بر کلونی زایی سلول‌های بنیادی اسپرماتوگونی در هم کشتی در محیط آزمایشگاه

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چکیده

زمینه مطالعه: فرایند اسپرماتوژنز بوسیله‌ی فاکتورهای مختلفی تنظیم می‌شود. انجام مطالعات بر روی سلول‌های بنیادی اسپرماتوگونی ابزار بسیار مهمی را برای بهبود ژنتیک گله و همچنین برای بهبود در زمینه‌های مختلف دامی فراهم می‌کند. ۰/۲ تا ۰/۳ ٪ از کل سلول‌های لوله‌های سمینیفر شامل سلول‌های بنیادی اسپرماتوگونی می‌باشند. برای بررسی و دستکاری زیستی این سلول‌ها، در ابتدا باید تکثیر و میزان زنده مانی این سلول‌ها در محیط آزمایشگاه افزایش داده شوند. **هدف:** در مطالعه حاضر، اثرات هورمون تستسترون بر روی کلونی‌زایی سلول‌های اسپرماتوگونی گوساله در محیط آزمایشگاه مورد بررسی قرار گرفته است. **روش کار:** سلول‌های سرتولی و بنیادی اسپرماتوگونی از گوساله‌های ۵–۳ ماهه جداسازی شده اند. شناسایی سلول‌های بنیادی اسپرماتوگونی از طریق آنالیز ایمونوسیتوشیمی تایید شده است. هم کشتی سلول‌های سرتولی و بنیادی اسپرماتوگونی بوسیله‌ی دوزهای مختلف $0/2 \mu mol/L$، $0/4 \mu mol/L$ و $0/8 \mu mol/L$ هورمون تستسترون قبل از آنالیز کلونی‌ها درمان شده‌اند. **نتایج:** این مطالعه نشان می‌دهد که هورمون تستسترون موجب کاهش تکثیر سلول‌های بنیادی اسپرماتوگونی نمی‌شود. **نتیجه‌گیری نهایی:** هورمون تستسترون می‌تواند به منظور کلونی‌زایی سلول‌های اسپرماتوگونی در محیط کشت استفاده شده شود.

واژه‌های کلیدی: هم‌کشتی، گوساله، تستسترون.

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