Isolation of bovine spermatogonial cells and co-culture with prepubertal sertoli cells in the presence of colony stimulating factor-1

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Abstract:

BACKGROUND: Spermatogonial stem cells (SSCs) are infrequent self-renewing cells among the type A spermatogonia within the seminiferous tubules and are the basis of spermatogenesis in mammalian testis. An adequate number of SSCs is a primary requirement for the study of their behavior, regulation, and further biomanipulation. **OBJECTIVES:** In this paper, we studied the development of the primary co-cultures of type A spermatogonia and prepubertal bovine sertoli cells in the presence of Colony Stimulating Factor 1 (CSF1), a potential contributor in the SSC niche. **METHODS:** The effect of different concentrations of CSF1 (0, 10, 50 and 100 ng/mL) on the colonization activity of spermatogonial cells was assessed 4, 7 and 11 days after the beginning of the culture by counting the total number of colonies and measuring their area in each group of the present experiment. Immunofluorescent staining against OCT4 and vimentin led to the confirmation of the nature of both the SSCs and sertoli cells. **RESULTS:** Results showed that the total number of colonies from day 4 to 11 increased significantly in all groups, independent of CSF1 concentration. In addition, the total number and total area of colonies were higher (not significant) in 10 and 50 ng/mL CSF1 treatments than the control and 100 ng/mL CSF1 groups in all the three evaluations during the experiment. However, this difference was only significant ($p<0.05$) between the total area of colonies in the control and 10 ng/mL CSF1 groups at day 4 of co-culture. **CONCLUSIONS:** It was concluded that CSF1 can be a suitable growth factor for improving SSCs colonization in vitro, particularly during the first days of culture where accompanying sertoli cells still have not proliferated sufficiently to support the propagating spermatogonial cells.

Introduction

Mammalian spermatogenesis produces millions of spermatozoa each hour for the reproductive lifetime of the animal. This highly efficient process requires maintenance of a small population of spermatogonial stem cells (SSCs). SSCs are capable of both self-renewal and giving rise to differentiating daughter spermatogonia. This dual capacity of spermatogonial stem cells ensures the long-lasting ability of the testes to produce spermatozoa (Aponte et al., 2005; Oatley et al., 2006; Dann et al., 2008). The regulation of the SSC self-renewal process and the mechanisms that maintain a balance between dif-

ferentiation and self-renewal are largely undefined (Aponte, et al., 2008; Dann et al., 2008; Kostereva and Hofmann, 2008). To study the characteristics of SSCs, the populations of pure SSCs must be large enough to be isolated, but SSCs are present in extremely low numbers. One way to reach this goal is to propagate these cells in vitro. Various attempts have been made to achieve such cells in the vitro system (Aponte et al., 2005; Oatley et al., 2006; Aponte et al., 2008).

In the mammalian, the testis Spermatogonial stem cells (SSCs; as spermatogonia) and their direct descendants (Apr and Aal spermatogonia) reside within specialized microenvironments in the seminiferous tubules called 'niches'. In the stem cell niche, self-renewal will be preferred and outside of the niche, differentiation is the preference. The main components of the niche include the sertoli cell, growth factors that this nursing cell produces, and the basement membrane (Kostereva and Hofmann, 2008).It is known that the SSCs niches are preferentially located in those areas of the seminiferous tubules that border on the interstitial tissue. Fewer of these cells are present in the tubule areas directly bordering on another tubule (De Rooij, 2009). Sertoli cells, the only somatic cells within the seminiferous tubules, are able to produce growth factors that stimulate the self-renewal of SSCs (GDNF and FGF2) (Kubotaet al., 2004; Hofmann, 2008; Oatley and Brinster, 2008)and differentiation of them (activin A, BMP4, and SCF) (De Rooij et al., 1999; Nagano et al., 2003).Since no difference has been observed between the characteristics of sertoli cells in the SSCs niches and the other parts of the seminiferous tubules, other factors coming from outside the tubules (i.e. interstitial tissue and blood vessels) should determine, directly or indirectly via sertoli cells, whether either the self-renewal of SSCs or differentiation will be preferred in a particular area (De Rooij, 2009; Caires et al., 2010).

One of the factors that link the niche to the interstitial tissue is FSH which has the highest levels near the blood vessels within the interstitial tissue. Therefore, the parts of seminfreous tubules reside on it. The FSH stimulates GDNF production by the sertoli cells and consequently increases the SSCs self-renewal (Tadokoro et al., 2002).

Another factor which could be considered as a link between the niche and the interstitial tissue is colony stimulating factor1 (CSF1), a stimulator of stem cell proliferation (De Rooij, 2009). Leydig cells in the interstitial tissues of the testis produce CSF1 (Ryan et al., 2001). It has been recently observed (Kokkinaki et al., 2009) that SSCs strongly express the colony stimulating the factor 1 receptor (CSF1R). Therefore, this growth factor might be an important factor in the establishment of the niche as it might favor the balance between self-renewal and differentiation in the direction of self-renewal.

In the present study, we evaluated the effects of different concentrations of CSF1 on the colonization activity of bovine spermatogonial cells in a short term in vitro co-culture with testicular sertoli cells.

Materials and Methods

Animals: In this study, 5 Holstein bull calves, aged between 3 to 5 months, were used as donors. At the time of biopsy, their scrotal circumference (SC) approximately ranged from 13 to 15 cm. Animals were handled and treated according to the guidelines of the Animal Ethics Committee at the University of Tehran.

Obtaining Testicular Biopsy: 15 minutes before surgery, animals were sedated with xylazine, the scrotal skin was completely shaved and prepared aseptically. Biopsy was performed under local anesthesia with lidocaine injected into spermatic chord as well as under scrotal skin. Spermatic chord was covered with tampons and temporarily legated by an Alice forceps. Scrotum, both layers of tunica vaginalis and tunica albugina were incised with a surgical blade in the equatorial region of the testis, and the protruded testicular tissue was cut from the incision site (dimensions: 1x1x1 Cm) with a sterile scissor. Tunica albugina and tunica vaginalis were sutured separately with USP 0 polyglactin 910, and then the scrotal skin was closed with USP 1 Nylon. Post-surgery follow up with anti-inflammatory drugs and systemic antibiotics was considered for 5 days. All animals recovered from the biopsy procedure without any complications.

Cell Isolation: The testicular biopsy sample was placed into a 15 mL tube containing Dulbecco's Minimal Essential Medium (DMEM, GIBCO), 10% Fetal Bovine Serum (FBS, Sigma) and 100 iu/ mL-

100 µg/ mL Penicillin-Streptomycinon (GIBCO) and then transferred on ice to the laboratory within 2 hours. Individual tubular cells were isolated in a two-step enzymatic isolation procedure, as previously described by Izadyar et al., (2002)and Van Pelt et al., (1996) with minor modifications. In brief, the obtained testis tissue was washed three times in DMEM with antibiotics with 3 minutes intervals and was minced into small pieces as much as possible by means of a sterile scissor. Then, it was incubated in DMEM containing antibiotics including 1 mg/mL collagenase type IV, 1 mg/mL hyaluronidase type II and 1 mg/mL trypsin (all from Sigma) at 37 °C in a shaker incubator operated at 80 cycles per minute for approximately 60 minutes. In this stage most of the connective and interstitial tissues were digested and after three to four times of washing with DMEM, mostly seminiferous tubules remained. Seminiferous tubules/cords were again incubated at 37°C in DMEM containing antibiotics including 1 mg/mL collagenase type IV and 1 mg/mL hyaluronidase type II and excluding trypsin. This step was continued until fragments of seminiferous tubules were deconstructed and their comprising cells (i.e. sertoli and spermatogonial cells) were separated. Individual cells were separated from the remaining tubule fragments by centrifugation at30 g for 2 min. After filtration through 77 and 55 mm nylon filters, the cells were pelleted. The pellet (consisting of spermatogonia and sertoli cells) was re-suspended in the DMEM solution containing an antibiotic and 10 % FBS. As the co-culture of spermatogonial cells with testis somatic cells (sertoli cells) was considered in this study, differential plating, part of the isolation procedure described by Izadyar et al., (2002), was not performed.

Viability Assay: Viability of the primary isolated cells was evaluated by trypan blue staining and the total number of the cells present in suspension was determined using a hemocytometer under a light microscope.

Short-term Culture: Cells were seeded in DMEM with antibiotics and 10% FBS, and cultured in a 24-well cell culture-treated plate (TPP®, Switzerland) at a concentration of 300,000 cells/well (88.2±3% viability) and incubated at 37°C in a humidified atmosphere with 5% CO_2. Macrophage-Colony Stimulating Factor (M-CSF or CSF1) human recombinant (Sigma, St. Louis, MO, USA) was added to the culturing medium in different concentrations (0, 10, 50 and 100 ng/mL). The cultures were refreshed every 3 days for 2 weeks.

Evaluation of SSCs Colonization: Spermatogonial colonies in each well were counted using a differential interference contrast microscope (Olympus, IX71® inverted microscope) at days 4, 7 and 11. Also, the area of these colonies was measured using the same microscope equipped with eyepiece graticule.

Immunofluorescent Staining against OCT-4 and vimentin: For immunostaining, cells (from the cell suspension acquired from step-2 of enzymatic digestion) were plated in 4-well chamber slides with each chamber containing 50,000 cells in DMEM with antibiotics and 10% FBS and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cultures were stopped and fixed after 5 days. Primary and secondary antibodies used for detecting OCT4 in SSCs were rabbit anti-OCT4 (polyclonal antibody ab18976, Abcam, Cambridge, UK) and FITC-conjugated goat anti rabbit IgG. Primary and secondary antibodies used for observing vimentin in sertoli cells were mouse anti-vimentin (monoclonal antibody ab8069, Abcam, Cambridge, UK) and fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG. Cell staining was performed according to the procedure previously described by Qasemi-Panahi et al., (2011).

Statistical Analysis: The data from 4 independent experiments were analyzed using one-way ANOVA and Duncan's multiple range tests. All statistical analyses were performed using the SPSS statistical software, version 16 (SPSS Inc., Chicago, USA) with a P-value of less than 0.05 considered as significant.

Results

Short-term Coculture: During the first week of culture, sertoli cells in all groups would keep developing so that 4 days after the incubation they partially covered the well and around day 6 or 7 formed a complete monolayer on top of which germ cells could proliferate. Spermatogonial colonies started to appear around day 4 and increased their number through time in all the experimental groups (Figure 1). The increase in the total number of colonies in each group was significant when compar-

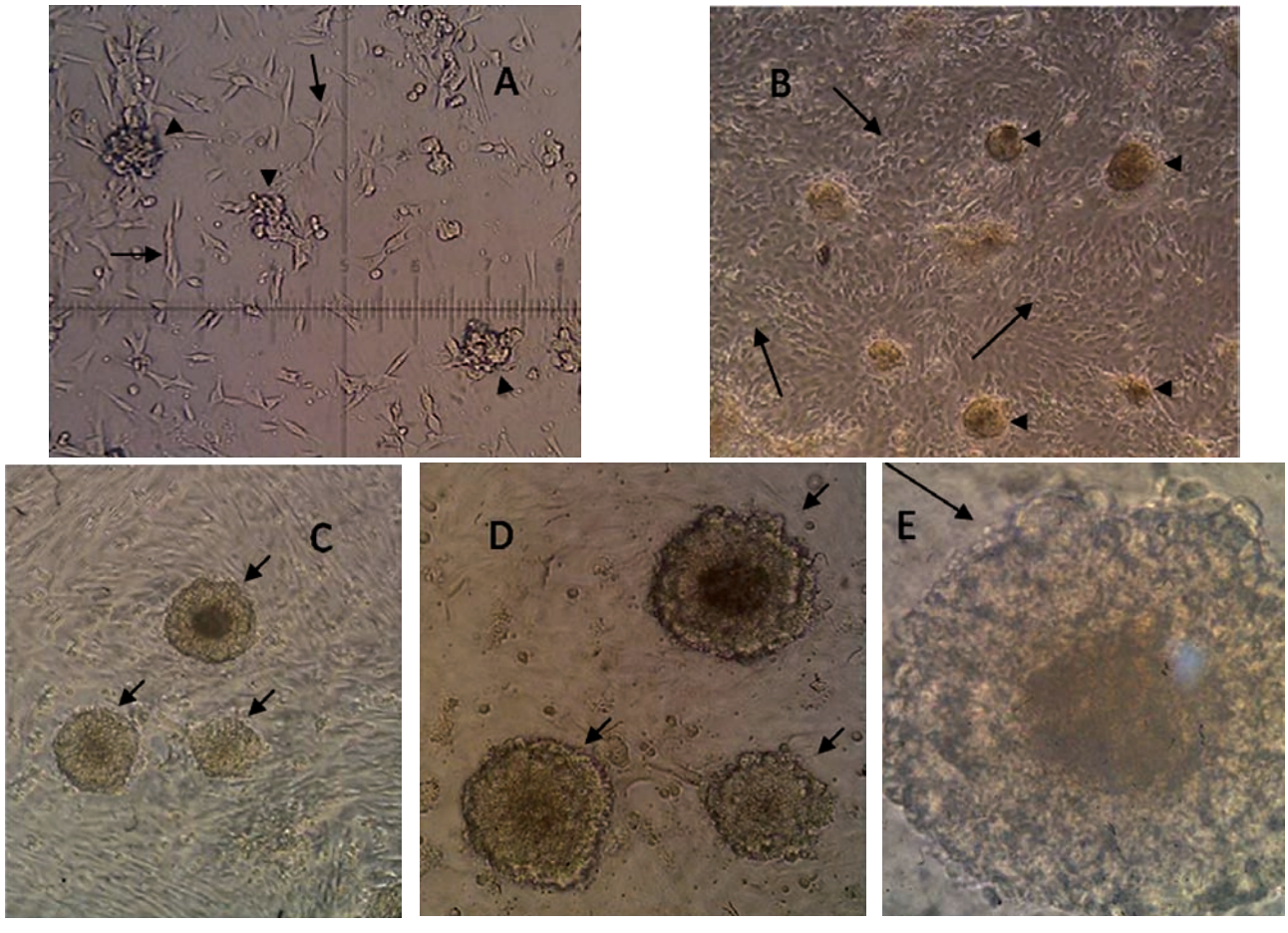

Figure 1. (A)Developing sertoli cells (arrows) and small spermatogonial colonies (arrow heads) at day 4 of culture (magnification ×100). (B) Day 11 of culture, Sertoli cells has formed a complete layer (arrows) covering the well and Spermatogonial colonies (arrow heads) have grown on top of it (mag. ×40). (C) Spermatogonial colonies (arrows) at day 14 of culture (×40). (D,E) same colonies (arrows) at higher magnifications (×100 and ×400).

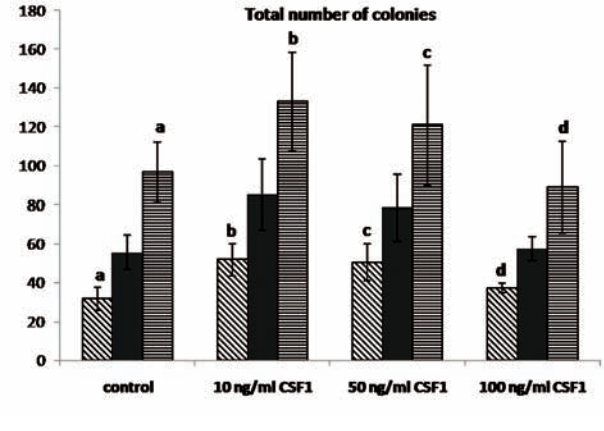

Figure 2. Total number of colonies (Mean±SE) in each experimental group in different evaluation days. Similar letters represent significant difference ($p<0.05$). day 4 day 7 day 11

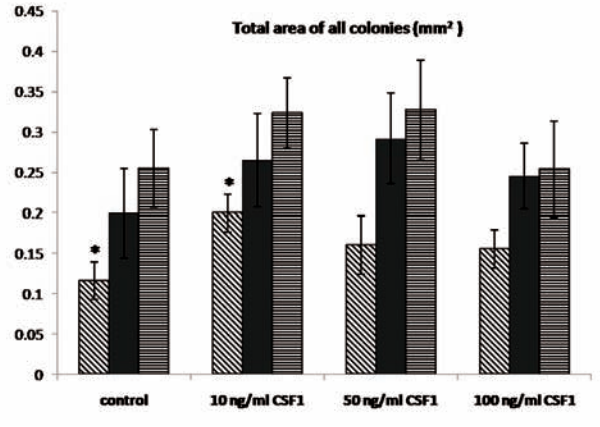

Figure 3. Total area of colonies (Mean±SE) in each experimental group in different evaluation days. Asterisks represent significant difference ($p<0.05$). day 4 day 7 day 11

ing day 4 with day 11 of the culture. However, the change was not significant from day 4 to day 7 and from day 7 to day 11 in none of the groups (Figure 2). It was also observed that the total number of colonies

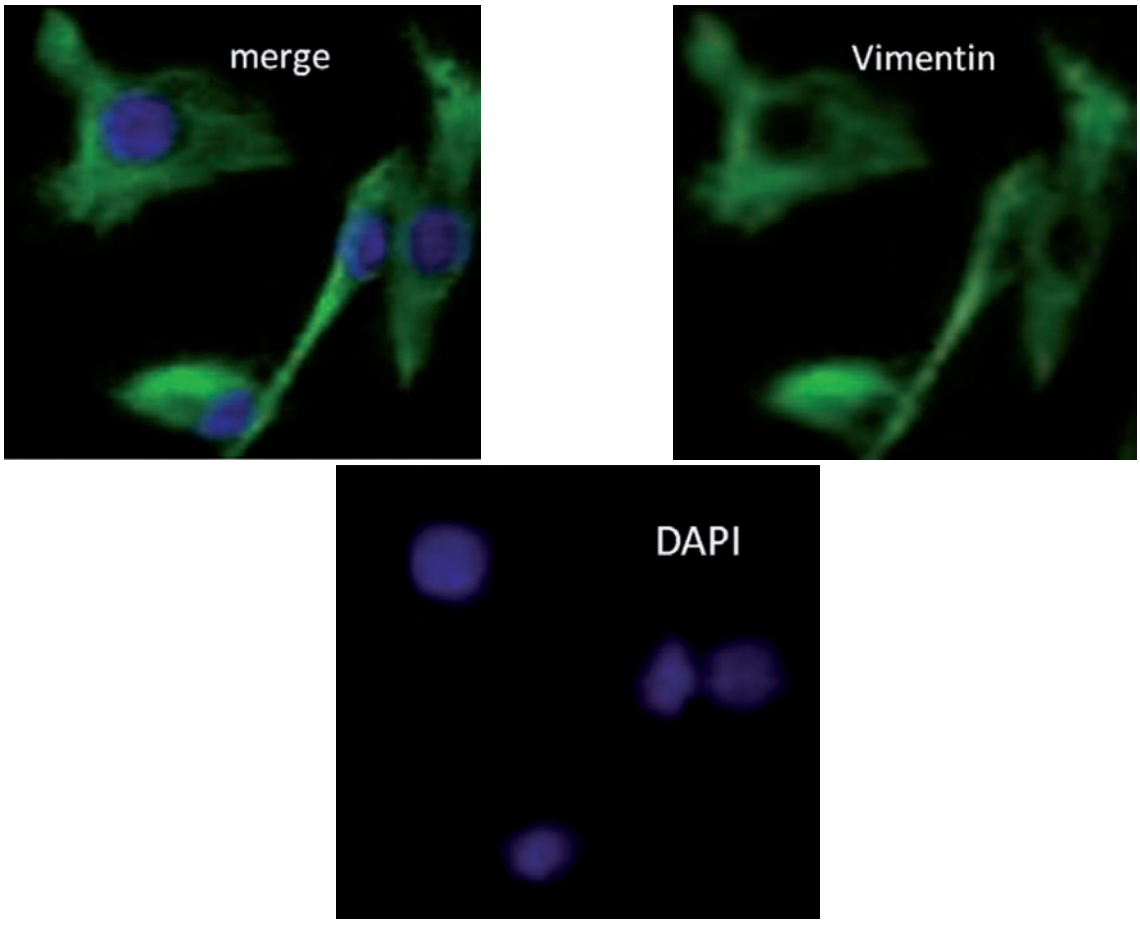

Figure 4. Immunofluorescent staining of Sertoli cells for vimentin (green) at day 5 of culture. DAPI staining is used to demonstrate the nuclei (blue) of sertoli cells.

in cultures with 10ng/mL and 50 ng/mL CSF1 was higher than the cultures with 100 ng/mL CSF1 and the control groups. Such a difference was not significant when the comparison was made at days 4, 7 and 11 of the experiment (Figure 2).

The total area of colonies (mm2) in each well (i.e., each experimental group) increased through the incubation period but these changes were not significant in any of the groups ($p>0.05$) (Figure 3). Similar to the total number of colonies, the total area of colonies was also higher in cultures with 10 ng/mL and 50 ng/mL CSF1 than 100 ng/mL CSF1 and the control groups at days 4, 7 and 11 but this difference was only significant between CSF1 10 ng/mL and the control at day 4 of the culture (Figure 3).

Changes in the mean area of colonies throughout the experiment were not significant in none of the treatments and control groups. Similarly, no significant difference was observed in the mean area of colonies between the groups.

Immunofluorescent Staining: At day 5 of culture, vimentin was detected in sertoli cells which developed a feeder monolayer (Figure 4), and OCT4 was demonstrated in the spermatogonial colonies (Figure 5).

Discussion

In this study, it was observed that during the co-culture of bovine spermatogonial cells with sertoli cells isolated from the testis of prepubertal Holstein calf, sertoli cells proliferated and formed a feeder layer on top of which, SSCs colonized. This is an expected result according to previous studies in this context (Izadyar et al., 2003; Oatley et al., 2004; Aponte et al., 2006; Aponte et al., 2008). In all the treatment groups considered in this study (0, 10, 50 and 100 ng/mL CSF1), the total number and total area of the colonies per culture well increased during the culture. This increase was significant on the total

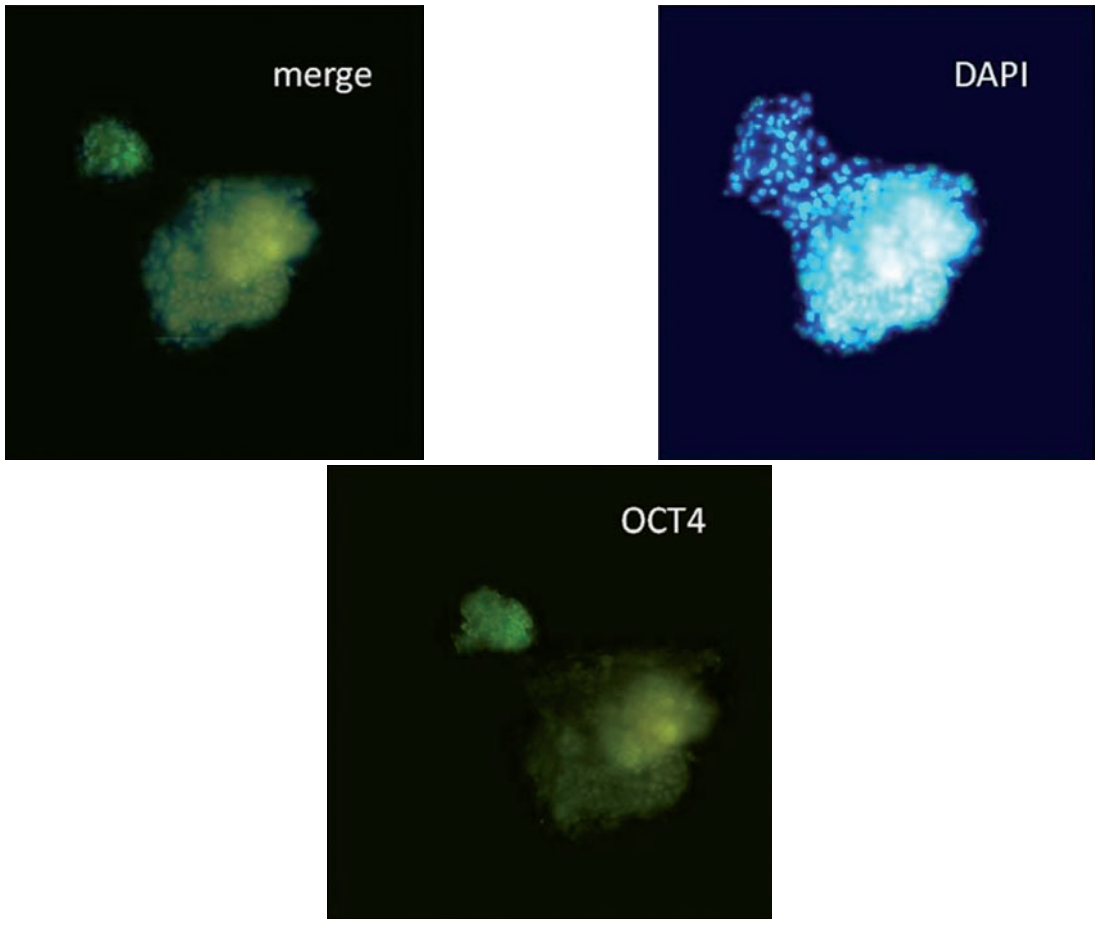

Figure 5. Immunofluorescent staining of SSCs for OCT4 (green) at day 5 of culture. DAPI staining is used to demonstrate the nuclei (blue) of SSCs.

number of colonies per well in all groups when day 4 and day 11 of the experiment ($p<0.05$) are compared together.

In each evaluation (day 4, 7 and 11), the total number of colonies was higher in 10 and 50 ng/mL CSF1 treatments than that of the control and 100 ng/mL CSF1groups, but this difference was not statistically significant. Similarly, the total area of colonies was higher in 10 and 50 ng/mL CSF1 treatments than that of the control and 100 ng/mL CSF1 groups. Statistical analysis revealed that the difference in the total area of colonies is only significant between the 10 ng/mL CSF1 treatment and the control. This means that adding CSF1 to the culturing medium of SSCs improves the colonization of these cells. The reason why a significant difference was not observed between the control and other treatments in these evaluations, except for day 4, might be associated to the status of sertoli cells. At day 4, the number of sertolli cells was not enough to form a complete feeder layer, so their physical and chemical support for developing spermatogonial colonies was lower compared to day 7 and 11. This means that at day 7 and 11, the effect of adding CSF1 to the culturing medium might be covered, at least, partially by the effects of sertoli cells. Further experiments are necessary to investigate this possibility.

To this date, no study has been carried out to investigate the effects of CSF1 on culturing bovine SSCs.. However, Kokkinaki et al., (2009) showed that CSF1 stimulates the proliferation of GFRα1 positive spermatogonia (As and Apr spermatogonia) isolated from mouse testis during in vitro culture. Oately et al., (2004) observed that CSF1R (CSF1 receptor) is highly expressed in Thy1 positive spermatogonial cells (including SSCs) on mouse SSCs. They also showed that CSF1 does not increase the overall proliferation of SSCs in vitro, although it increases the self-renewal mitotic divisions of these cells.

In this study, no significant change was observed in the mean size of colonies either in each treatment group during the culture period or between the groups in any of the evaluation days. This observation was not in agreement with results obtained from previous studies (Aponte et al., 2006; Aponte et al., 2008). As the size of certain colonies would grow in all groups, inconsistence between the results of this study and previous studies regarding the mean size or area of colony may be associated to the method used for calculating these parameters; where, in this study, both newly formed small colonies and larger growing colonies would be used simultaneously for calculating the mean colony size.

In conclusion, the results of the present investigation suggests that CSF1 can be a suitable growth factor for improving the SSCs colonization during vitro culture, especially during the first days of culture when accompanying sertoli cells would not still sufficiently proliferate to support the propagating spermatogonial cells.

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جداسازی سلول‌های اسپرماتوگونی از بیضه گوساله نابالغ و همکشتی با سلول‌های سرتولی در محیط کشت حاوی CSF1

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چکیده

زمینه مطالعه: سلول‌های بنیادی اسپرماتوگونی (SSCs) گروه کوچکی از سلول‌های اسپرماتوگونی نوع A هستند که می‌توانند سلول‌هایی کاملاً مشابه خود ایجاد نموده (خودسازی) و همچنین تمایز یابند و در پایان روند اسپرماتوژنز، اسپرم را تولید نمایند. فرآیند خودسازی SSCs به طور کامل شناخته نشده است. برای فراهم شدن امکان مطالعه خصوصیات و عملکرد این سلول‌ها که منجر به خودسازی و تمایز آنها می‌گردد، دسترسی به تعداد کافی از آنها ضروری است. **هدف:** در این مطالعه اثر دوزهای مختلف CSF1 روی کلونیزاسیون SSCs در همکشتی با سلول‌های سرتولی بررسی شد. CSF1 از عواملی است که پیشنهاد شده ممکن است در SSC niche (جایگاه قرارگیری SSCs در لوله‌های سمینیفروس) که در آن خودسازی به تمایز برتری دارد، نقش داشته باشد. **روش کار:** تعداد و مساحت کلونی‌های اسپرماتوگونی ایجاد شده در حضور دوزهای متفاوت CSF1 (۰، ۱۰، ۵۰ و ۱۰۰ ng/mL) طی روزهای ۴، ۷ و ۱۱ کشت ارزیابی گردید. توسط رنگ‌آمیزی ایمیونوسیتوفلورسنت علیه مارکرهای OCT4 و vimentin ماهیت SSCs و سلول‌های سرتولی تأیید شد. **نتایج:** تعداد کلونی‌ها از روز ۴ تا روز ۱۱ در تمامی گروه‌ها مستقل از حضور و یا دوز CSF1 به‌شکل معنی‌داری افزایش نشان داد. مشاهده گردید که تعداد کل کلونی‌ها و مجموع مساحت آنها در گروه‌های ۱۰ SCF1 و ۵۰ng/mL نسبت به گروه‌های ۱۰۰ CSF و ۰ (کنترل) بالاتر است اما این تفاوت تنها در مورد مقایسه مجموع مساحت کلونی‌ها بین گروه ۱۰ ng/mL CSF1 و کنترل و تنها در روز ۴ معنی‌دار بود ($p<0/05$). **نتیجه‌گیری نهایی:** نتایج به دست آمده از این مطالعه پیشنهاد می‌کند CSF1 می‌تواند فاکتور رشد مناسبی برای بهبود کلونیزاسیون SSCs در محیط کشت باشد، به خصوص در روزهای ابتدایی که سلول‌های سرتولی هنوز به اندازه کافی برای حمایت از کلونی‌های اسپرماتوگونی، رشد و تکثیر نیافته‌اند.

واژه‌های کلیدی: سلول‌های بنیادی اسپرماتوگونی، همکشتی، CSF1، اسپرماتوگونی گاو

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