# Bovine serum albumin improves *in vitro* development of caprine preantral follicles

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## Abstract

This work investigated the effects of different protein supplements (fetal calf serum (FCS) and bovine serum albumin (BSA)) on the *in vitro* development of caprine preantral follicles. Preantral follicles ($\geq 150$ μm) were isolated from ovarian cortex fragments and individually cultivated in α-MEM medium in an incubator at 37°C for 24 days with 5% atmospheric $CO_2$, and supplemented with either BSA at 1.25 or 3.0 mg/ml or FCS at 5 or 10%. An evaluation of follicular development was conducted based on survival rate, antrum formation, increase in follicular diameter, oocyte viability and obtainment of fully-grown oocytes. It was observed that from the 12th cultivation day, the percentage of surviving follicles under treatment with BSA at 3.0 mg/ml was greater than that of the other treatments (P < 0.05). As for follicular growth, although on the 6th day of cultivation FCS-treated follicles had a greater mean diameter (P < 0.05) when compared to those treated with BSA, on the last day of cultivation the mean diameter and antrum formation of follicles treated with BSA at 3.0 mg/ml were greater than those of follicles under other treatments (P < 0.05). With oocyte growth, the percentage of oocytes cultivated with BSA (3.0 mg/ml) that were destined for *in vitro* maturation (IVM; $\geq 110$ μm diameter) was higher than that of other treatments. Moreover, under this treatment, 86% of oocytes presented a germinal vesicle and 14% restarted meiosis, out of which 3% were mature (metaphase II). In conclusion, supplementing cultivation medium with BSA at 3.0 mg/ml not only improves follicular development but also provides meiotically-competent oocytes after *in vitro* cultivation of caprine preantral isolated follicles.

**Keywords**: albumin, goat, maturation, oocytes, preantral follicles, serum.

## Introduction

*In vitro* cultivation of preantral follicles has been described as an important tool to maximize *in vitro* embryo production. Significant advances in *in vitro* cultivation of preantral follicles have already been described for some animal species and the most satisfactory results were obtained with mice, where the *in vitro* maturation of oocytes from cultivated preantral follicles led to the birth of healthy offspring (O'Brien *et al.*, 2003). However, in domestic species like goats, such results are still difficult to obtain. In our study group, only one oocyte restarted meiosis and reached the stage of metaphase II after preantral follicle cultivation (Silva *et al.*, 2010), which reflects the need to improve the cultivation system. Thus, many growth factors, hormones, and energy and protein supplements have been widely tested in the cultivation of precocious preantral follicles (Bruno *et al.*, 2008; Lima-Verde *et al.*, 2009; Silva *et al.*, 2010).

Among the protein supplements commonly used with the goal of improving *in vitro* cultivation efficiency (Basso *et al.*, 2007), bovine serum albumin (BSA) and fetal calf serum (FCS) should be highlighted. However, the variation in the composition of BSA and FCS used to prepare semi-defined (Itoh *et al.*, 2002) and undefined medium (Telfer *et al.*, 2000), respectively, has given conflicting results (Rose *et al.*, 1992). In murines, improved oocyte meiosis restart rates were obtained after *in vitro* cultivation of preantral follicles with 10% FCS, when compared with BSA (Abedelahi *et al.*, 2008). Conversely, in felines, the replacement of FCS (10%) with BSA (3 mg/ml) provided a 30% increase in normal preantral follicles after *in vitro* cultivation (Jewgenow, 1998). Until now, however, the effect of these supplements (BSA or FCS) on *in vitro* cultivation of caprine preantral isolated follicles has not been assessed.

Thus, this study investigated the effects of different concentrations of BSA or FCS on the *in vitro* development of caprine preantral isolated follicles.

## Materials and Methods

### Chemicals

Unless otherwise mentioned, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co., St Louis, MO, USA.

### Source of ovaries

Ovaries (n = 32) were collected at a local slaughterhouse from sixteen adult (1-3 years old), non-pregnant mixed-breed goats, making a total of four

replicates (4 goats/replicate). The animals were cyclic (showing ovaries containing corpus luteum) and were in good body condition (destined to meat production). Immediately postmortem, the ovaries were washed in 70% alcohol followed by two rinses in Minimum Essential Medium (MEM) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin. The ovaries were transported within 1 hour to the laboratory in MEM at 4°C (Chaves *et al*., 2008).

*Isolation and in vitro culture of preantral follicles*

In the laboratory, the ovaries were stripped of surrounding fat and fibrous tissue. After removal of the medulla, the ovaries were transferred to MEM + HEPES supplemented with antibiotics (100 mg/l penicillin- G, 100 mg/L streptomycin sulfate) and were cut into small pieces (1-2 mm in diameter) using a surgical blade under sterile conditions. Intact preantral follicles (≥150 µm in diameter) were mechanically isolated from the cortex of the ovaries using 26G needles, with a clump of stromal tissue attached. After isolation, their diameters were measured by using an ocular micrometer inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) at 100X magnification. Only follicles with a visible oocyte, surrounded by two or three granulosa cells layers, and with an intact basement membrane and no antral cavity, were selected for culture.

The culture medium was composed of α-MEM (pH 7.2 - 7.4) supplemented with 1% ITS (insulin 1.0 mg/mL, transferrin 0.55 mg/ml, and selenium 0.5 µg/ml), 2 mM glutamine, 2 mM hypoxanthine, 50 µg/ml of ascorbic acid and 100 ng/ml recombinant follicle stimulating hormone (rFSH) (hereafter called α-MEM⁺). Selected preantral follicles were randomly distributed in α-MEM⁺ alone or α-MEM⁺ associated with two different concentrations of FCS (5 or 10%) or with two different concentrations of BSA (1.25 or 3.0 mg/ml). From each treatment approximately 40 preantral follicles were utilized. Follicles were individually transferred into a 100 µl drop of culture medium under 7 ml of pre-equilibrated mineral oil and cultured at 39°C in a humidified atmosphere of 5% $CO_2$ for a maximum of 24 days. Every other day, 60 µl of the culture medium drop were replaced with fresh medium.

*Follicular growth and morphological evaluation*

The follicular growth and the presence or absence of an antral cavity was evaluated every 6 days during the culture. An antral cavity was defined as a visible, translucent area within the granulosa cell mass. Follicles were scored for their morphology, and those showing morphological signs of degeneration (i.e., darkness of oocytes and surrounding cumulus cells) or those with misshapen oocytes, were discarded. The survival rates of preantral follicles and antrum formation rate were calculated as follows: 1 -

Survival rate (%) = (No. of degenerated follicles/viable follicle cultured) x 100. 2 - Antrum formation rate (%) = (No. of follicles with antrum after day 24 of culture/initial no. of follicles) x 100. Follicle growth was scored as an increase in diameter. The diameter was measured only in healthy follicles in the *x* and *y* dimensions (90°) by using an ocular micrometer (100 X magnifications) inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan).

*In vitro maturation and recovery rate of fully-grown oocytes from in vitro cultured follicles*

For oocyte recovery at the end of the culture period (day 24), all of the healthy follicles were carefully opened mechanically using 26G needles under a stereomicroscope. Only oocytes≥( 110 µm) with a homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for *in vitro* maturation (IVM). The selected oocytes were washed three times in maturation medium composed of TCM 199 supplemented with 10% FCS, 100 µg/ml of Luteinizing Hormone (LH), 5 µg/ml of rFSH, 1 µg/ml of 17β-estradiol, 1 ng/ml of Epidermal Growth Factor (EGF) and 2 mM of pyruvate. After washing, the oocytes were transferred to 50 µl drops of maturation medium under mineral oil and then incubated for 26 h at 39ºC with 5% atmospheric $CO_2$. For a more precise evaluation of the oocytes' viability and chromatin configuration, oocytes were incubated in 100 µl droplets of MEM containing 4 µm calcein-AM and 2 µm ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) and 10 µm Hoescht 33342 at 37°C for 15 min. Afterwards, the oocytes were washed three times in TCM 199Hepes and examined under a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan). The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were collected at 488 and Hoescht was collected at 568 nm. Oocytes were considered alive if the cytoplasm was stained positively with calcein-AM (green) and if the chromatin was not labeled with ethidium homodimer-1 (red).

The recovery rate of the oocytes was calculated as follows: the total number of oocytes selected (≥110 µm) for maturation divided by the total number of cultured preantral follicles.

*Statistical analyses*

Data from follicular survival, antrum formation, and maturation after *in vitro* culture were compared using the Chi-square test and the results were expressed as percentages. The follicular diameter and growth rate after culture showed a normal distribution and homoscedasticity after running an ANOVA, followed by a Student's *t* test for comparison of means (SAS, 1999). Results were expressed as mean ± SEM and differences were considered to be significant when P < 0.05.

## Results

*Effect of BSA and FCS on survival and antrum formation of goat preantral follicles*

Follicular survival was evaluated according to morphology before and after *in vitro* culture. Starting on the 6th day of cultivation, it was observed that treatments with BSA (1.25 and 3.0 mg/ml) resulted in a significantly greater follicular survival rate when compared with treatments using FCS (Fig. 1). On day 12, treatment with BSA at 3.0 mg/ml resulted in a significantly higher follicular survival rate when compared to all other treatments (Fig. 1). When comparing cultivation days, BSA treatments exhibited a significant reduction in follicular survival rate starting on day 12 (Fig. 1), while the FCS group exhibited a similar reduction as early as day 6.

The results of the different effects of protein supplementation on antrum formation are shown in Fig. 2. Antrum formation rate was significantly greater in the group treated with BSA at 3.0 mg/ml.
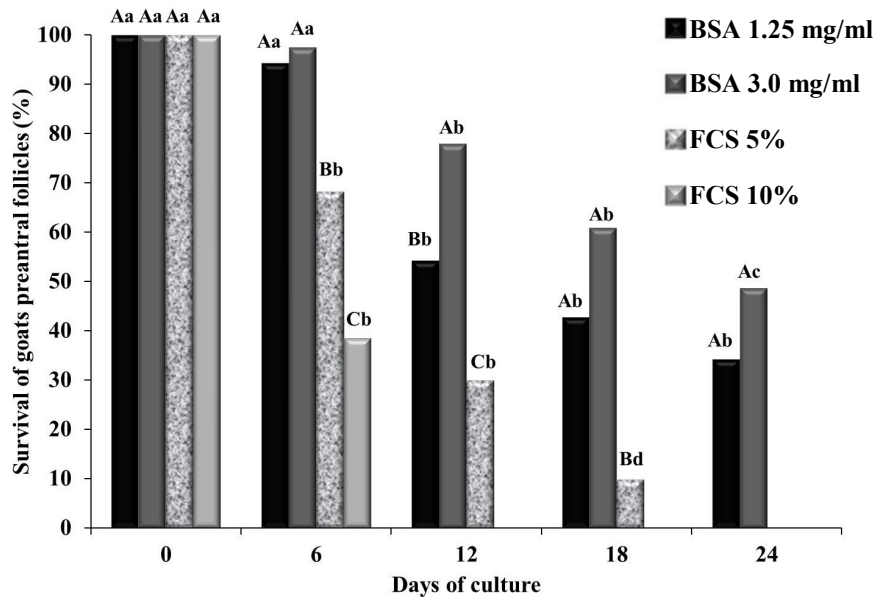


Figure 1. Effect of BSA and FCS at different concentrations on survivability of caprine isolated preantral follicles cultivated for 24 days. [A,B,C]Differ among treatments (P < 0.05). [a,b,c]Differ among days within each treatment (P < 0.05).
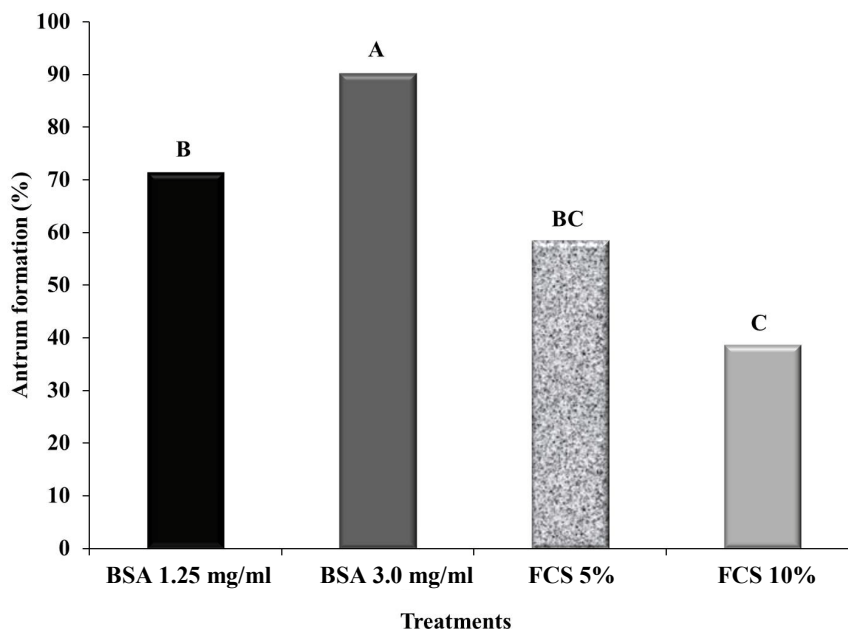


Figure 2. Effect of BSA and FCS on antrum formation. [A,B,C]Differ among treatments (P < 0.05).

*Effect of BSA and FCS on the growth of preantral follicles*

As for the increase in follicular diameter during cultivation, a significant progress in follicular growth from days 0 to 18 was observed in the BSA-treated (3.0 mg/ml) group.

On the 6th day, follicles cultivated with FCS had a significantly greater mean diameter than those cultivated with BSA. However, starting on day 12, follicles treated with BSA at 1.25 mg/ml had a significantly higher mean diameter, when compared with follicles treated with 5% FCS. At the end of the cultivation period, follicles treated with BSA at 3.0 mg/ml had significantly larger diameters when compared to all other treatments (Fig. 3).
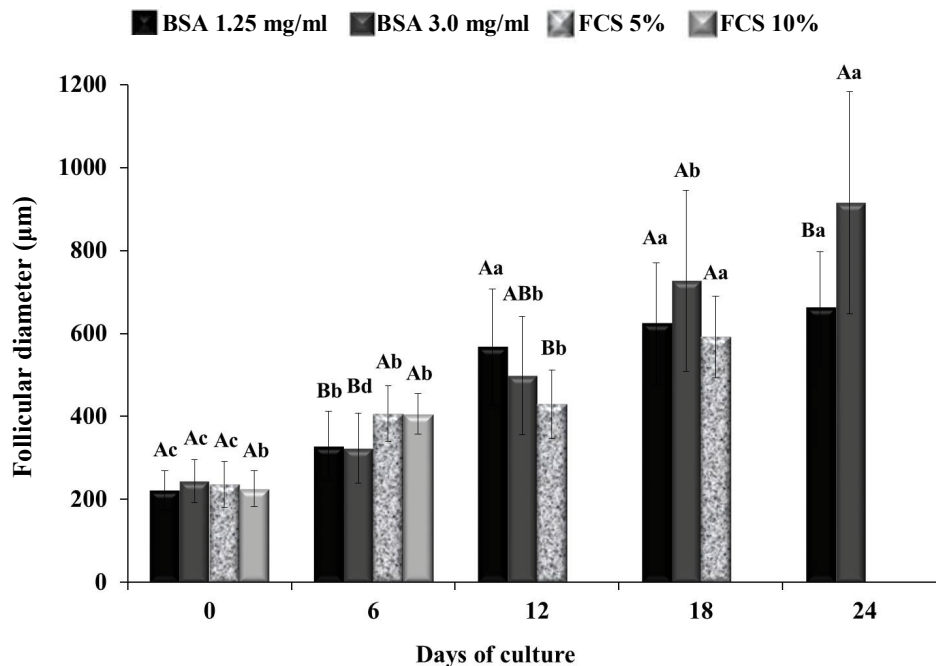


Figure 3. Effect of BSA and FCS at different concentrations on *in vitro* growth of caprine isolated preantral follicles. [A,B]Differ among treatments ($P < 0.05$). [a,b,c]Differ among days within each treatment ($P < 0.05$).

*Effect of different concentrations of BSA and FCS on the meiotic competence of oocytes from goat preantral follicles*

The use of BSA at a concentration of 3.0 mg/ml compared with 1.25 mg/ml provided a significant increase in the number of oocytes destined for IVM (≥110 μm diameter). Alternatively, all of the oocytes obtained from treatments with FCS were degenerated, making it impossible to perform IVM.

The oocyte viability analysis showed that oocytes obtained from treatment with BSA at 3.0 mg/ml, which were destined for IVM, were viable. After a chromatin configuration analysis, 14% of the viable oocytes underwent meiotic resumption (Fig. 4B), out of which 3% were mature (metaphase II; Fig. 4C). Under treatment with BSA at 1.25 mg/ml, all of the oocytes were viable and presented an intact germinal vesicle (GV; Fig. 4A).

Table 1. Effect of different concentrations of BSA and FCS on meiotic competence of oocytes from caprine preantral follicles cultivated *in vitro*.

| Treatments | Preantral follicles n | Oocytes for the IVM n (%) | VG (%) | Meiotic resumption (%) | MII (%) |
|---|---|---|---|---|---|
| BSA 1.25 mg/ml | 35 | 15 (42.86)[B] | 15 (100) | 0 | 0 |
| BSA 3.0 mg/ml | 41 | 28 (68.9)[A] | 24 (86) | 3 (11) | 1 (3) |
| FCS 5% | 41 | 0[C] | 0 | 0 | 0 |
| FCS 10% | 44 | 0[C] | 0 | 0 | 0 |

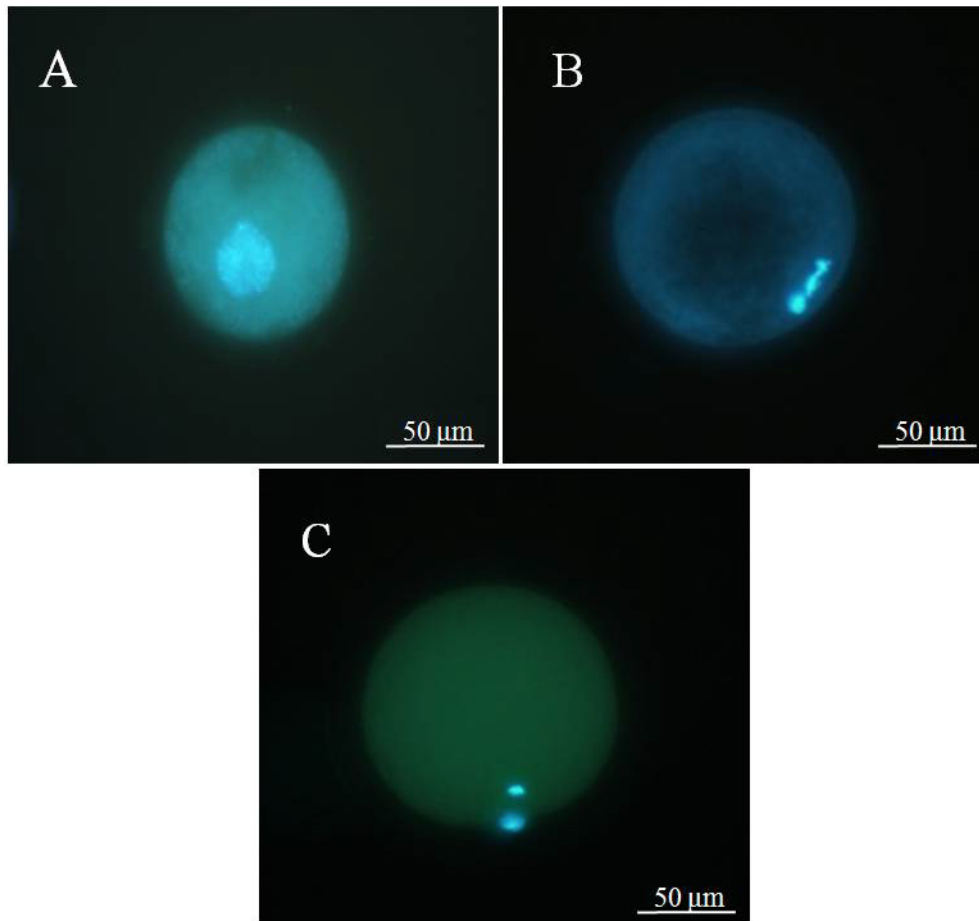[A,B,C]Differ among treatments ($P < 0.05$).

Figure 4. Representation of meiotic stages of caprine oocytes after *in vitro* maturation. (A) viable oocyte at GV stage; (B) oocyte presenting germinal vesicle breakdown (GVBD) and (C) oocyte at stage MII.

### Discussion

This study evaluated for the first time the effect of different protein supplements (BSA or FCS), at different concentrations, on the *in vitro* development of caprine preantral isolated follicles. Several studies have shown that follicular development is positively (Newton *et al.*, 1999; Telfer *et al*., 2000) or negatively (Hulshof *et al.*, 1995; Thomas *et al.*, 2001) influenced by protein supplements. In our study, it was observed that medium supplementation with BSA in a concentration-dependent manner, when compared with FCS, provided improved results for all parameters evaluated.

Our results showed that after 24 days of cultivation, medium supplementation with BSA maintained follicular survivability, in contrast to what was observed after the addition of FCS. This may be the result of albumin's surfactant property, which prevents cell adherence to plastic or glass surfaces (Bavister *et al*., 1981; Pinyopummintr and Bavister, 1991). Some studies have shown that the maintenance of follicular structure during *in vitro* cultivation favors follicular survivability (Jin *et al*., 2009). Itoh *et al.* (2002) cultivated bovine preantral follicles with BSA at 1 mg/ml

and observed that the normal tridimensional structure of follicles was maintained after 13 days of cultivation. McCaferry *et al*. (2000) obtained similar results after a 6-day cultivation of bovine preantral follicles in medium containing 3.0 mg/ml of BSA. In felines, the replacement of FCS (10%) with BSA at 3 mg/ml resulted in a 30% increase in normal preantral follicles cultivated *in vitro* (Jewgenow, 1998). In addition, albumin may act as a sequestrator of reactive oxygen species, thereby reducing the risks of oxidative stress (Natsuyama *et al*., 1993).

With BSA treatment at 3.0 mg/ml, an elevated antrum formation rate was observed. Such results are similar to those observed by Wu and Tian (2007), who obtained an 89% rate of antrum formation after *in vitro* cultivation of swine preantral follicles in a BSA-supplemented medium (3.0 mg/ml). Additionally, Gutierrez *et al*. (2000) obtained, for the first time, antrum formation in bovine preantral follicles that had been cultivated for 28 days in BSA-supplemented medium. In cultivation systems, antrum formation may be impaired when follicles adhere to the substrate, which makes them lose their tridimensional shape (Mitchell *et al*., 2002). We believe that the improved

antrum formation is related not only to the property of BSA in maintaining cell architecture, but also to its ability to enhance the absorption of liposoluble substances (Kane and Headon, 1980) such as FSH, which is added to our basic medium and has been considered essential for antrum formation (Mitchell *et al*., 2002; Silva *et al*., 2010).

Concerning follicular diameter, the association of BSA and FSH positively influenced follicular growth, as was observed for antrum formation. On the other hand, our results showed that FCS promoted an accelerated growth until the 6th day of cultivation. Such precocious follicular growth may be related to an overstimulation of follicular cells, due to the presence of several growth factors and hormones at undefined concentrations. Ultimately, overstimulation was not beneficial, as all of the FCS-treated follicles were degenerated by day 18.

For meiotic competence, we found that the percentage of oocytes that reached a diameter of ≥110 µm was greater with BSA treatment at 3.0 mg/ml. In addition, under this same treatment, meiosis restart was observed and 3% of the oocytes reached the stage of metaphase II. These results were better than those previously achieved by our group, where, after cultivating caprine preantral follicles for 24 days, we only obtained oocytes in metaphase I (Silva *et al*., 2010). Our findings show that adequate protein supplementation positively influenced our cultivation system and favored the acquisition of meiotically-competent oocytes from isolated preantral follicles. In spite of this advance, achieving meiosis restart in caprine preantral follicles grown *in vitro* still represents a great challenge.

In conclusion, cultivation medium supplemented with BSA at 3.0 mg/ml not only improves follicular development, but also results in meiotically-competent oocytes after *in vitro* cultivation of caprine isolated preantral follicles.

## Acknowledgments

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