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Comparison of Medium Supplementation on Proliferation and Hormone Production of Bovine Granulosa Cells in a Defined Culture System

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Authors' contributions

This work was carried out in collaboration between all authors. All authors were involved in data and information gathering, analysis, organization, manuscript writing and critical reviewing. All authors read and approved the final manuscript

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ABSTRACT

Aims: The objective of this study was to develop a serum-free bovine granulosa cell (GC) culture system in which estradiol (E2) production could be maintained in a defined media, with polyvinyl alcohol (PVA), insulin and insulin-like growth factor-1 (IGF-1) and without FSH and compared the effects of two different macromolecules (PVA vs, BSA) on steroids output and cell proliferation during in vitro culture.
Study Design: Bovine granulosa cell culture.
Place and Duration of Study: Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil, 2011.
Methodology: Bovine ovaries were collected from adult cows at a local abattoir and were transported in warm saline solution. Small follicles were dissected according to their vascularization and follicular fluid conditions. GC were cultured in α-MEM containing IGF-I,

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insulin, androstenedione, 0.1% PVA or 0.1% BSA, without FSH. After 48, 96 and 144 h of culture were analyzed GC morphology and secretion of E2 and P4. The relationship among cell shape, cell proliferation, and GC time course of steroidogenesis in vitro was further explored.

Results: In the presence of PVA and BSA, E2 production reached its highest production at 144 h. There was a significant increase on P4 production on the medium containing BSA at 48 and 96 h. The changes in E2 production/P4 production ratio in PVA-cultures indicate that there was a larger increase in E2 production by these cells at 48 and 96 hrs than by cells from medium with BSA. The results of the cellular proliferation demonstrated significant tritiated thymidine incorporation in both PVA and BSA cells cultured at 144 hours.

Conclusion: These results demonstrate the development of a relevant culture system for bovine GC under defined conditions with PVA. This chemically defined culture system will enable us to study the factors that regulate the physiological control of GC proliferation, differentiation and steroidogenic characteristics.

Keywords: Steroidogenesis; granulosa cells; bovine; polyvinyl alcohol; reproduction.

1. INTRODUCTION

Traditionally, studies examining the control of ovarian follicular development and oocyte maturation have focused mainly on the endocrine regulation of the hypothalamic-pituitary-ovarian axis. However, it has long been recognized that intra-ovarian factors are also important in regulating follicular development in paracrine or a autocrine manner [1].

The relationship between the circulating hormones and ovarian cells during reproductive cycles in vivo is complicated by a diversity of local factors. Thus, in vitro models such as cell culture provide an ideal approach by which the interactions of specific factors can be more precisely evaluated. Previous bovine granulosa cell (GC) culture systems have been characterized by a decline in E2 production with time in culture [2,3], lost of aromatase activity [4] while P4 synthesis increases, suggesting the onset of luteinization [2]. This might be due to interference of the serum: the most commonly used medium supplementation.

Thus, it is desirable to use a completely defined culture medium, which does not contain serum. If the medium adds known serum factors, the use of serum may also introduce nonspecific contaminants into the culture, which has been shown to affect cellular processes [2,3,5,6]. Using a serum-free defined medium with bovine serum albumin (BSA) Campbell [7] and Gutierrez et al. [8], developed a GC culture system that expresses and maintains aromatase activity, remaining responsive to physiological concentration of FSH and growth factors [8]. However, BSA may also present contaminating factors as hormones and growth factors [9] that could work as confounding factors when local ovarian factors are evaluated in the granulosa cell culture system. In this scenario, inert molecules such as polyvinyl alcohol (PVA) could potentially replace BSA in a well supplemented medium. Polyvinyl alcohol is a synthetic polymer, with a molecular weight of 30,000-70,000, used in cell culture media for the stabilization of osmotic pressure and as surfactants and heavy metal chelating agent [10]. This polymer is able to replace the oncotic characteristic of proteins in vitro maturation (IVM) of oocyte without impairing any physiologic development [11,12,13,14,15]. In addition, supplementation of PVA in the media of follicle section incubations promoted steroid production and enhanced steroidogenic enzyme expression [16]. Piccinato et al. [17] show a

strictly defined culture system supplemented with PVA based on a CG culture that is valuable for studying intraovarian factors without the interfering effects of serum or contaminated serum replacers. In this study, the direct effect of norepinephrine as paracrine/juxtacrine regulators of steroidogenesis evaluated in a model where E2 was maintained high and P4 synthesis was clearly modulated by this local factor.

Although an improvement has been described in supplementing PVA in in vitro models that evaluated hormone production [16,17], no previous study has formally compared the effects of PVA versus the widely used BSA as serum replacer. The aim of the present study was to examine cell proliferation and E2 and progesterone production, the key markers of healthy and developing follicles, in the proposed culture system compared to the previously described model that uses BSA as supplemental factor. The results described in the present study highlight the advantage of using a chemically defined culture system and reinforce this system as an ideal model to study local factors that regulate GC proliferation and steroidogenesis.

2. MATERIALS AND METHODS

2.1 Materials

α-MEM (alpha-Minimum Essential Medium) culture medium, sodium bicarbonate, Hepes, antibiotics (penicillin/streptomycin), sodium selenite, transferrin, insulin, human recombinant IGF-1, non-essential amino acids. These materials were obtained from Gibco BRL (Grand Island NY, USA). PVA, bovine serum albumin (BSA), Trypan blue and androstenedione (A4) were purchased from Sigma Chemical Co, St. Louis, MO, USA; tritiated thymidine and androstenedione were purchased from Armshan, USA.

2.2 Granulosa Cell Isolation

Bovine ovaries (Bos Taurus) at various stages of the estrus cycle were obtained from a slaughterhouse, transported to the laboratory in PBS, and maintained at 37ºC. Follicles were selected according to their size (3-5 mm), shape (spherical), fluid appearance (clear), and vascularization (well vascularized). Thus, most follicles were expected to be non-atretic and to have emerged after a new follicular wave, although prior to the selection of the single dominant follicle. The GC isolation was performed according to the methodology described by Gutierrez et al. [8], with slight modifications. The ovaries were washed in 70% ethanol for 30 seconds and rinsed in alpha-MEM. The selected follicles were transferred into a Petri dish with modified medium, were split in halves, and gently washed 3 times. Cells were washed twice using low-speed centrifugation (500 x g) for 10 minutes followed by resuspension in alpha-MEM and cell concentration and viability were assessed by Trypan blue exclusion method. Briefly, a sample of cell suspension was diluted (1:10) in 0.2% solution of Trypan blue (Trypanblau, Merck, Germany) and analyzed on Neubauer chamber at 200 times magnification, on a Zeiss Axioscope microscope (Carl Zeiss Inc., USA).

2.3 Cell Culture and Steroid Measurement

Cells were cultured in 24 well plates in the modified (α-MEM supplemented with 10 nM sodium bicarbonate, 20 nM Hepes, 5 µg/mL transferrin, 1.4 ng/mL sodium selenite, 11 nM non-essential amino acids, 10 ng/mL human recombinant IGF-1, 0.1 µg/mL insulin, 1000 U/mL penicillin and 1000 µg/mL streptomycin, without FSH) supplemented with BSA

(control) or 0,1% PVA. The E2 precursor, androstenedione, cannot be synthesized by granulosa cells and also added to the medium (10^{-7} M) in physiological concentration. Each well of the plates had 1mL of culture medium and approximately 1,000,000 of viable granulosa cells. This cell density was established in preliminary experiments to optimize cell proliferation as well as the E2 synthesis [32]. Cells were cultivated with 5% CO_2 at 37 °C for 144 hours (6 days) with change 70% of the medium every 48 hours. The collected medium was kept at -20°C for posterior analysis of E2 and P4 by radioimmunoassay [18], modified in our laboratory [19]. Based on the values of E2 and P4, the ratio between E2 and P4 was calculated (E2 values multiplied by 1000 and divided by P4 values at each time point). A total of 6 different sets of culture experiments were designed to determine the effects of steroid hormone production in bovine CG. Analyses were carried out in duplicate, without extraction, and each of the two steroids was analyzed in a single assay to avoid inter-assay variability. The intra-assays coefficients of variation were less than 15% for estradiol and 10% for progesterone. The analytical sensitivities of the estradiol and progesterone were 15pg/mL and 0.1ng/mL, respectively. All experiments were done in accordance to the institutional Research Ethics and Animal Care Committee at School of Medicine of Ribeirão Preto (São Paulo University).

2.4 Tritiated Thymidine Incorporation

Three separated cell cultures, with 6 replicates, were used according to the methodology described by Hirshfield [20], to compare cell growth between GC cultivated in PVA and BSA supplemented medium. In each well of a 96 well-plate 200 µL of culture medium were added plus 200,000 viable cells and 50 µL of tritiated thymidine solution (50 µL thymidine/well). This solution was added to the plates 12 h before collecting stopping the culture at all evaluated time points (at 0, 48, 96 and 144 h).

2.5 Immunocytochemistry

The enzyme 3β-hydroxysteroid dehydrogenase (HSD3β1) was determined by immunocytochemistry at T0 h, T48 h and T96 h. The antibodies to the enzyme were kindly provided by Professor Yves Tremblay and Professor Van Luu Thé (CHUL – Quebec – Canada), and were produced in rabbits against human. At the appropriate time the medium was replaced with phosphate buffered saline (PBS) simultaneously and the coverslips were washed twice in PBS. Fixation and permeabilization were performed using 20% paraformaldehyde dissolved in methanol. In the next step the cells were washed twice with PBS, followed by washing in PBS with 0.1M glycine and subsequent washing with PBS plus 1% BSA. The next procedure was incubation with the first antibody (1:100, PBS + 1% BSA+0.01% saponin) for 1 hour. The preparation was then washed again and incubated for 30 minutes with the second antibody (Alexa Flúor 488 F(ab')), associated with goat anti-rabbit IgG made at 1:300 concentration in PBS at room temperature. After incubation, cells were washed in PBS and mounted on slides for preview.

2.6 Statistical Analysis

All data analyses were performed using general linear model (Proc GLM) of SAS software version 6.11 (Statistical Analysis System Institute Inc., Cary, NC, USA). Final concentration of hormones synthesized by GC (E2 and P4) was calculated adding up the concentrations measured at each time point. In addition, mathematical calculations were performed to account for the remaining 30% of medium left in the wells. Thus, the statistical model

included experimental culture, time, groups (BSA vs. PVA cultures), and interactions. The values were expressed as mean±standard deviation (SD) and P=0.5 was considered statistically significant.

3. RESULTS AND DISCUSSION

Fig. 1 shows the mean E2 production in GC cultures from bovine ovaries in culture medium with BSA or PVA during the 144 hours of incubation. The maximum average production of E2 in PVA cultures occurred at 96 hours of culture (1.36±0.65 ng/mL) and was high up to 144 h. Production of E2 in BSA supplemented cells only increased at 144 h (P=0.5). The media supplemented with PVA reached 1ng/mL of E2 production earlier than BSA supplemented GCs at 48 h. The production of E2 in PVA cultures at 48 h (1.01±0.5 ng/mL) and 96 h (1.86±0.3 ng/mL) were significantly different from BSA (0.26±0.04 ng/mL (48 h) and 0.65±0.1 ng/mL (96 h)) cultures (P=0.5).

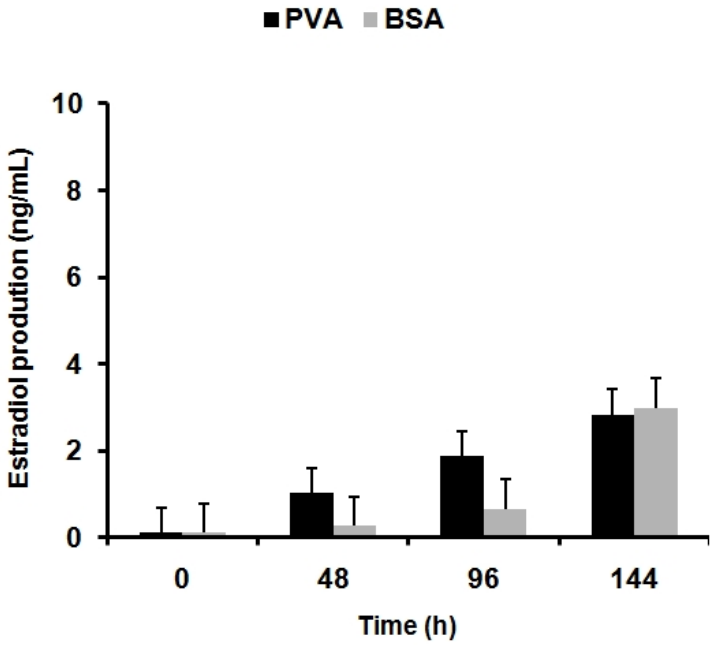

Fig. 1. Estradiol production by bovine granulosa cells from small follicle cultured for up to 144 h, in the presence of PVA and BSA, IGF-1, and insulin and absence of FSH. Values are least-square means±SD of 6 independent cultures.
** P=0.5*

Progesterone production in GC cultures is presented in Fig. 2. Both treatment groups presented a significant peak of P4 production at 96 h (49.21±10.2 ng/mL (PVA) and 93.91±28.22 ng/mL (BSA) (P=0.5). When PVA and BSA supplemented cultures were compared, there was a significant (P=0.5) greater P4 production by BSA supplemented GC at 48 h (70.15±22.39 ng/mL and 39.57±7.08 ng/mL (PVA)), at 96 h (93.91±28.22 ng/mL and 49.21±10.2 ng/mL (PVA)) and 144 h (73.83±20.9 ng/mL and 46±8 ng/mL (PVA)).

The mean ratio between E2 and P4 (E2:P4) reflects the production of E2 and P4 by GC under the tested supplemental medium. As shown in Fig. 3, the E2:P4 ratio was elevated for

PVA cultures starting at 48 h (25.52±1.34) and 96 h (37.39±1.5), whereas BSA presented significant higher ratio only later in culture at 144 h (61.3±2.4).

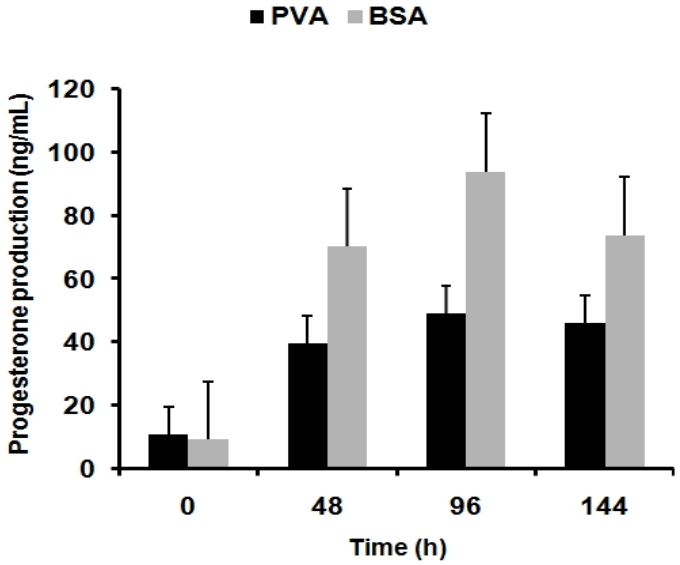

Fig. 2. Progesterone production by bovine granulosa cells from small follicle cultured for up to 144 h, in the presence of PVA and BSA, IGF-1, and insulin and absence of FSH. Values are least-square means±SD of 6 independent cultures.

** P=0.5*

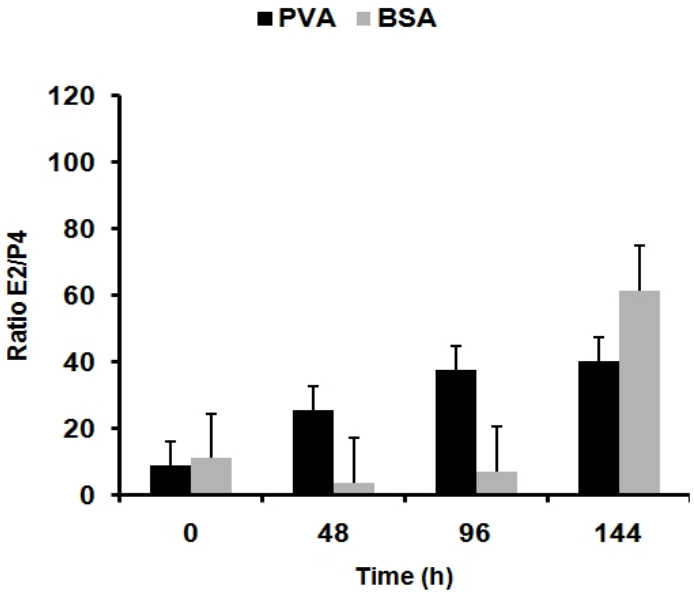

Fig. 3. Ratio of estradiol/progesterone production by bovine granulosa cells from small follicle cultured for up to 144 h, in the presence of PVA and BSA, IGF-1, and insulin and absence of FSH. Values are least-square means±SD of 6 independent cultures

** P=0.5*

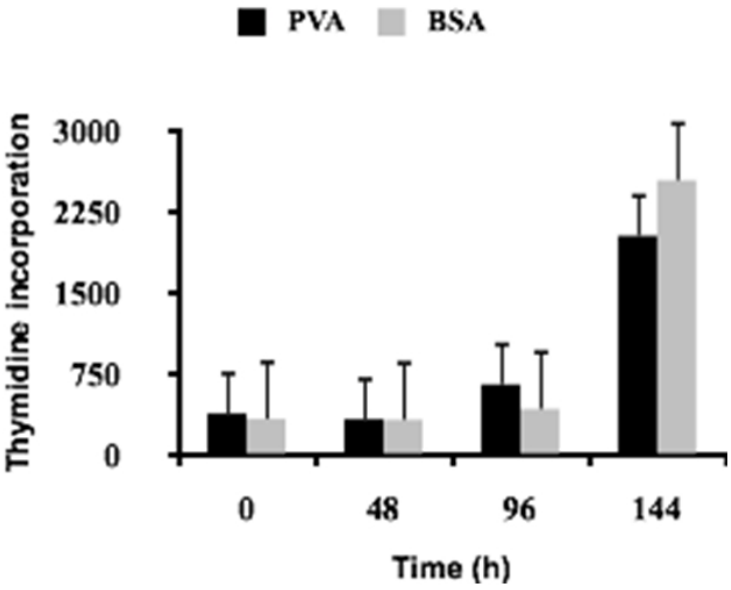

Fig. 4. Thymidine incorporation by bovine granulosa cells from small follicle cultured for up to 144 h, in the presence of PVA and BSA, IGF-1, and insulin and absence of FSH. Values are least-square means±SD of 6 independent cultures.

In order to monitor the proliferation of the GC with different supplementation, the incorporation of tritiated thymidine was evaluated. Fig. 4 summarizes the results of tritiated thymidine incorporation by GC cultures with PVA or BSA media supplementation for 144 h. On both treatments, we could detect an increase in thymidine incorporation with time, with no difference between groups. The significant peak of incorporation was detected at 144 h for both PVA (2029.12±328.69) and BSA (2543.25±234.85).

Figs. 5 and 6 illustrate morphological features of GC cultivated under defined chemical conditions with PVA supplemented media. The initial seeding of bovine GCs in medium supplemented with 0.1% PVA provided cell aggregation into clusters with some other cells attached to the well in the first 12 h (Fig. 5). Fig. 6 demonstrates round cell populations during 48, 96, and 144 h of culture. The toluidine blue staining highlights lipid droplets in the cytoplasm of GC throughout culture time.

To verify the activity of some steroidogenic enzime, we performed the immunocytochemistry for HSD3β1 (indicative of the dominance process) in GC culture systems using PVA. The Fig. 7 shows the immunocytochemistry of the steroidogenic enzyme evaluated at T0 h (a), T48 h (b) and T96 h (c) in granulosa cell cultures, which showed the presence of HSD3β1. The presence of the cell cluster observed in Fig. 7(d) is characteristic of this defined culture model [8]. The staining for HSD3β1 was maximum at T0 h and decreased thereafter.

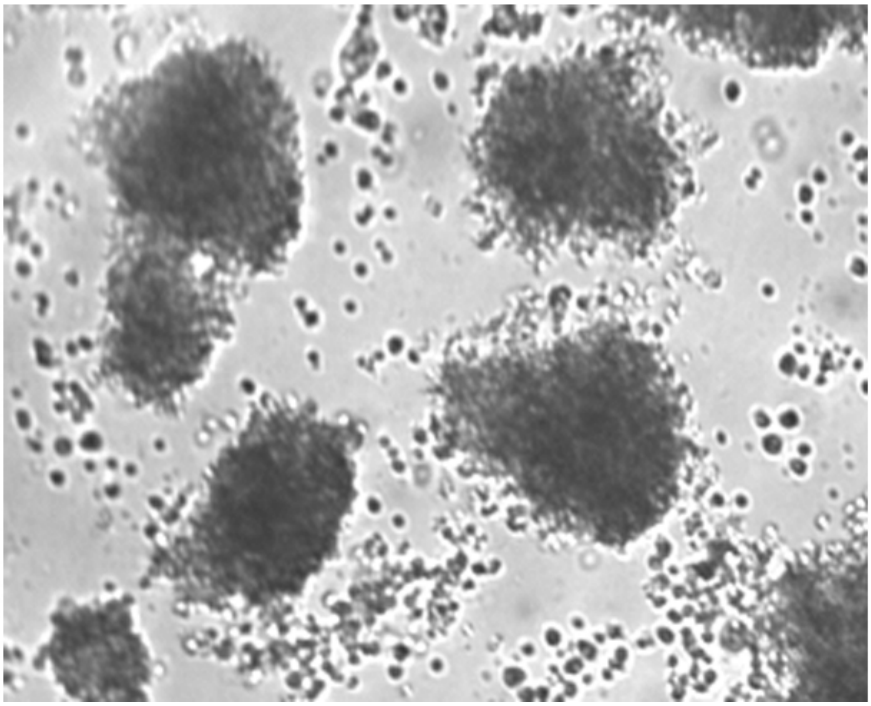

Fig. 5. Morphologycal feature of bovine granulosa cells under chemically defined conditions using 0.1% PVA supplementation for 96 h. Round GCs are aggregated in clusters sustained by elongated cells. Magnificance: 400x.

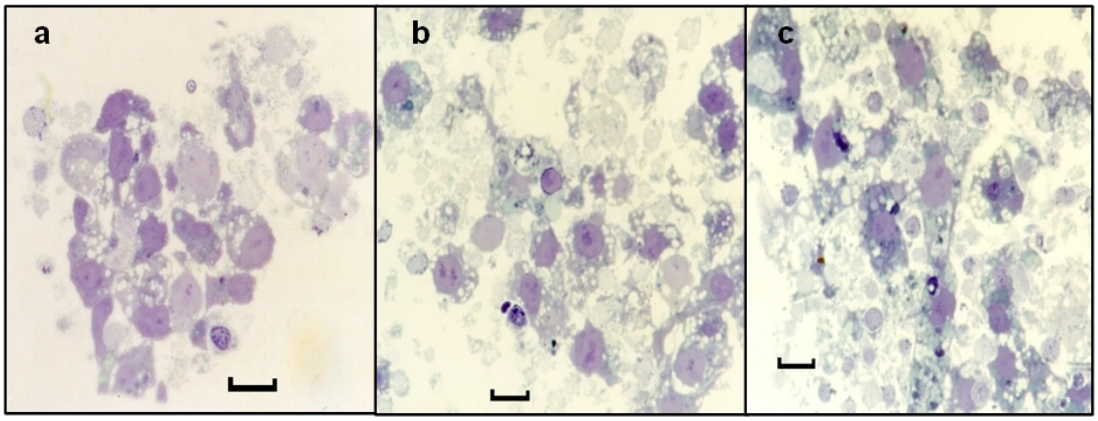

Fig. 6. Round bovine granulosa cells stained with Tiluidine blue 1% at 48 (a), 96 (b), and 144 h (c) chemically defined conditions using 0.1% PVA supplementation. The round blue areas in the cytoplasm of the cells are lipid droplets. Magnificance: 1071x.

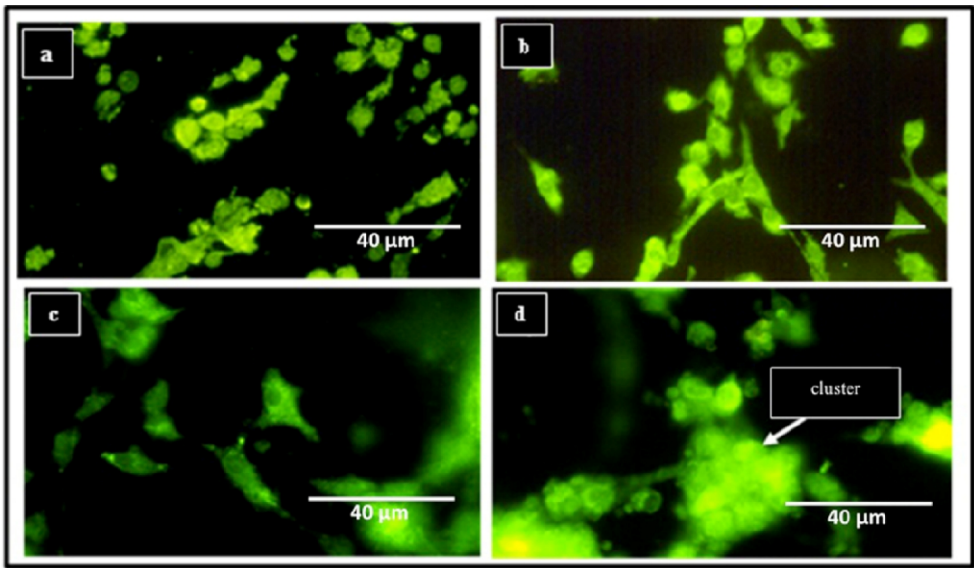

Fig. 7. Immunocytochemistry for 3β-hydroxysteroid dehydrogenase enzyme (HSD3β1) at T0 h(a), T48 h (b), T96 h (c), and a cell extending and cluster formation (d).

3. DISCUSSION

The major function of the female gonad is the differentiation and release of the mature oocyte for fertilization and successful propagation of the species. Additionally, the ovary produces steroids that allow the development of female secondary sexual characteristics and support pregnancy [21]. The efforts of biologists to understand how ovarian follicular regulated have been focused on the later stages of follicular development [22]. In the present study, we were able to show that GC cultivated in medium supplemented with PVA had a better performance on steroid production that cells in BSA supplemented medium. This reinforces previous findings that medium supplemented with PVA might offer an interesting alternative in order to achieve completely defined conditions in models that represent steroidogenesis and folliculogenesis. In the present study, the term “defined medium” was used to refers a medium without addition of undefined components from any kind of serum and serum replacers, including BSA.

We showed a culture of GC system that is able to maintain E2 and P4 production and cell proliferation in a long term culture (144 hours) under a strictly defined culture medium using PVA as macromolecule, without FSH. Although high E2 production by GC cultured in the presence of BSA or other serum replacers has also been described in earlier studies [17], the present study was able to directly test that replacement with PVA (and additional defined supplements) allow for greater and earlier E2 production by GC when compared with BSA. This might be due to interferences of BSA. The significant delay in E2 production by BSA supplemented GC cultures may be due to the known contaminants of BSA (such as steroids and growth factors) [9], since for instance, the presence of androgens or progestagens may interfere with E2 and P4 production. Although attempts to propagate bovine GC using serum replacers have succeeded in some cases [15,27,28], some of those serum replacers were contaminated with steroids and other interfering factors [18]. Serum and some serum replacers are commonly used as a protein source in culture media, but it may be

contaminated with various defined and undefined molecules such as steroids, cholesterol, peptides, antibiotics, and proteases, and with extrinsic agents such as bacteria, virus, antibodies, and prions [24,25].

The proposal of utilization of PVA is to create a strictly defined medium since it is a synthetic polymer used in the cell culture medium for the stabilization of osmotic pressure and as protective agent and chelant of heavy metal ions [14,10,15]. Although there are few reports regarding their physicochemical properties, this polymer affects the properties of cell adhesion, protein adsorption and diffusion of paracrine and autocrine factors in the culture medium. PVA have been added to the medium as substitute of serum [10] or BSA during IVM of bovine oocytes [13,14,15] also showing a good performance. For instance, the expression of conexin-43 was increased in 8 to 16 cells embryos in medium containing PVA, which correspond to the moment of activation of the embryo development [23]. Also, our group has shown that bovine oocytes were matured in serum- and gonadotrophin-free medium, supplemented with PVA and growth factors or with PVA without growth factors and hormones, with no changes in post-cleavage development or in the relative abundance of stress-associated genes such as Hsp-70 and Bax [15]. In additon, the same defined medium with PVA and in absence of FSH enhances steroidogenic enzymes expression and maintains E2 production by co-culture of GC and theca cells (TC) present in follicle wall sections cultured in vitro [16]. Vasconcelos et al. [26], show that in vitro serum-free follicular wall section culture in the defined medium is able to maintain cell morphology, steroid profile, and enhances levels of steroidogenic enzymes mRNAs, also allows construction of an in vitro model of a growing and/or dominant follicle, in which both follicular wall and oocyte may be present, to study the crosstalk between the germinal and somatic compartments involved in the in vitro regulation of oocyte maturation.

The P4 concentrations in PVA medium were not significantly different during the latter times culture, that is, it may be suggested that in this culture medium, the GC are keeping steroidogenic characteristics of follicles in follicular phase, when the E2 production is increasing along time and P4 production is not. These data could be confirmed when we analyzed the correlations between E2:P4. It may be observed that the E2:P4 ratio is significantly greater for the culture medium kept with PVA during initial 96 hours of the experiment. There is not a significant expression of the steroidogenic enzymes P450 aromatase and cholesterol side-chain cleavage enzyme (CYP11A1) in GC of follicles with less than 4 mm diameter [27]. However, there are circumstances in which P450 aromatase mRNA levels and E2 concentrations are not so closely associated. For example, significant decreases in follicular E2 concentration were observed before decreased P450 aromatase mRNA levels; and during final preovulatory follicle maturation, there is increased follicular E2 content in the absence of increased P450 aromatase mRNA levels [28].

Increase in cell differentiation in vitro has been demonstrated in ovine GC [7], but the steroidogenic capacity of the GC from small follicles (< 3.5 mm of diameter) after 6 days of culture is still smaller than the production of the larger follicles (> 3.5 mm). IGF-1 and insulin were enough to stimulate estradiol production and cell proliferation in granulosa cell from small follicle (< 4 mm in diameter) in the culture medium with FSH [8]. Considering tritiated thymidine incorporation, our datas indicate that GC proliferation was stimulated at 144 hours of culture for both treatment (BSA and PVA). The time course of differentiation of bovine GC in this defined culture system occurs in parallel to the physiological events in vivo.

At the present study, the addition of insulin and IGF-I without FSH stimulated steroidogenesis since 48 hours of culture in PVA supplemented cultures, but the cell

proliferation was attained only at 144 hours of culture. The IGF's are potent mitogens and stimulate the mitosis of cultured theca and granulosa cells; they increase the synthesis of androgens and E2, and modulate gonadotropin action on those cells [30,31]. The FSH action on GC differentiation depend on the presence of IGF-1 and an active IGF-1 receptor. IGF-1 is involved in the expression of FSH-receptor expression in GC [29].

Our findings regarding GC morphology demonstrate that such cells cultivated under defined conditions preserve characteristics similar to those of in vivo or in vitro GC [8]. After initial seeding in medium supplemented with 0,1% PVA, bovine GC aggregated into attached clusters. Cluster formation allowed interactions between cells possibly favoring GC polyhedral shape, as well as their high nucleus/cytoplasm ratio, smooth and rough endoplasmic reticulum and free ribosomes in the cytoplasm of clustered cells, mitochondria with tubulovesicular cristae and lipid droplets were randomly distribute through the cytoplasm and dispersed cytoplasmic lipid droplets [17]. Since cell architecture and structure appear to be involved in cell signal transduction and may determine the ability of the cells to respond to stimulatory factors, culture systems that have physiological relevance must preserve normal granulosa cell morphology [8]. In fact, the maintenance of epithelial characteristics and more precisely their conformation permitted extensive communication between them by junctions such as tight junctions. These junctions and cell machinery allowed the continuous production of E2 and P4. In a previous work from our group, Piccinato et al. [17], show that morphologic characteristics of highly estrogenic GC cultivated in PVA supplemented medium were similar to that described by Gutierrez et al. [8], that use the same medium but add BSA and FSH, reinforcing the importance of the structure: function relationship.

The immunocytochemistry of HSD3β1 in GC culture showed the presence of the enzyme for 96 h these cultures and reflected the hormonal production.

Changes in gene expression of gonadotrophin receptors, steroidogenic enzymes (including StAR), IGF and IGF binding proteins (IGFBP) in bovine follicles during recruitment, selection, dominance and atresia have been already characterized in studies investigating the roles of each one in follicular development and steroidogenesis. Changes in gene expression of steroidogenic enzymes in dominant bovine follicles [33] or during the differentiation of dominant follicles in the preovulatory period [34] have been shown in some studies. Therefore, the ability of follicles to secret rising E2 concentrations is due to a specific increase of enzyme activity, with CYP11A1 and HSD3β1 being involved in steroid biosynthesis [35].

Many researchers have used the ratio between the concentrations of E2 to P4 in follicular fluid as an indicator of atresia. However, when the follicles are smaller than 5 mm, like ours, they do not express aromatase activity and this method to analyze atresia is unsuitable [36]. Studies have shown increased CYP11A1 mRNA expression in GC from dominant follicles and observed that this increase was less marked than that of aromatase [37]. Moreover, granulosa cell expression of HSD3β1 was detected by in situ hybridization only upon the onset of follicle dominance in one study [38], but other studies have indicated that enzyme activity [39] and mRNA levels as measured by quantitative RT-PCR [40] did not change with follicle development. Our results show that the enzyme HSD3β1, important for the steroidogenic pathway, was present in GC cultured according to the present methodology, using PVA as a macromolecular donor.

In 1995, McGee and colleagues [41] reported that HSD3β1 activity depended on FSH and that insulin and/or IGF-1 just acted synergically, but Eirmel and Orly [42] showed that FSH did not alter HSD3β1 expression. In our culture system, using only IGF-1 and insulin in our medium, we noted that HSD3β1 was present independently of FSH. Bao and colleagues [38] showed that only follicles selected for dominance expressed HSD3β1 in GC. Based on this affirmation and on the presence of HSD3β1 in our cells, we suggest that GC cultivated in our defined medium mimic GC from growing follicles.

4. CONCLUSION

Therefore, this paper demonstrates the effectiveness of synthetic macromolecules in maintaining the estrogenic potential of bovine GC from small bovine follicles cultured in a chemically defined medium supplemented with insulin and IGF-1 in the absence of FSH. Moreover, immunocytochemistry was efficient for the detection HSD3β1 in this model of GC culture. Thus, we may suggest that the presence of HSD3β1 and an increasing E2:P4 ratio indicate that culture GC mimic GC from follicles selected for dominance. So, this system is of singular relevance for studies related to the growth, differentiation and the steroidogenic capacity of bovine granulosa cells, allowing studies related to local factors of growth, directly or indirectly related to the ovarian physiology and follicle development. It can potentially be used as a tool for the research on the interrelationship of the somatic cells with the germ cell in the follicle.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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