Small Ruminant Research 169 (2018) 62–66

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Short communication

# Goat *in vitro* follicular response to insulin concentration is affected by base medium and follicular stage

V.M. Paes[a,*], L.A. Vieira[a], J. Cadenas[a], H.H. Correia[a], N.A. Sá[a], B.G. Alves[a], F. Brandão[b], R.R. Santos[c], A.P.R. Rodrigues[a], J.R. Figueiredo[a]

[a] *Laboratory of Manipulation of Oocyte and Preantral follicles (LAMOFOPA), State University of Ceara, Fortaleza, CE, Brazil*
[b] *Department of Animal Reproduction, Faculty of Veterinary, Federal University Fluminense, Rio de Janeiro, RJ, Brazil*
[c] *Laboratory of Wild Animal Biology and Medicine, Federal University of Pará, Castanhal, Pará, Brazil*

ARTICLE INFO

*Keywords:*
Antral follicles
Base media
Insulin
In vitro culture
Goat
Preantral follicles

ABSTRACT

Different culture media are used for the culture of preantral (PFs) and antral (AFs) follicles, among them the αMEM and TCM199. Also, culture media supplementation with insulin may improve the in vitro development of goat PFs and AFs. However, there is no information on the indicated concentration of insulin and which basis culture medium is the most suitable for caprine PFs and AFs. This study aimed to assess if the culture of PFs and AFs can be affected by the basis medium, and if its supplementation with two different insulin concentrations will improve follicular growth and oocyte development. Isolated PFs and AFs were in vitro cultured for 18 days in αMEM, or TCM199 supplemented either with 10 ng/mL or 10 μg/mL insulin. A higher degeneration rate ($P < 0.05$) of PFs was observed for TCM199 compared to αMEM within the same insulin concentrations. Preantral follicles cultured in αMEM with insulin 10 μg/mL presented the greatest ($P < 0.05$) daily growth, as well as increased ($P < 0.05$) antrum formation, while the daily growth rate of AFs was highest ($P < 0.05$) in αMEM supplemented with insulin at both tested concentrations. Furthermore, the development of oocytes recovered from PFs and AFs was improved when culture was performed in αMEM supplemented with insulin 10 ng/mL.

## 1. Introduction

Immature oocytes from preantral (PFs) and antral follicles (AFs) have been used to develop reproductive biotechnologies in human and other species (Palma et al., 2012; Monniaux et al., 2014). Although the caprine species has been described as a good model for human ovary due to similarities in oocyte diameter and length of folliculogenesis (Figueiredo et al., 2011), advances are limited to the production of a low and variable number of mature oocytes and embryos (Silva et al., 2015). Many factors affect the outcome of the in vitro culture (IVC) of follicles, e.g., supplements (Silva et al., 2015), basis medium (Rossetto et al., 2012), and follicular developmental stage (Cadenas et al., 2017). The most used media for follicular IVC are αMEM and TCM199 (Rossetto et al., 2012; Arunakumari et al., 2010). Among the supplements, insulin is often used because of its key role in steroid production and embryo development. Ferreira et al. (2016) reported that 10 μg/mL insulin stimulates oocyte growth when the base medium contains growth hormone and follicle-stimulating hormone (FSH). Nevertheless, it is still unknown if follicular survival and growth will depend on their stage of development, the base medium used for IVC, and insulin concentration. Therefore, the present study aimed to determine the adequate base medium (αMEM vs. TCM199) and insulin concentration (10 ng/mL vs. 10 μg/mL) for isolated caprine preantral and antral follicles.

## 2. Material and methods

All chemicals used in the present study were purchased from Sigma Chemical Co (St. Louis, MO) unless otherwise indicated.

All experiments were performed according to the recommendations of the Committee of Animal Handling and Ethical Regulation from the State University of Ceara, Fortaleza, Ceara, Brazil. The study does not involve any human samples.

Ovaries (n = 72) from 36 adult mixed-breed goats were collected from a local slaughterhouse and submitted to microdissection for the recovery of isolated advanced PFs and early AFs follicles (~200 μm and

* Corresponding author at: Laboratório de Manipulação de Oócitos e Folículos Pré-Antrais (LAMOFOPA). Universidade Estadual do Ceará (UECE). Av. Silas Munguba, 1700, Campus do Itaperi. Fortaleza, CE, 60740 903, Brazil.
*E-mail address:* macedovictor_ef@yahoo.com.br (V.M. Paes).

https://doi.org/10.1016/j.smallrumres.2018.11.001
Received 26 October 2017; Received in revised form 25 October 2018; Accepted 2 November 2018
Available online 03 November 2018
0921-4488/ © 2018 Elsevier B.V. All rights reserved.

~350 µm, respectively). Follicles with a visible oocyte surrounded by granulosa cells and intact basement membrane were selected for IVC. For this, PFs and AFs were individually cultured in 25 µL drops of culture medium on Petri dishes (60 × 15 mm, Corning, USA) (Araújo et al., 2011). Base media (αMEM and TCM199) were supplemented with 3 mg/mL bovine serum albumin (BSA), 2 mM glutamine, 2 mM hypoxanthine, 5.5 µg/mL transferrin, 5 ng/mL selenium and 50 µg/mL ascorbic acid (Chaves et al., 2012), and after that referred to $\alpha MEM^{+}$ and $TCM199^{+}$. A total of 193 PFs and 167 AFs were cultured in $\alpha MEM^{+}$ or $TCM199^{+}$ supplemented with 10 ng/mL or 10 µg/mL insulin named as $\alpha MEM^{+}$ 10 ng/mL, $\alpha MEM^{+}$ 10 µg/mL, $TCM199^{+}$ 10 ng/mL and $TCM199^{+}$ 10 µg/mL. The experiments were replicated six times, and approximately 48 follicles of each follicular category (PF or AF) were used per treatment (8 follicles/replicate/treatment). The total number of follicles in each treatment was: PFs ($\alpha MEM^{+}$ 10 ng/mL; n = 46, $\alpha MEM^{+}$ 10 µg/mL; n = 45, $TCM199^{+}$ 10 ng/mL; n = 52, $TCM199^{+}$ 10 µg/mL; n = 50) and AFs ($\alpha MEM^{+}$ 10 ng/mL; n = 41, $\alpha MEM^{+}$ 10 µg/mL; n = 41, $TCM199^{+}$ 10 ng/mL; n = 46, $TCM199^{+}$ 10 µg/mL; n = 39). All groups were cultured for 18 days at 39 °C and 5% $CO_2$. Every other day, 5 µL of fresh culture medium was added to every drop, with a final volume of 65 µL on day 18 (Araújo et al., 2011). The conditioned culture media were collected on day 18, and stored at -80 °C for assessing hormone content. After the culture period, oocytes were retrieved from follicles and immediately destined to in vitro maturation (IVM). Furthermore, as non-cultured control 19 PFs and 34 AFs were fixed in glutaraldehyde (1%) for further analysis of oocyte meiotic stage and morphometry before culture (Day 0).

### 2.1. Follicular morphology and development

Before and after IVC (days 0 and 18), follicles were classified according to their morphological features. Degenerated follicles were scored when the cytoplasm of the oocyte and surrounding cumulus cells were dark or misshapen. Antral cavity formation (defined as a visible translucent cavity within the granulosa-cell layers) and the diameters of healthy follicles were also recorded. Follicle diameter was determined as the mean of two perpendicular measurements of each follicle using a stereomicroscope (SMZ 645 Nikon) with an ocular micrometer (100× magnification). The growth rate was calculated as follows: the final diameter minus the initial diameter of viable follicles (on Day 0), divided by the days of IVC (on Day 18).

### 2.2. Hormone assays

Estradiol (E2) concentrations were analyzed using radioimmunoassay kits (MP Biomedicals, LLC – Orangeburg, NY, USA). The assay sensitivity and intra-assay coefficient were 5 pg/mL and 7%, respectively.

### 2.3. In vitro maturation of oocytes

At the end of the culture period, the cumulus-oocyte complexes (COCs) were recovered from the cultured AFs and PFs using 26-gauge needles. The COCs were washed twice in IVM medium (TCM199 supplemented with 0.5 µg/mL recombinant FSH (bovine), 5 µg/mL LH, 1 µg/mL 17β-estradiol, 10 ng/mL EGF, 1 mM pyruvate, 100 µM cysteamine, 50 ng/mL recombinant IGF-1 and 1% BSA). All COCs were individually matured in 10 µL drops of IVM medium on culture dishes (60 × 15 mm, Corning, USA) under mineral oil for 32 h (Ferreira et al., 2016).

### 2.4. Assessment of oocyte viability and chromatin configuration

After 32 h IVM, oocytes were mechanically denuded and individually incubated for 30 min in 10 µL drops of PBS supplemented with 4 mM, calcein-AM, 2 mM ethidium homodimer-1, and 1% glutaraldehyde, for viability analysis under a fluorescence microscope (Eclipse 80i, Nikon, Japan). Oocytes were considered viable if the cytoplasm stained with calcein-AM and if the chromatin was not labeled with ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany). The emitted fluorescent signals of calcein-AM (green) and ethidium homodimer-1 (red) were measured using 488 nm excitation. Oocyte chromatin was labeled with 10 mM Hoechst 33342 (emission at 479 nm), and the chromatin configuration was analyzed from intact germinal vesicles to meiotic resumption (germinal-vesicle breakdown, metaphase I and metaphase II). Oocyte diameter was determined as the mean of two perpendicular measurements of each oocyte excluding the zona pellucida.

### 2.5. Statistical analysis

All statistical analyses were performed using Sigma Plot 11 (Systat Software Inc., USA). Comparisons of means among treatments were analyzed by Kruskal–Wallis test. The proportion variables among treatments and days of culture were evaluated by chi-square or Fisher's exact test. A probability of $P < 0.05$ indicated that difference was significant.

## 3. Results

### 3.1. Follicular morphology and growth in vitro

For both follicle categories $\alpha MEM^{+}$ had a more positive effect than $TCM199^{+}$, including using higher (10 µg/mL) concentration of insulin. The percentages of atretic follicles after 18 days IVC are depicted in Fig. 1A. The significantly highest degeneration rates were observed when PFs were cultured in $TCM199^{+}$. Antral follicles cultured in $TCM199^{+}$ with insulin at 10 ng/mL presented higher ($P < 0.05$) atresia rates than with insulin 10 µg/mL. The growth of PFs was significantly improved if the 18 days IVC was performed in $\alpha MEM^{+}$ with 10 µg/mL insulin. For AFs, IVC in $\alpha MEM^{+}$ resulted in significantly higher growth, independently on the insulin concentration in the culture medium (Fig. 1B).

### 3.2. Antrum formation in vitro

The rate of antrum formation in vitro was measured based on observations every six days until reach day 18. After 18 days of in vitro culture, antrum formation was significantly higher if PFs were cultured in the presence of $\alpha MEM^{+}$ at 10 µg/mL insulin compared to $TCM199^{+}$ with 10 µg/mL insulin. These differences were not affected by insulin concentration in the medium (Fig. 2).

### 3.3. Oocyte viability, diameter and chromatin configuration

Regardless the follicular category, the percentage of viable oocytes (Table 1) was lower ($P < 0.05$) in the cultured groups than in the non-cultured ones (control). Regardless the treatment, after culture mean oocyte diameter was greater ($P < 0.05$) in cultured follicles than non-cultured control except for AFs cultured in $TCM199^{+}$ with insulin 10 µg/mL treatment. When the base media were analyzed for the same insulin concentration, despite the follicular category, oocyte diameters were higher ($P < 0.05$) in $\alpha MEM^{+}$ than in $TCM199^{+}$. It is important to emphasize that except for PFs cultured in $TCM199^{+}$, the largest ($P < 0.05$) oocyte diameters were obtained when PFs and AFs were previously cultured in the presence of insulin 10 ng/mL. As expected, at onset the of culture (non-cultured - control) oocyte diameter from AFs was greater ($P < 0.05$) than that from PFs. AFs cultured in $\alpha MEM^{+}$ with insulin at 10 ng/mL had greater ($P < 0.05$) percentage of meiotic resumption in relation to $TCM199^{+}$ treatment combined with insulin, regardless its concentration. It is important to highlight that only $\alpha MEM^{+}$ treatments yielded metaphase I and II oocytes.

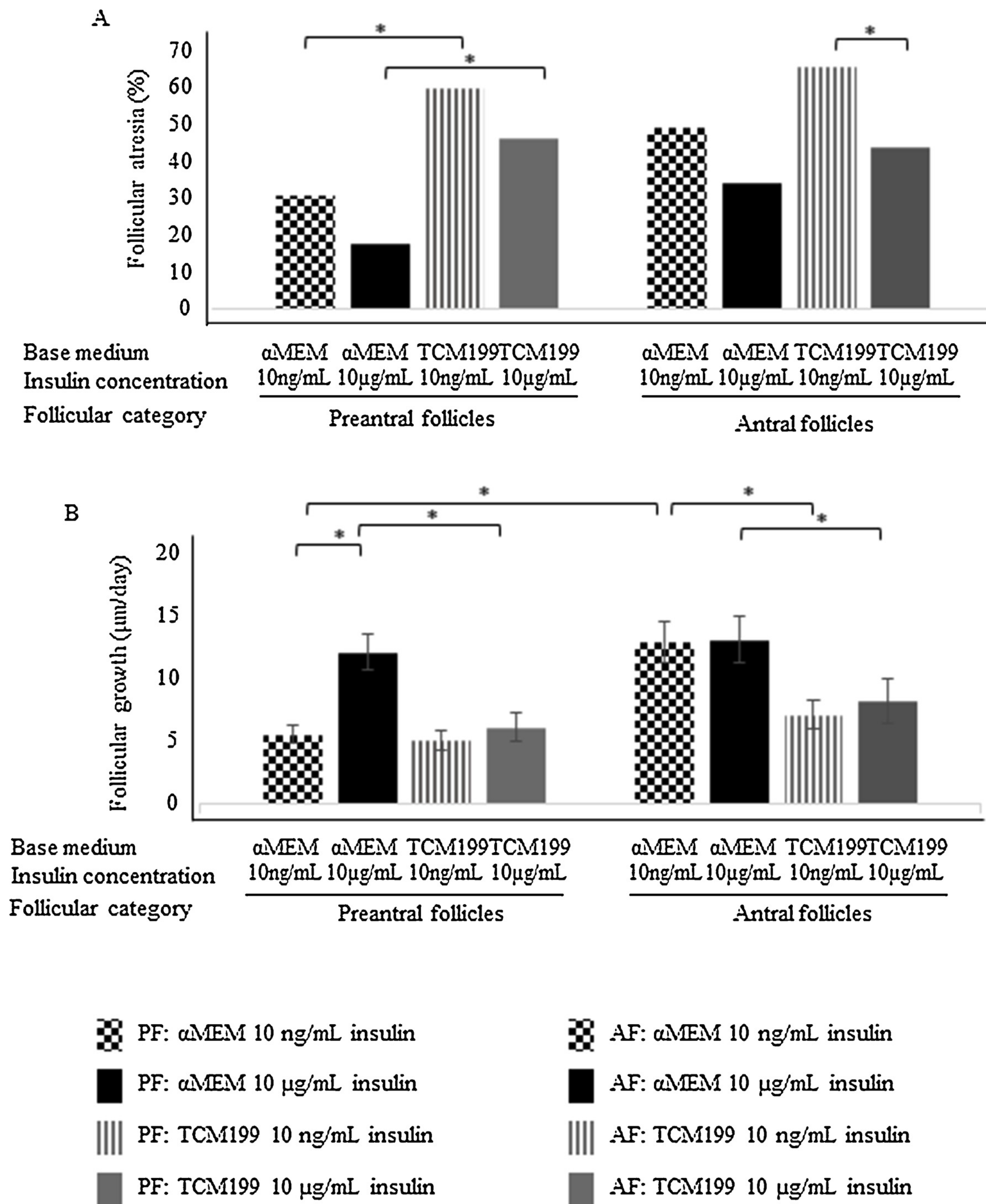


**Fig. 1.** Effect of base medium and insulin concentration on atresia (1 A) and follicular growth (1B). * significant difference among treatments ($P < 0.05$).

### 3.4. Estradiol production

Estradiol production from individual follicles was assessed at the end of IVC (Table 2). A large individual variation (46 to 14,000 pg/mL) was observed in the estradiol secretion among the cultured follicles, and no differences were found among the tested treatments regardless the base medium and insulin concentration.

## 4. Discussion

As observed in the present study, $\alpha MEM^+$ is more suitable than $TCM199^+$ for the in vitro growth of preantral and antral follicles, as well as the development of oocytes enclosed in these follicles. Both $\alpha MEM^+$ and $TCM199^+$ are culture media with a rich composition, including amino acids, vitamins and salt. However, $\alpha MEM^+$ has suitable levels of ascorbic acid, which in turn would ensure appropriate redox state, as well as basement membrane turnover to support follicular development (Silva et al., 2011).

The effect of insulin concentration on follicle development depended on the base medium and follicular category, where $\alpha MEM^+$ with a 1000-fold higher concentration (10 μg/mL vs. 10 ng/mL) of insulin resulted in a significant increase in the growth of PFs. Similar results were obtained by other authors after the IVC of isolated caprine PFs (Ferreira et al., 2016; Silva et al., 2017). Furthermore, in vitro cultured AFs in the presence of insulin 10 μg/mL using $TCM199^+$, reduced atresia compared to insulin 10 ng/mL. Granulosa cells have a

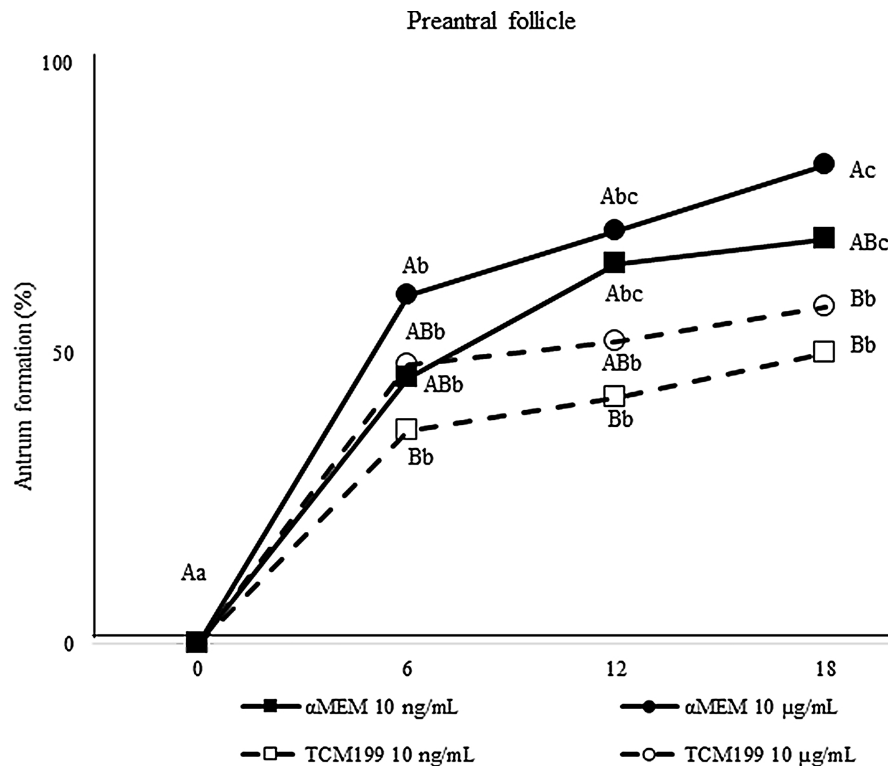


**Fig. 2.** Antrum formation rate of preantral follicles cultured in distinct base media with different insulin concentrations. [a,b,c] significant difference among days of the culture in the same treatment ($P < 0.05$). [A,B] significant difference among treatments in the same day of the culture ($P < 0.05$).

high glycolytic capacity (Munakata et al., 2016). Since insulin will affect the glucose consumption by granulosa cells (Itami et al., 2017), it was already expected that at the highest tested concentration, the granulosa cells proliferation and subsequent follicular growth should be increased. Only in the presence of αMEM$^+$, PFs and AFs responded differently to insulin concentrations for follicular growth. Our team has shown that follicle requirements differ throughout the IVC according to their developmental stage (Cadenas et al., 2017). Furthermore, it was already demonstrated in vivo that the complex transition from PFs to AFs leads to the up- or down-regulation of 2466 genes involved in three metabolic pathways, which may explain the different outcomes according the follicular category (Magalhães-Padilha et al., 2013). Insulin stimulates steroid production (Frank et al., 2008). Unfortunately, the huge variation in the estradiol levels produced by the follicles did not allow such observation in the present study. In a previous study, Araújo et al.,(2015) reported higher estradiol levels when bovine PFs were cultured in αMEM than in TCM199. Besides the species-specific differences, these later authors performed the in vitro culture for a longer period (32 days) and added recombinant FSH to the culture medium.

**Table 2**
Estradiol production in αMEM$^+$ or TCM199$^+$.

| Treatment* | E2 (pg/mL) |
|---|---|
| αMEM$^+$ | 1466.1 ± 957.7 |
| TCM199$^+$ | 284.7 ± 149.5 |

* Ovarian follicles were grouped within αMEM+ or TCM199+ regardless follicular category and insulin concentration. Between treatments ($P > 0.05$).

Regarding oocyte development, in general, there was an improved

**Table 1**
Oocyte development in αMEM$^+$ or TCM199$^+$ with insulin at 10 ng or 10 μg/mL.

| | Treatment | n | % Viable | Oocyte diameter | % GV† | % GVBD† | % MI† | MII† | Meiotic Resumption† |
|---|---|---|---|---|---|---|---|---|---|
| Preantral | Non-culture | 19 | 94.7 (18/19)A | 74.1 ± 2.7A | 100.0 (18/18)A | ND | ND | ND | ND |
| | αMEM$^+$ 10 ng/mL | 46 | 60.9 (28/46)B | 97.4 ± 2.1B | 67.9 (19/28)BC | 25.0 (7/28)A | 7.1 (2/28) | ND | 32.1 (9/28)A |
| | αMEM$^+$ 10 μg/mL | 45 | 60.0 (27/45)B | 91.2 ± 1.8C | 81.5 (22/27)AB | 11.1 (3/27)A | ND | 7.4 (2/27) | 18.5 (5/27)A |
| | TCM199$^+$ 10 ng/mL | 52 | 46.1 (24/52)B | 85.8 ± 2.2CD | 75.0 (18/24)BC | 25.0 (6/24)A | ND | ND | 25.0 (6/24)A |
| | TCM199$^+$ 10 μg/mL | 50 | 46.0 (23/50)B | 83.9 ± 2.7D | 69.6 (16/23)BC | 30.4 (7/23)A | ND | ND | 30.4 (7/23)A |
| | **Treatment** | **n** | **% Viable** | **Oocyte diameter** | **% GV†** | **% GVBD†** | **% MI†** | **MII†** | **Meiotic Resumption†** |
| Antral | Non-culture | 34 | 91.2 (31/34)A | 94.4 ± 2.4A* | 96.8 (30/31)A | 3.2 (1/31)A | ND | ND | 3.2 (1/31)A |
| | αMEM$^+$ 10 ng/mL | 41 | 58.5 (24/41)B | 119.3 ± 2.8B* | 16.7 (4/24)B* | 12.5 (3/24)AB | 37.5 (9/24)A* | 33.3 (8/24)A | 83.3 (20/24)B* |
| | αMEM$^+$ 10 μg/mL | 41 | 43.9 (18/41)B | 109.3 ± 2.6C* | 33.3 (6/18)BC* | 22.2 (4/18)BC | 33.3 (6/18)A | 11.1 (2/18)A | 66.7 (12/18)BC* |
| | TCM199$^+$ 10 ng/mL | 46 | 52.2 (24/46)B | 107.4 ± 3.0C* | 45.8 (11/24)CD | 54.2 (13/24)D* | ND | ND | 54.2 (13/24)C* |
| | TCM199$^+$ 10 μg/mL | 39 | 56.4 (22/39)B | 98.1 ± 3.4A* | 54.5 (12/22)C | 45.4 (10/22)CD | ND | ND | 45.4 (10/22)C |

A,B,C Within a column in the same follicular category ($P < 0.05$).
* Difference between follicular category in the same base medium and insulin concentration ($P < 0.01$).
† Values calculated out of the viable oocytes. ND, not detected. n Total number of oocytes in each treatment. Abbreviations: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

when insulin was added at the lowest tested concentration, i.e. 10 ng/mL, regardless if the oocyte was harvested from PFs or AFs. In previous studies (Chaves et al., 2012; Ferreira et al., 2016), no difference was observed regarding oocyte growth between culture media containing high (10 µg/mL) or low (10 ng/mL) insulin concentration. Unlike these authors, we have used an IVC system with culture medium replacement protocol, which is the most efficient for the oocyte growth (Araújo et al., 2011). Importantly, it was reported before that the higher the insulin level in the medium, the higher the proliferation of granulosa cells (Itami et al., 2017). However, these authors also showed that this effect has, as cost, the exhaustive use of the glucose sources for oocyte development. As in the present study we did not increase glucose levels according the tested insulin level, we suggest that the highest tested insulin concentration resulted in a reduction in energy for the oocyte development.

It was demonstrated a similar efficiency of the IVC in the promotion of oocyte growth in both follicle categories. Nevertheless, the ratio follicle diameter/oocyte diameter was different at the onset of IVC of PFs (follicle ~ 214 µm / oocyte ~ 74 µm) and AFs (follicle ~ 340 µm / oocyte ~ 94 µm). Otoi et al (1997) have already reported a positive relationship, at least in vivo, between follicle and oocyte diameters, and the ability of the oocyte to resume meiosis. In our findings, oocyte meiotic progression to MI and MII occurred only in the presence of αMEM$^+$. Thus, we hypothesize that the better performance showed by αMEM$^+$ compared to TCM199$^+$, as well as AFs better than PFs is related to the ability of the base medium to stimulate oocyte growth in vitro, and to follicle diameter at the onset of the culture, respectively.

In conclusion, follicular development and oocyte growth better outcomes were obtained with αMEM basic medium. Moreover, αMEM in the presence of 10 ng/mL insulin yielded larger oocyte diameter regardless follicular category.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgements

We acknowledge core support provided by the National Council for Scientific and Technological Development (CNPq-79/2013) for the study. Victor Macêdo Paes is the recipient of a grant from CAPES (Brazil).

## References

Araújo, V.R., Chaves, R.N., Duarte, A.B.G., Celestino, J.J.H., Silva, G.M., Fernandes, D.D., Matos, M.H.T., Campello, C.C., Figueiredo, J.R., 2011. Effect of culture medium replacement protocol on the in vitro development of isolated caprine secondary follicles. Small Rumin. Res. 95, 139–143.

Araújo, V.R., Gastal, M.O., Wischral, A., Figueiredo, J.R., Gastal, E.L., 2015. Long-term in vitro culture of bovine preantral follicles: effect of base medium and medium replacement methods. Anim. Reprod. Sci. 161, 23–31.

Arunakumari, G., Shanmugasundaram, N., Rao, V.H., 2010. Development of morulae from the oocytes of cultured sheep preantral follicles. Theriogenology 74, 884–894.

Cadenas, J., Leiva-Revilla, J., Vieira, L.A., Apolloni, L.B., Aguiar, F.L.N., Alves, B.G., Lobo, C.H., Rodrigues, A.P.R., Apgar, G.A., Smitz, J., Figueirado, J.R., Maside, C., 2017. Caprine ovarian follicle requirements differ between preantral and early antral stages after IVC in medium supplemented with GH and VEGF alone or in combination. Theriogenology 87, 321–332.

Chaves, R.N., Duarte, A.B.G., Rodrigues, G.Q., Celestino, J.J.H., Silva, G.M., Lopes, C.A.P., Almeida, A.P., Donato, M.A.M., Peixoto, C.A., Moura, A.A.A., Lobo, C.H., Locatelli, Y., Mermillod, P., Campello, C.C., Figueiredo, J.R., 2012. The effects of insulin and follicle-stimulating hormone (FSH) during in vitro development of ovarian goat preantral follicles and the relative mRNA expression for insulin and FSH receptors and cytochrome P450 aromatase in cultured follicles. Biol. Reprod. 87, 1–11.

Ferreira, A.C.A., Maside, C., Sá, N.A.R., Guerreiro, D.D., Correia, H.H.V., Leiva-Revilla, J., Lobo, C.H., Araújo, V.R., Apgar, G.A., Brandão, F.Z., Figueiredo, J.R., Campello, C.C., 2016. Balance of insulin and FSH concentrations improves the in vitro development of isolated goat preantral follicles in medium containing GH. Anim. Reprod. Sci. 165, 1–10.

Figueiredo, J.R., Rodrigues, A.P.R., Silva, J.R.V., Santos, R.R., 2011. Cryopreservation and in vitro culture of caprine preantral follicles. Reprod. Fertil. Develop. 23, 40–47.

Frank, S., Stark, J., Hardy, K., 2008. Follicle dynamics and anovulation in polycyctic ovary syndrome. Hum. Repod. 14, 367–378.

Itami, N., Munakata, Y., Shirasuna, K., Kuwayama, T., Iwata, H., 2017. Promotion of glucose utilization by insulin enhances granulosa cell proliferation and developmental competence of porcine oocyte grown in vitro. Zygote 25, 65–74.

Magalhães-Padilha, D.M., Geisler-Lee, J., Wischral, A., Gastal, M.O., Fonseca, G.R., Eloy, Y.R., Geisler, M., Figueiredo, J.R., Gastal, E.L., 2013. Gene expression during early folliculogenesis in goats using microarray analysis. Biol. Reprod. 89, 1–12.

Monniaux, D., Clement, F., Dalbies-Tran, R., Estienne, A., Fabre, S., Mansanet, C., Monget, P., 2014. The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: what is the link? Biol. Reprod. 90, 1–11.

Munakata, Y., Ichinose, T., Ogawa, K., Itami, N., Tasaki, H., Shirasuna, K., Kuwayama, T., Iwata, H., 2016. Relationship between the number of cells surrounding oocytes and energy states of oocytes. Theriogenology 86, 1789–1798.

Otoi, T., Yamamoto, K., Koyama, N., Tachikawa, S., Suzuki, T., 1997. Bovine oocyte diameter in relation to developmental competence. Theriogenology 48, 769–774.

Palma, G.A., Arganaraz, M.E., Barrera, A.D., Rodler, D., Mutto, A.A., Sinowatz, F., 2012. Biology and biotechnology of follicle development. Sci. World J. 2012, 1–14.

Rossetto, R., Saraiva, M.V.A., Santos, R.R., Silva, C.M.G., Faustino, L.R., Chaves, R.N., Brito, I.R., Rodrigues, G.Q., Lima, I.M.T., Donato, M.A.M., Peixoto, C.A., Figueiredo, J.R., 2012. Effect of medium composition on the in vitro culture of bovine pre-antral follicles: morphology and viability do not guarantee functionality. Zygote 21, 125–128.

Silva, G.M., Araújo, V.R., Duarte, A.B.G., Chaves, R.N., Silva, C.M.G., Lobo, C.H., Almeida, A.P., Matos, M.H.T., Tavares, L.M.T., Campello, C.C., Figueiredo, J.R., 2011. Ascorbic acid improves the survival and in vitro growth of isolated caprine preantral follicles. Anim. Reprod. 8, 14–24.

Silva, G.M., Rossetto, R., Chaves, R.N., Duarte, A.B.G., Araújo, V.R., Feltrin, C., Bernuci, M.P., Anselmo-Franci, J.A., Xu, M., Woodruff, T.K., Campello, C.C., Figueiredo, J.R., 2015. In vitro development of secondary follicles from pre-pubertal and adult goats cultured in two-dimensional or three-dimensional systems. Zygote 23, 475–484.

Silva, G.M., Brito, I.R., Sales, A.D., Aguiar, F.L.N., Duarte, A.B.G., Araújo, V.R., Vieira, L.A., Magalhães-Padilha, D.M., Lima, L.F., Alves, B.G., Silveira, L.B.R., Lo Turco, E.G., Rodrigues, A.P., Campello, C.C., Wheleer, M.B., Figueiredo, J.R., 2017. In vitro growth and maturation of isolated caprine preantral follicles: influence of insulin and FSH concentration, culture dish, coculture, and oocyte size on meiotic resumption. Theriogenology 90, 32–41.