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Interval in the replacement of *in vitro* culture medium affects the integrity and development of equine preantral follicles[1]

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ABSTRACT.- Bizarro-Silva C., González S.M., Búfalo I., Lindquist A.G., Sarapião F.D. & Seneda M.M. 2018. **Interval in the replacement of *in vitro* culture medium affects the integrity and development of equine preantral follicles***. Pesquisa Veterinária Brasileira 38(12):2284-2288.* Laboratório de Reprodução Animal, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid PR-445 Km 380, Cx. Postal 10.011, Campus Universitário, Londrina, PR 86057-970, Brazil. E-mail: camilabizarros@gmail.com

The efficiency of a culture system is related to the elaboration and replacement of a medium with conditions suitable for follicular development. Recent investigations suggested that *in vitro* culture medium should be replaced after specific time periods in various species. However, the suitable interval for the exchange of *in vitro* culture medium has not yet been established in equine species. The objective of this investigation was to evaluate the effect of medium exchange intervals of 24 hours (T24) or 48 hours (T48) for *in vitro* culture of preantral follicles at 2 or 6 days. At the end of the culture period, the fragments were processed using classical histology. Equine preantral follicles were classified according to morphological integrity and developmental stage. Data analysis was performed using Fisher's test with a significance level of $p<0.05$. Out of a total of 399 follicles evaluated, 174 (43.6%) were primordial follicles, 225 (56.4%) were in development, and 63.76% were morphologically intact. In the *in vitro* culture performed over two days, there was no significant difference in relation to follicular integrity after medium replacement ($p>0.05$). Compared to the medium replacement at six days of culture, there was a statistically significant difference for T24 (68.9%, $p<0.05$). Therefore, we suggest changing the medium for equine species at 48 hours after the start of culture followed by subsequent daily replacements.

INDEX TERMS: *In vitro* culture, equine preantral follicles, ovary, horses, culture *in vitro* period, follicular morphology.

RESUMO.- [**Intervalo na substituição do meio de cultivo *in vitro* afeta a integridade e o desenvolvimento de folículos pré-antrais equinos.**] A eficiência de um sistema de cultivo está relacionada à elaboração e substituição do meio de cultivo com condições adequadas ao desenvolvimento folicular. Pesquisas recentes sugerem que o meio de cultivo *in vitro* deve ser substituído após períodos de tempo específicos para várias espécies. No entanto, o intervalo adequado para a troca de meio de cultivo *in vitro* ainda não foi estabelecido na espécie equina. O objetivo desta investigação foi avaliar o efeito de intervalos de troca média de 24 horas (T24) ou 48 horas (T48) para cultivo de folículos pré-antrais aos 2 ou 6 dias. No final do período de cultivo, os fragmentos foram processados usando histologia clássica. Os folículos pré-antrais equinos foram classificados de acordo com a integridade morfológica e o estágio de desenvolvimento. A análise dos dados foi realizada utilizando o teste de Fisher com um nível de significância de $p<0,05$. De um total de 399 folículos avaliados, 174 (43,6%) foram folículos primordiais, 225 (56,4%) estavam em desenvolvimento e 63,76% estavam morfologicamente intactos. No cultivo *in vitro* realizado ao longo de dois dias, não houve diferença significativa em relação à integridade folicular após a substituição do meio ($p>0,05$). Comparado com a substituição média aos seis dias de cultivo, houve

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diferença estatisticamente significativa para T24 (68,9%, p<0,05). Portanto, sugerimos alterar o meio para as espécies equinas às 48 horas após o início da cultura, seguindo as subsequentes substituições diárias.

TERMOS DE INDEXAÇÃO: Cultivo *in vitro*, folículos pré-antrais, equinos, ovário, período de cultivo *in vitro*, morfologia folicular.

INTRODUCTION

Reproductive biotechnologies allow people to overcome several obstacles of reproductive physiology, optimize the genetic material of animals and accelerate and facilitate genetic improvement. This technology has been especially useful for mitigating the high level of follicular loss that occurs naturally throughout the lives of mammals (Carmo et al. 2002). Since the ovarian reserve of female mammals consists of 90% primordial follicles in the ovarian tissue, approximately 99% of these follicles do not reach the ovulatory phase (Markström et al. 2002).

In this context, the *in vitro* culture (IVC) of preantral follicles has been used widely to minimize the apoptotic fate or follicular loss by degeneration that occurs through the physiological process of atresia (Haag et al. 2013). Therefore, several researchers have sought the efficient development of the in vitro culture systems in the various species, such as sheep (Magalhães et al. 2011, Bandeira et al. 2015), goats (Magalhães et al. 2011, Duarte et al. 2013, Pessoa et al. 2014), bovines (Andrade et al. 2012, Sun & Li 2013), buffalos (Gupta et al. 2008), humans (Telfer et al. 2008), canines (Serafim et al. 2015) and in mice (Demeestere et al. 2002), with the purpose of finding clarification related to the follicular loss, making the follicles that would never reach ovulation can be used through this tool.

The efficiency of the culture system is related to the elaboration and substitution of a base medium that allows for the conditions necessary for follicular development. This system is indispensable for producing substances that are favorable and/or harmful for cultured cells. However, it has not yet been possible to standardize protocols for replacing the medium during culture. In equines, the IVC and the medium replacement interval has not been fully established, and great efforts are required to advance the technique, which is extremely important due to some reproductive peculiarities that the species present (Gomes et al. 2015).

In the last decade, the expansion of reproductive biotechnologies applied to Equidae has promoted an increase in the equestrian industry, directly reflecting the interest in this species (Gomes & Seneda 2013). Thus, it is notable the execution of applied research to continue the advancement in this segment. Therefore, the objective of the present investigation was to verify the effect of the medium replacement protocol after 24 and 48 hours at 2 or 6 days of *in vitro* culture using equine preantral follicles.

MATERIALS AND METHODS

Collection and transportation of ovaries. Ovaries (n=5) from five mares in seasonal anestrus of unknown age, body condition and reproductive status were collected from a slaughterhouse located approximately 40 km from the laboratory (latitude 23°17'34" S and longitude 51°10'14" W). At the slaughterhouse, the ovaries were washed in 70% alcohol followed by a wash in PBS (PBS; Embriolife®, Vitrocell, Brazil). The ovaries were transported to the laboratory in a temperature-controlled container, following the method used by Gomes & Seneda (2013).

Experimental protocol. Each ovary was carefully dissected to remove adipose and connective tissue. The ovary was sectioned along the sagittal plane, and ovaries containing abundant CL or antral follicles were discarded. The portion of the parenchyma (internal) of the five selected ovaries was cut into fragments of approximately 3x3x1mm. A fragment from each ovary was randomly selected and immediately fixed in Bouin. The remaining fragments (n=8) were individually cultured in 1ml aliquots of the culture medium in 24-well dishes with a 5% CO2 atmosphere in air and saturated humidity at 38.5°C.

The base culture medium used was the minimal essential medium (MEM, Gibco BRL, Rockville/MD, USA; osmolarity 300mOsm/L, pH 7.2) supplemented with ITS (insulin 6.25mg/mL, transferrin 6.25ng/mL, and selenium 6.25ng/mL), 0.23mM pyruvate, 2mM glutamine, 2mM hypoxanthine, 1.25mg/mL bovine serum albumin (BSA Gibco BRL, Rockville/MD, USA), 20 IU/ml of penicillin and 200mg/ml of streptomycin. Two fragments were intended for *in vitro* culture for two or six days, and the medium was completely replaced according to the treatment at either 24 (T24) or 48 (T48) hours. After the culture period, the ovarian fragments were evaluated using the classical histology technique. The staining was performed with Schiff's periodic acid (PAS) and hematoxylin.

Follicular classification. All sections were examined using light microscopy (Nikon®, Tokyo, Japan). Equine preantral follicles were classified according to the integrity of their structure as morphologically intact or degenerate, according to Andrade et al. (2012). Briefly, follicles were classified according to the integrity of their structure in morphologically normal (an intact oocyte, surrounded by granulosa cells well organized in one or more layers and absence of pycnotic nuclei) or degenerate (atretic follicles: pynotic nucleus and/or withdrawn, disorganization of the granulosa cells and cytoplasmic vacuoles. Preantral follicles were also evaluated for their development according to Gomes et al. (2015); these were classified as primordial or developing (primary or secondary) follicles. Briefly, primordial follicles (a layer of granulosa cells flattened around the oocyte) or primary (a single layer of granulosa cuboid cells), or secondary (two or more layers of granulosa cuboid cells).

Morphometric analysis. The slides were examined, and images were captured using a MOTICAM 2500 digital camera (5.0 M Pixel) then analyzed using the Motic Images Plus 2.0 ML software. The measurements of follicles and oocytes were recorded, according to Silva-Buttkus et al. (2008), and the oocyte and follicular diameters were calculated from the arithmetic mean of two perpendicular measurements.

Statistical analysis. Follicular integrity data from the five replicates were submitted for analysis of normality and homoscedasticity. The multiple comparisons between the experimental groups were performed using Fisher's test. The follicular development data in the five replicates were compared using Student's t-test. All analyses were performed using the software Action 3.1 version of R 3.0.2 (Campinas/SP, Brazil). The level of statistical significance was set at $p<0.05$.

RESULTS

The follicles were present in only 5.8% (217/3,750) of the fragments at different stages of development (Fig.1). Of the 399 follicles evaluated, 174 (43.6%) were primordial

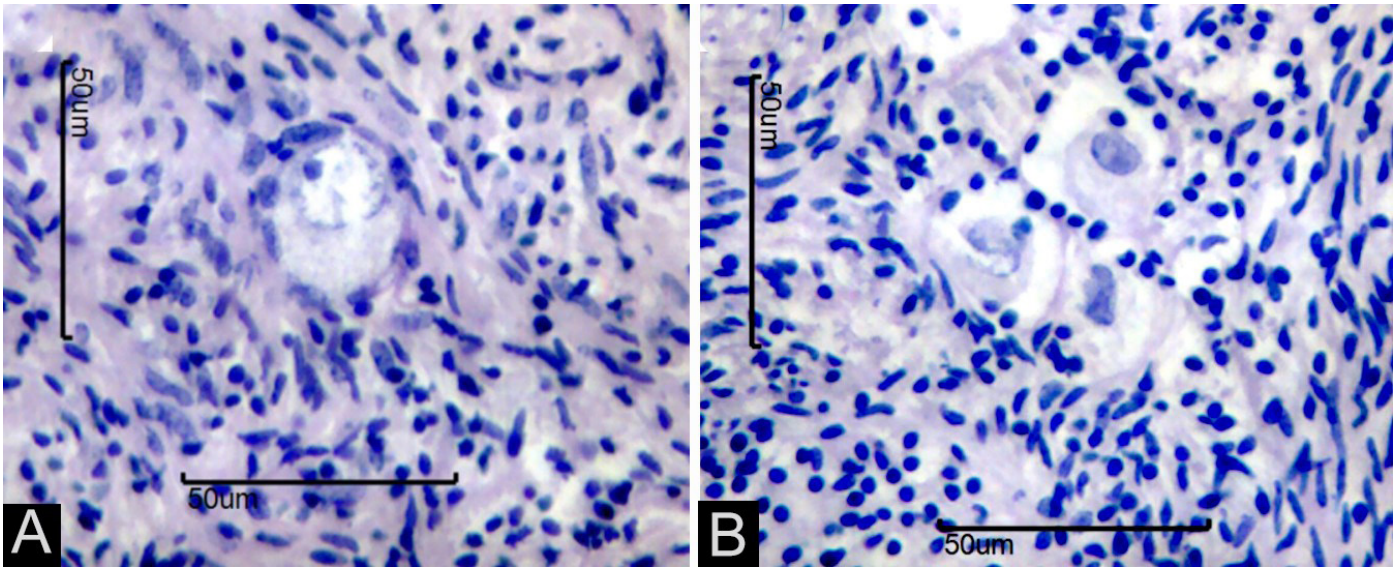

Fig.1. Morphological aspects of preantral follicles. (**A**) Intact primordial follicles and (**B**) degenerate primary follicles. PAS and hematoxylin, bar = 50µm.

follicles, 225 (56.4%) were in development and 63.76% were morphologically intact. On average, 91.8 follicles were found per treatment.

When the different protocols for medium replacement were analyzed, there was a significant difference in relation to the follicular integrity in the replacement of the medium at T48 compared to the daily exchange (T24) after two days of culture ($p<0.05$; Fig.2). When comparing the types of medium replacement over a six-day culture period of the intact ovarian follicles, the daily intervention demonstrated statistical significance ($p>0.05$; Fig.2). In this way, the culture over a two-day period presented the best result as a percentage of preantral follicles intact compared to the other treatments (T48 D2, T24 D6).

The morphometric analysis was performed on the follicle and oocyte (Fig.3). The mean follicle and oocyte diameter was measured at days two and six of the culture. The mean diameter of the follicles was found predominantly at the primary stage of development in most treatments. After the culture period, the mean diameter of the preantral follicles was similar for T24 and T48 replacement treatments after 2 days of culture (27.6±2.6µm, 29.2±2.3µm, respectively; $p>0.05$). While the mean follicle diameters did not show significant differences, replacements T24 h and T48 h showed mean diameters of 24.1±3.2 and 24.5±4.5µm, respectively ($p<0.05$). Compared to the diameter of the oocytes present in the follicles, there was no statistically significant difference according to the mean replacement and the culture period ($p>0.05$).

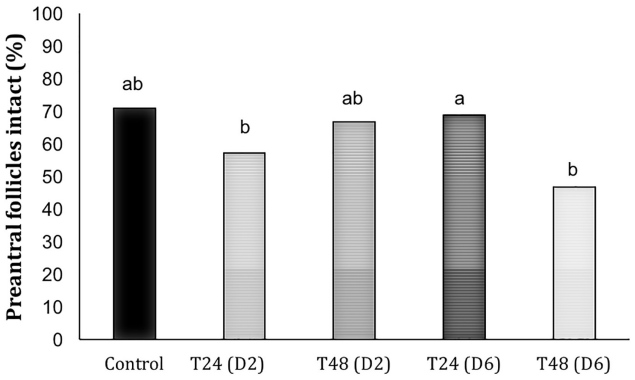

Fig.2. Percentage of preantral follicles cultured *in vitro* according to morphological integrity in relation to medium replacement in the following groups: daily exchange of the medium for two days (T24 D2), exchange of the medium every two days until the second day (T48 D2), daily exchange of the medium for six days (T24 D6) and exchange of the medium every two days until the sixth day (T48 D6). Values followed by lower case letters (a, b) differ statistically ($p<0.05$).

DISCUSSION

We evaluated the efficiency of replacing *in vitro* culture medium for preantral follicles in the ovarian tissue. We propose two methods of total substitution of the culture medium that consist of medium exchange every 24 and 48 hours. Our results showed a difference in the percentage of total follicles at six days after total daily culture medium exchange. On the other hand, during the first days of *in vitro* culture, the medium replacement could be done at 24h or 48h intervals for without causing interference in the morphological integrity of the follicle.

After 6 days of culture, the daily exchange treatment was the only treatment that could maintain the integrity of the cultured follicles (68.9%). Haag et al. (2013) performed *in vitro* culture for 7 days using ovarian fragments obtained by biopsy. They completely changed the medium every two days and obtained 65.5% of total intact follicles. Our research found similar results (59.2%); however, in the culture period (D6), which was similar to the study by Haag et al. (2013), we found that daily exchange was beneficial and had repercussions on the maintenance of follicular integrity. In contrast, similar studies in cattle (Andrade et al. 2012), sheep (Bandeira et al. 2015) and buffaloes (Gupta et al. 2008) obtained positive results

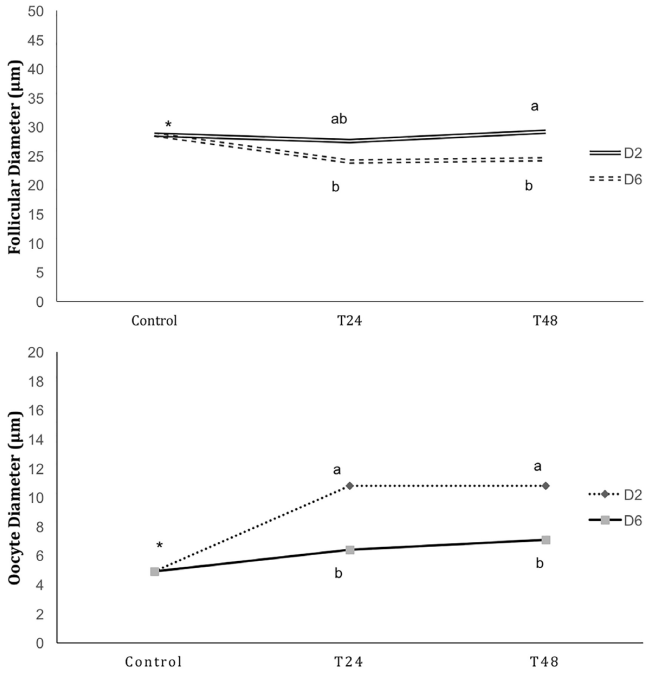

Fig.3. Percentage of mean diameter follicular and oocyte the preantral follicles (primordial and primary) and equine oocytes in fragments of uncultured ovarian tissue (control) and after two or six days in medium supplemented with different methodologies of total medium replacement: 24 and 48 hours exchange. *Not compared; values followed by lower case letters (a, b) differ statistically ($p<0.05$).

with the replacement of the medium every two days. In the present study, the replacement of the medium every 48 hours was beneficial for maintaining follicular integrity only at two days of culture.

The medium exchange interval affected the development of isolated cultured follicles after replacement every 2 days in caprine species (Magalhães et al. 2011). Our study showed that the replacement of the medium every two days did not have harmful repercussions for the ovarian follicles of equine species, and it could contribute to the development of these follicles. This finding opposes what was proposed in the abovementioned study.

For the in vitro culture of ovarian follicles, it is considered essential to replace the medium. The medium refreshment allows the withdrawal of the substances metabolized by the growing follicles, as well as the inclusion of new components to the follicles keep the development (Figueiredo et al. 2002).

We can infer that the follicular diameter observed on days 2 and 6 of *in vitro* culture did not change. This can be justified by the absence of substances in the culture medium that provide for the increase in follicular diameter. Replacement of culture medium is considered essential for *in vitro* culture technology using ovarian follicles since it washes away substances metabolized by the growing follicles and brings in new culture medium to support development (Figueiredo et al. 2002).

In most *in vitro* culture systems, the medium is totally or partially replaced with fresh medium every two days regardless of the culture period (O'Brien et al. 2003, Muruvi et al. 2005, Matos et al. 2007). In our work, we found that the total exchange of equine ovarian follicle in *in vitro* culture medium can initially be performed daily or every two days and subsequently done daily.

We identified few preantral follicles in this study, even after the removal of internal fragments of the equine ovary. This difficulty finding preantral follicles was also reported by Driancourt et al. (1982) and in recent investigations proposed by Gomes et al. (2015), Alves et al. (2015) and Gonzalez et al. (2017), in which they found little quantitative homogeneity of follicles in the evaluated fragments.

CONCLUSIONS

We found that the total exchange of *in vitro* culture medium for equine ovarian follicles can be performed daily in cultures up to 6 days. However, it is possible to carry out an exchange on the two days of cultivation and to continue the replacement with daily intervals.

This information contributes to the standardization of *in vitro* culture protocols through the adequate replacement of the medium during the culture of preantral follicles.

Conflict of interest statement.- The authors have no competing interests.

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