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Effect of fixative type and fixation time on the morphology of equine preantral ovarian follicles

Efeito do tipo de fixador e tempo de fixação na morfologia de folículos pré-antrais equinos

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Abstract

The aim of this study was to investigate the efficacy of the tissue fixatives Bouin, Carnoy and 10% Formaldehyde in equine ovarian fragments. Ovaries (n=4) from mares of mixed breeds were obtained at a local slaughterhouse and transported at 20 ºC in a thermo container. Immediately after collection, the ovaries were washed with a modified PBS solution (Cultilab®, Campinas-SP, Brazil) and divided into nine fragments with approximately 5x5x1 mm, removed from the parenchyma of each ovary. The ovarian fragments were then immersed in three different fixatives, Bouin (B) Carnoy (C) or 10% Formaldehyde (F) for 6, 12 or 24 hours. Each fragment was individually immersed in a 20 mL tube containing 20 times the volume of fixative solution. After this period, the fragments were held in 70% ethanol for 24 hours. Each procedure was performed in four replicates. For histological analysis, the specimens were dehydrated in increasing concentrations of alcohol, submitted to diaphanization in xylol and embedded in paraffin. Serial sections of 5 µm were made with the use of a rotating microtome (Leica® type, Wetzlar, Germany), followed by slide mounting and staining with periodic acid-Schiff (PAS) and hematoxylin. A total of 540 slides with 1,620 sections were evaluated, which contained 465 preantral follicles that were classified as normal or degenerated. Follicles were considered as degenerated when presented at least one of the following aspects: cytoplasm retraction, pyknotic nucleus, cytoplasmic vacuoles, displacement of granulosa cells and/or disruption of the basal membrane. A logistic regression test was used for statistical analysis, and differences were considered significant when $P<0.05$. The Carnoy fixative, when used for 24 hours, provided the best conditions of morphological integrity (53.3%; 32/60) compared to all others, and the use of Boiun for 24 hours was considered the worst treatment (19.1%; 9/47). The other treatments lead to the following results: C12h 50% (30/60), C6 H 40% (24/60), F24h 37.8% (17/45), F12h 35.1% (13/37), F6h 32% (16/50), B12h 30.5% (18/59) and B6h 24.4% (11/45). Therefore, we suggest that fixation of equine ovarian tissue with Carnoy for 24 hours is the most suitable protocol for morphological preservation of pre-antral follicles.

Key words: Bouin, Carnoy, equine, preantral follicles, 10% formaldehyde, ovary

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Resumo

O objetivo deste estudo foi investigar a eficácia dos fixadores teciduais Bouin, Carnoy ou Formol 10% em fragmentos ovarianos equinos. Ovários (n=4) de éguas, sem raça definida, foram obtidos de abatedouro local e transportados em recipiente térmico a 20 ºC. Imediatamente após a coleta, os ovários foram lavados com solução de PBS modificado (Cultilab®, Campinas-SP, Brasil), e divididos em nove fragmentos com aproximadamente 5x5x1 mm, retirados do parênquima de cada ovário. Em seguida, os fragmentos ovarianos foram imersos em um dos três diferentes fixadores, Bouin (B), Carnoy (C) ou Formol 10% (F), por 6, 12 ou 24 horas. Cada fragmento foi acondicionado individualmente em um frasco contendo aproximadamente 20 vezes o volume da solução fixadora. Após este período, foram mantidos em álcool 70% por 24 horas. Para cada fixador e tempo foram realizadas quatro réplicas. No processamento histológico, os fragmentos foram desidratados em concentrações crescentes de álcool, diafanizados em xilol e incluídos em parafina. Em seguida, foram feitos cortes seriados de 5 µm em micrótomo rotativo (Leica®, Wetzlar-Alemanha), seguidos da montagem de lâminas e coloração com ácido periódico de Schiff (PAS) e hematoxilina. Foram avaliadas 540 lâminas com 1.620 cortes histológicos, contendo 465 folículos pré-antrais que foram classificados como íntegros ou degenerados. A degeneração foi detectada pela presença de pelo menos um dos seguintes aspectos: retração do citoplasma, núcleo picnótico, vacúolos citoplasmáticos, deslocamento das células da granulosa e/ou rompimento da membrana basal. Um teste de regressão logística foi utilizado para a análise estatística, e as diferenças foram consideradas significativas quando $P<0,05$. O fixador Carnoy utilizado por 24 horas proporcionou as melhores condições de integridade morfológica (53,3%; 32/60) em relação aos demais, sendo Boiun por 24 h o tratamento menos eficaz (19,1%; 9/47). Os demais tratamentos apresentaram os resultados a seguir: C12h 50% (30/60), C6h 40% (24/60), F24h 37,8% (17/45), F12h 35,1% (13/37), F6h 32% (16/50), B12h 30,5% (18/59) e B6h 24,4% (11/45). Portanto, sugerimos que a fixação de tecido ovariano equino com Carnoy por 24 horas é o mais indicado para preservação morfológica de folículos pré-antrais.

Palavras-chave: Bouin, Carnoy, equinos, folículos pré-antrais, formol 10%, ovário

Introduction

In the last decade, the Brazilian horse herd was greatly improved, considering both the number of animals and the excellence of the breeding stock. The interest in research related to reproductive biotechnologies that apply to the equine species has constantly grown, thus facilitating and accelerating genetic improvement (CARMO et al., 2002; GOMES; SENEDA, 2013). In this context, the study of folliculogenesis is considered essential for understanding the mechanisms and factors involved in female reproductive physiology, leading to an increase in the reproductive performance of the herd.

The mammalian ovary is constituted by a follicular reserve pool that is formed by millions of preantral follicles. Each ovarian follicle is a morphofunctional unit comprising one oocyte involved with granulosa and theca cells that exerts gametogenic and exocrine activities (FIGUEIREDO et al., 2007). Although the ovary has tens or even hundreds of thousands of follicles, most of those follicles (around 99.9%) never reach ovulation due to a degeneration process named atresia (MARKSTRÖM et al., 2002). Many biotechnologies can be used to achieve a wide exploitation of the female gametes, such as cloning, intracytoplasmic sperm injection (ICSI) and in vitro culture of preantral ovarian follicles (FIGUEIREDO et al., 2008). In the equine species, reproductive biotechnologies present many shortcomings, especially due to the scarcity of ovaries that are available for research due to the reduced number of equine slaughterhouses in Brazil (OLIVEIRA et al., 2012). Therefore, equine ovaries should be used with the greatest possible efficiency, maximizing the knowledge about equine ovarian physiology.

Many techniques are available for morphological and ultrastructural ovarian analysis, especially for evaluation of preantral ovarian follicles. Most studies use basic histology as the main technique of follicular evaluation, considering that this method allows for a quantitative analysis. The histology processes is based on fixation, dehydration, diaphanization or clarification, infiltration and inclusion in liquid paraffin, as well as microtomy and staining of the slides for microscopic examination (MATOS et al., 2007; JUNQUEIRA; CARNEIRO, 2008). Once the ovary is obtained for experimental purposes, the best method for fixation must be established. The ovaries may be fixed shortly after the death of the mare or after the procedures related to the biotechnology of interest are performed.

Tissue fixation aims to preserve the original morphology and composition of the tissue and to inhibit enzymatic digestion, avoiding hardening of the fragments or other undesirable changes. Fixation is achieved with the use of different solutions named tissue fixatives. The duration of each protocol varies according to the size and constitution of the sample, as well as the fixative capacity of the substance and time of action (CULLING et al., 1985; SANTOS et al., 2012). Fixatives of alcoholic nature (10% Formaldehyde and Carnoy) require less time of fixation, accelerating the entire process; however, they can induce coagulation of proteins and nucleic acids, thus leading to tissue shrinkage, collapse and hardening the tissue (SANTOS et al., 2012).

In the chemical fixation, the fragments are immersed in solutions that are diffused through the tissue and promote preservation of the cellular ultrastructure and cellular matrix (JUNQUEIRA; CARNEIRO, 2008). However, if fixation is not correctly performed, the cells suffer retractions and morphological distortions that are clearly seen during histological evaluation. Several fixatives are used for tissue fixation, among these: Bouin, Carnoy and 10% Formaldehyde (BAUMGÄRTNER et al., 1988). The 10% Formaldehyde is commonly used for tissues assigned to evaluation by optical microscopy and is viable for different tissue types (FOX et al., 1985). Good results were obtained with 4% Paraformaldehyde (SILVA et al., 2004), Bouin (ANDRADE et al., 2005) and Carnoy (MATOS et al., 2004) for fixation of ovarian tissue obtained from sheep and cattle. However, data regarding the use of these fixatives in equine ovarian tissue are scarce. The objective of this study is to compare the maintenance of morphological integrity of equine ovarian fragments after fixation with 10% Formaldehyde, Bouin or Carnoy for a period of six, twelve or twenty-four hours.

Material and Methods

Collection and processing of ovaries

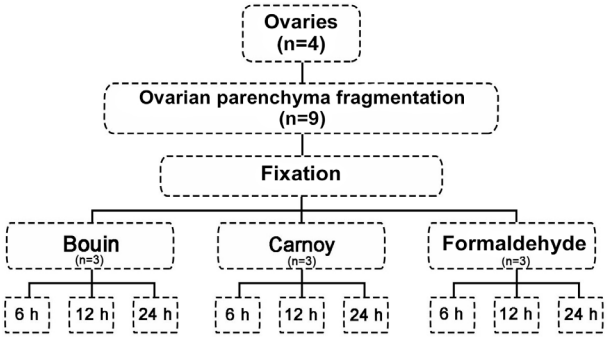
Ovaries (n=4) from four adult mares of mixed breeds were obtained from a local slaughterhouse and transported to the laboratory (latitude 23° 17' 34" S and longitude 51° 10' 24" W) located 40 km (30 minutes) away from the laboratory. The ovaries were transported in a thermal container at room temperature (20 ºC). Immediately after collection, each ovary was washed with a modified PBS solution (Cultilab®, Campinas-SP, Brazil) and then divided into 9 fragments of approximately 5x5x1 mm. These fragments were immersed in three different fixatives: Bouin, Carnoy and 10% Formaldehyde, during 6, 12 or 24 hours (B6h, B12h, B24h; C6h, C12h, C24h; F6h, F12h, F24h; Figure 1). The fragments were individually held in tubes containing 20 times the volume of histological piece.

Histological processing

After by 6, 12 or 24 hours of fixation, the fragments were immersed in 70% alcohol for 24 hours. For histological analysis, the fragments were dehydrated in increasing concentrations of alcohol,

diaphanized in xylene and embedded in Paraplast Plus (Ted Pella, Inc, CA, USA). Subsequently, serial sections of 5 μm were performed with a rotary microtome (Leica®, Wetzlar, Alemanha), mounting of the slides and staining with periodic acid-Schiff (PAS) and hematoxylin. The slides were evaluated under an optic microscopy (Nikon®, Tokyo, Japan), by a single evaluator. In each treatment, all follicles found were evaluated. Preantral follicles were morphologically classified as normal or degenerated. The following aspects were considered as degeneration signs: disruption of the basement membrane, shrinking of the cytoplasm, cytoplasmic vacuoles, pyknotic nucleus and displacement of granulosa cells (LUCCI et al., 2002).

Figure 1. Experimental protocol for assessment of fixatives for equine ovarian tissue, used in different periods of time.



Statistical analysis

The experimental design was fully randomized with four replications and 36 treatments. Data regarding follicular viability were submitted to normality (Shapiro) and homoscedasticity (Levenne) tests. The multiple comparisons of follicular viability between the experimental groups were carried out by a simple logistic regression analysis, with 5% of significance.

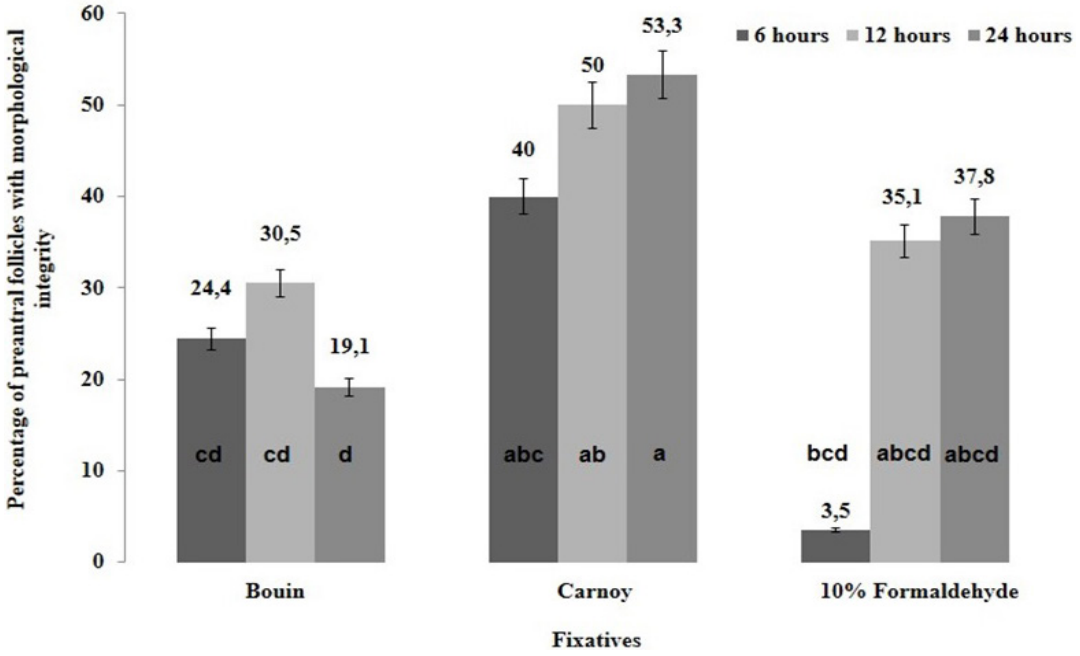
Results

This study evaluated 1,260 blades containing 3,780 histological cuts from 36 ovarian fragments, obtained from four mares. Follicles were found in only 10.6% (401/3,780) of the evaluated fragments, which were at different stages of development. In 89.4% of histological sections (3,379/3,780), from the inner portion of ovarian tissue, no follicles were observed. From the 463 evaluated follicles, 281 (60.7%) were primordial follicles and 182 (39.3%) were developing follicles, out of which 36.7% were morphologically normal.

The fixatives Bouin, Carnoy e and 10% Formaldehyde differed regarding the morphological integrity of the preantral follicles. The fragments fixed with Carnoy presented a greater percentage of normal follicles when compared to the other fixatives, especially when treatment lasted for 24 hours, which presented 53.3% (32/60) of normal follicles (Figure 2; $P<0.05$).

Treatment with Bouin showed the highest percentage of degenerated follicles, and the 24 hours period showed the lowest rate of intact follicles (19.1%). Fixation of ovarian fragments with 10% Formaldehyde showed satisfactory results in all periods tested; however, the proportion of follicles with normal morphological integrity was lower than the rate observed with the Carnoy treatment (Figure 3; $P<0.05$). The percentage of morphologically normal and degenerated preantral follicles observed in each treatment is presented in Table 1.

Figure 2. Percentage of morphologically normal preantral follicles found in fragments of equine ovarian tissue equine fixed with three different fixatives for 6, 12 or 24 hours.



a, b, c - lowercase letters indicate differences within the same fixative solution (P<0.05).

Figure 3. Histological illustration of equine preantral follicle morphology. (A) Normal primordial follicle fixed in Bouin; (B) Degenerated primordial follicle fixed in Bouin; (C) Normal primordial follicle fixed in Carnoy; (D) Degenerated primordial follicle fixed in Carnoy; (E) Normal primordial follicle fixed in 10% Formaldehyde and (F) Degenerated primordial follicle fixed in 10% Formaldehyde. The fragments were stained using periodic acid - Schiff and hematoxylin.

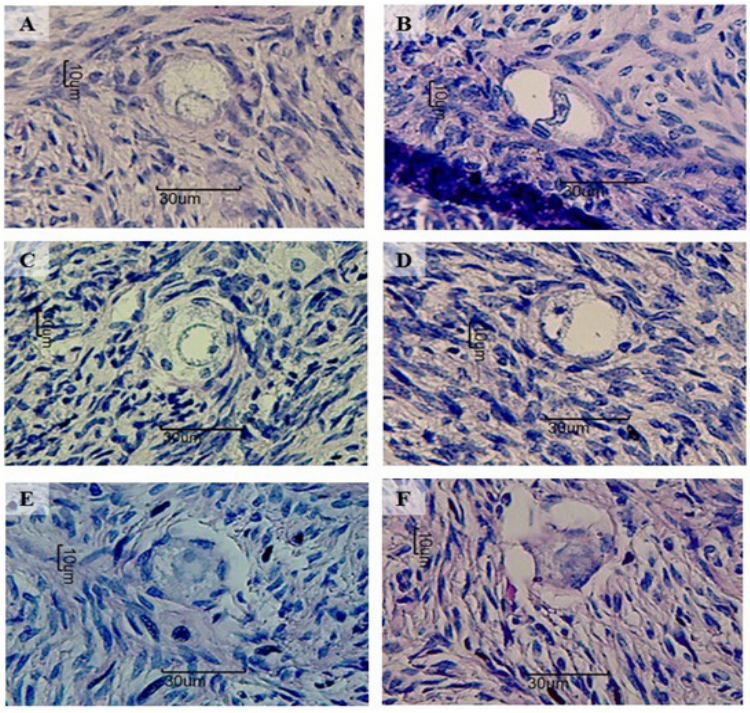


Table 1. Evaluation of different fixatives and periods of fixation of equine ovarian tissue according to the morphology of primordial follicles.

Treatments	Follicular morphological classification		
	Normal	Degenerated	Total
Carnoy 24H	32 (53.3%) [a]	28	60
Carnoy 12H	30 (50.0%) [ab]	30	60
Carnoy 6H	24 (40.0%) [abc]	36	60
10% Formaldehyde 24H	17 (37.8%) [abcd]	28	45
10% Formaldehyde 12H	13 (35.1%) [abcd]	24	37
10% Formaldehyde 6H	16 (32.0%) [bcd]	34	50
Bouin 12H	18 (30.5%) [cd]	41	59
Bouin 6H	11 (24.4%) [cd]	36	47
Bouin 24H	9 (19.1%) [d]	38	47

Values with different letters in the same column differ significantly ($P<0.05$).

Discussion

To the best of our knowledge, this is the first report of a comparison between three different tissue fixatives, used in three different fixing periods, for preservation of morphological ovarian integrity in the equine species. This study demonstrated that the Carnoy fixative, used for 24 hours, conserved the morphological integrity of preantral follicles. Puchtler et al. (1968) also described satisfactory outcomes of tissue preservation with the use of Carnoy, which efficiently stabilized cellular structure and minimized tissue shrinkage. Pereira et al. (2015) described Carnoy as an excellent choice for preservation of both animal and human tissue and demonstrated that this fixative can be used for immunohistochemistry. This fact is possible because of Carnoy's formulation, which contains ethanol, acetic acid and chloroform, generating a great conservative potential for conservation of mammalian tissues (MAKKUS et al., 1994).

In the fragments fixed with Carnoy and 10% Formaldehyde, a lower proportion of degenerated preantral follicles was observed in comparison with Bouin, indicated that the latter is not suggested for equine ovarian tissue preservation. In sheep, Carnoy effectively preserved the basement membrane of the cells, leading to 80% of normal follicles (MATOS et al., 2004). However, Santos et al. (2012) observed that the use of Bouin and Carnoy resulted in a better maintenance of bovine ovarian morphology. Therefore, Carnoy may be indicated as a fixative for ovarian tissue of cattle, horses and sheep; however, Bouin should not be indicated for equine ovarian tissue. These outcomes can be explained by Bouin's composition (picric acid, formaldehyde and acetic acid), which can cause serious damage to tissue proteins and result in loss of the homogeneous appearance of cells (COLE; SYKES, 1974; LOCQUIN; LANGERON, 1983).

In the present study, satisfactory results regarding the maintenance of cell structure of the equine ovary were obtained with the use of 10% Formaldehyde. Our study confirms the findings of Bruno et al. (2008), which observed about 76% of preantral follicles with satisfactory cell morphology in goats. In cattle, Santos et al. (2010) and Castro et al. (2014) obtained conflicting results, with approximately 70% of degenerated follicles.

A discrepancy regarding the number of follicles found in each treatment was observed in this study. Importantly, there is a wide individual variation concerning preantral follicular population in horses, as well as a great variation in the number of follicles in comparison to other species (DRIANCOURT et

al., 1982). Alves et al. (2015) suggested a possible variation in follicular distribution within the ovarian structure; also, the influence of genes that control primordial germ cells survival, proliferation, colonization and inclusion. This behavior was also reported by Gomes et al. (2015), which did not observe a quantitative homogeneity in the distinct treatments when studying in vitro culture of equine preantral follicles.

Conclusion

The Carnoy fixative, used for 6, 12 or 24 hours, is the most appropriate fixative solution to preserve the morphology and integrity of equine preantral follicles. Among all the groups tested, Carnoy used for 24 hours presented the best result regarding the proportion of normal follicles, and can be efficiently used in studies about reproductive technologies in the equine species.

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