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Short Communication

Methods for Equine Preantral Follicles Isolation: Quantitative Aspects

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Contents
The aim of this study was to test the use of mechanical and mechanical-enzymatic methods, saline solution (SS), and PBS solution for the manipulation and isolation of mare ovarian preantral follicles (PAFs). The ovaries were subjected to mechanical isolation (mixer) alone or in association with enzymatic digestion (collagenase). Incubation times of 10 and 20 min were employed. In the first group, 4.1 ± 4.9 PAFs were harvested with the mechanical-enzymatic method vs 71.1 ± 19.2 with the mechanical procedure, showing a significant difference between methods; using SS and PBS, these numbers were 35.7 ± 34.3 and 39.6 ± 39.6, respectively, with no significant difference between solutions. In the second group, there was significant difference between methods, with 7.1 ± 10.6 follicles harvested with the mechanical-enzymatic method vs 63.2 ± 22.9 with the mechanical procedure; using SS and PBS, means were 35.5 ± 36.4 and 34.9 ± 31.1, respectively. The mechanical method proved more effective than the mechanical-enzymatic approach. Both SS and PBS can be used as a media for equine PAFs preparation.

Introduction

Development of new biotechnologies is an important tool for conserving genetic material. Cryopreservation has been greatly employed in the field with this purpose. Preantral follicle (PAF) isolation allows a large number of oocytes to be harvested for cryopreservation of genetic material, using mechanical (Taru Sharma et al. 2009) and/or enzymatic methods (Itoh and Hoshi 2000).

These studies, however, are limited in horses. Enzyme-based methods in association with mechanical procedures are the most frequently used approaches for domestic animals such as sheep (Nandi et al. 2009). The main enzyme employed for this purpose is collagenase (Kristensen et al. 2011).

Mechanical methods were also tested. Tissue chopper is the device most often employed (Haag et al. 2013). Nevertheless, the lower cost of vertical mixers and its capacity of fragmentation are considerable factors to its use (Nuttinck et al. 1993).

Ovarian tissue fragmentation with mixer is possible by adding a solution that promotes dissociation while conserving follicular structures. Several media have been tested, including 0.9% saline solution (SS) (Munhoz and Luna 2008), PBS (Santos et al. 2002) and coconut water (Costa et al. 2002).

The purpose of the present study was to test a mechanical method and a mechanical-enzymatic procedure of follicular isolation, and the capacity of SS and PBS was to keep the follicle morphological structure after the manipulation.

Material and Methods

Ovaries

Equine ovaries (n = 20) were collected from an abattoir and immediately frozen. After thawing, the ovarian capsule was dissected and then each ovary was washed in SS and 70% alcohol. Antral follicles were punctured and corpora lutea excised. A sagittal sectioning of the gonad was performed.

Isolation of preantral follicles

Preantral follicle isolation was carried out using either a mechanical method (MM) employing a vertical mixer (Nuttinck et al. 1993) or a mechanical method associated with enzymatic digestion (ED) (collagenase, 200 IU/ml, Sigma Aldrich®, Sigma Aldrich do Brasil, São Paulo, Brasil). Two solutions – 0.9% SS and PBS, supplemented with 10% BSA – were tested for their effects on ovarian tissue fragmentation and manipulation.

The methods were tested assigned to two groups, with ten repetitions per group, as follows: Group 1, MM + ED-10 (10-min enzymatic digestion); Group 2, MM + ED-20 (20 min). Figure 1 depicts the experimental scheme.

The ED aliquot was incubated in a water bath at 37°C, and consecutive pipetting (100 times) was performed at 5-min intervals 2 (10 min) or 4 (20 min) times.

In both groups, the final volume was filtered in nylon mesh filters of 500 and 200 μm to select only PAF. The filtered volume was evaluated under bright field microscopy at 400 × magnification.

Statistical analysis

Data were subjected to analysis of variance to account for the individual effects of the methods of PAF isolation (mechanical or mechanical-enzymatic), solutions employed (SS or PBS) and the interaction between them. Means were compared by Student's *t*-test, and $p \leq 0.05$ was regarded as significant.

Results

The mean number of follicles recovered per ovary was 145.7, and the mean number of follicles isolated per

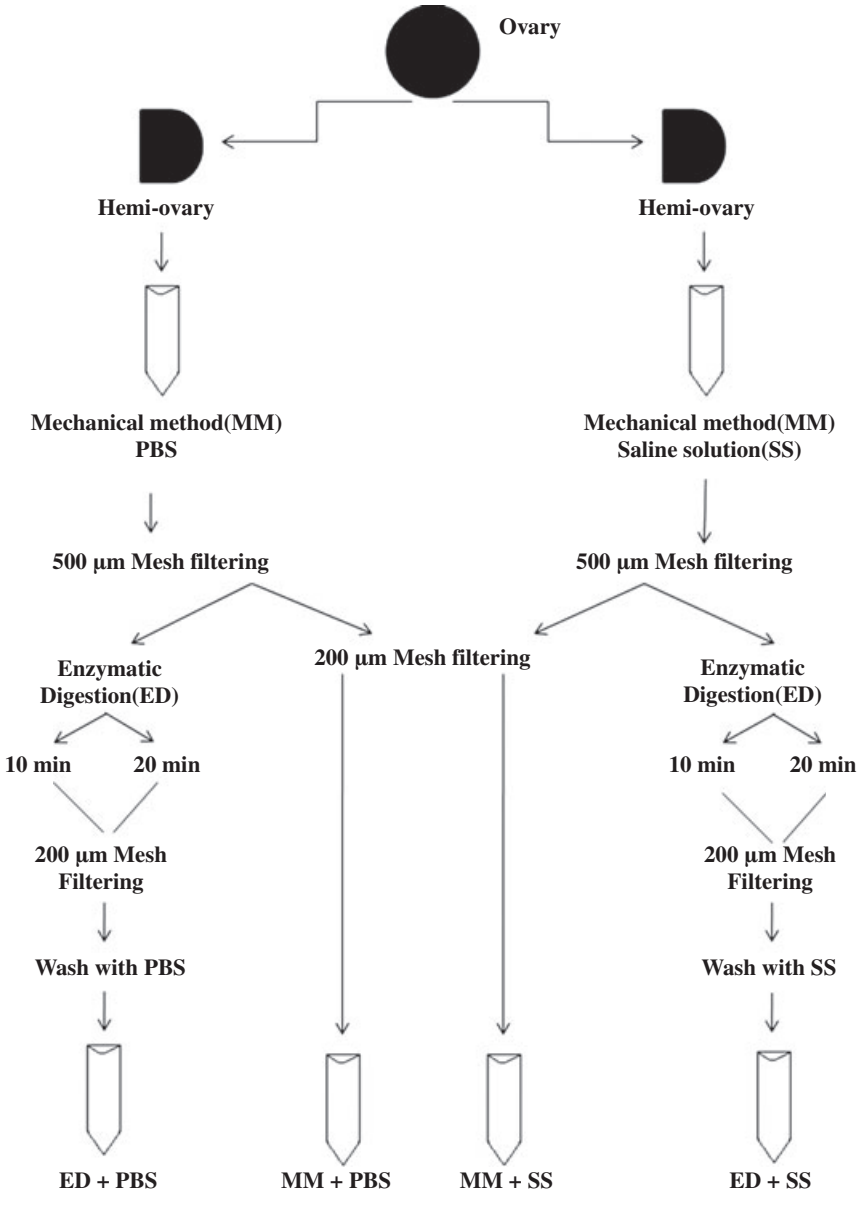

Fig. 1. Experimental design

gram of ovary was 9.93. The mean number of PAFs harvested in Groups 1 and 2 is given in Table 1.

It was revealed no significant effect of exposure time on the total number of PAFs harvested (4.15 ± 4.93 for 10-min vs 10.62 ± 7.15 for 20-min exposure).

The mean number of PAFs harvested using the different solutions is given in Table 2.

Table 1. Mean number (± SD) of equine preantral follicles isolated from ovaries by mechanical and mechanical-enzymatic methods

	Method employed for preantral follicle isolation	
Enzymatic digestion times*	Mechanical (mixer)	Mechanical-enzymatic
10 min (Group 1, n = 10)	71.15 ± 19.27[a]	4.15 ± 4.93[b]
20 min (Group 2, n = 10)	63.25 ± 22.97[a]	7.15 ± 10.62[b]

Different letters on the same row indicate significant difference ($p < 0.05$, Student's *t*-test).
*Collagenase (200 IU/ml).

Table 2. Mean number (± SD) of equine preantral follicles isolated from ovaries after manipulation of ovarian tissue in 0.9% saline solution (SS) and PBS

	Solutions employed	
Enzymatic digestion times[a]	SS	PBS
10 min (Group 1, n = 10)	39.60 ± 39.63	35.70 ± 34.35
20 min (Group 2, n = 10)	34.90 ± 31.11	35.50 ± 36.46

$p > 0.05$ (Student's *t*-test).
[a]Collagenase (200 IU/ml).

For both collagenase exposure times, ANOVA revealed a significant effect of the isolation method, but not of the solution employed ($p > 0.05$) or of the interaction between them. For 10-min enzymatic digestion, the coefficients of determination (R^2) and variation (CV) were 0.98 and 26.74, respectively; for 20 min, 0.95 and 37.04, respectively.

Discussion

In the present study, relatively few mare PAFs were recovered compared with studies on different domestic species. In one such study, 99 300 bovine PAFs were harvested using a mechanical-enzymatic procedure (Carambula et al. 1998). In fact, isolation of equine preantral follicles with tissue chopper revealed a high number of these structures; in 254.2 mg of mare ovarian tissue, 188 PAFs were recovered – range of 739.5 PAF per gram (Haag et al. 2013). However, in the present study, it was recovered only 9.93 PAFs per gram. With a collagenase-based enzymatic method, Telfer and Watson (2000) obtained fewer PAFs in mares, compared with other species although in mares, quiescent follicular populations are known to range from 6400 to 75 200, irrespective of breed (Driancourt et al. 1982).

Despite recovering a lower number of PAFs, mechanical isolation methods contribute to preserving follicles, particularly theca cells. Enzymatic isolation is known to damage ovarian structures more than mechanical methods (Rossetto et al. 2011). It is possible to attribute the lack of isolated follicles with collagenase in the present study to the fact mentioned, once the PAFs might have been degenerated.

Telfer and Watson (2000) tested collagenase for isolation and concluded that a greater number of follicles degenerated upon 90 and 120 min. The investigators attributed the low yield of the enzymatic method to degradation of structures, particularly the zona pellucida, by collagenase or to low effectiveness of the technique in mares. It can therefore be assumed that the number of PAFs isolated in the present study was a result of the degradation of follicular structures by the enzyme. Because the ovaries had been frozen, it can be hypothesized that PAFs with some degree of damage from freezing–thawing became more susceptible to collagenase and were digested over the incubation period.

Santos et al. (2002), evaluating the effectiveness of 0.9% SS and PBS in the conservation of goat PAFs, concluded that both solutions can be employed to retain the integrity of follicular structure. Other experiments revealed that SS did not differ from TCM-199 or coconut water (Costa et al. 2002) in its effect on PAF isolation. Both experiments mentioned are consistent with the results obtained in the present work, where no differences were observed between the solutions used.

The mechanical method is the best way to isolate PAFs from equine ovarian tissue concerning to number of follicles harvested. Both SS and PBS can be used with this aim. Research with female germ cells included in PAF should be encouraged, considering the great availability of these structures.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

The authors Leonel, Bento-Silva and Ambrozio contributed to the laboratorial development of this work (process of isolating and counting follicles). The authors Costa e Silva and Luna contributed to the research design and to the analysis and interpretation of data. The author Zúccari developed the research design and supervised the whole experiment, since the laboratory job until the final creation of the manuscript. The author Leonel was responsible to the manuscript development and submission to this journal.

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