Effect of sectioning on the number of isolated ovine preantral follicles

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Abstract

The aim of the present study was to test the effect of the interval of serial sections in the tissue chopper on the number of isolated ovine preantral follicles. Best results were obtained when the ovarian fragments were cut in the tissue chopper at interval of 87.5 µm (1592 preantral follicles per treatment). Histochemical analysis showed that the follicular morphology was preserved after mechanical isolation as demonstrated by the normality of oocytes and granulosa cells as well as by preservation of basement membrane. The percentages of isolated primordial, primary and secondary follicles were 92, 6, and 2%, respectively. The follicular sizes varied from 12.5 to 96.3 µm in diameter. In conclusion, a greater number of isolated preantral follicles were obtained when the method of isolation used the tissue chopper adjusted at 87.5 µm. Besides, this treatment does not affect the follicular integrity after the isolation procedure. ® 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

At birth, the mammalian ovary contains a large number of primordial follicles, but during the reproductive life span of the female just a few viable oocytes are produced. The vast majority of the follicles will become atretic during their growth and maturation and will not end up as a completely mature follicle (Taha and Schellander, 1992).

The development of reproductive technologies such as, in vitro maturation and fertilization, embryo transfer, gene transfer, embryo sexing and cloning offer an important tool for rescuing genetic material from valuable and endangered animals. Current methods of superovulation and in vitro maturation of oocytes from antral follicles have not provided sufficient material to allow rapid progress. Moreover, superovulation methods can not manipulate the large hormone insensitive population of immature oocytes.
Development of successful procedures to use the large number of preantral follicles could be a potential supply of mature oocytes for in vitro manipulation. However, these procedures are dependent upon efficient methods to isolate preantral follicles. Mechanical and/or enzymatic procedures for the isolation of preantral follicles have been described in several animals, such as cows (Jewgenow and Pitra, 1991; Figueiredo et al., 1993, 1995), pigs (Greenwald and Moor, 1989), mice (Eppig, 1976; Eppig and Schroeder, 1989; Carroll et al., 1990, 1991; Qvist et al., 1990), rats (Grob, 1964), domestic cats (Jewgenow and Pitra, 1993; Jewgenow and Göritz, 1995), nondomestic felids (Jewgenow et al., 1997), and marsupials (Butcher and Ullmann, 1996). Specially for ruminant species, Figueiredo et al. (1993) developed a mechanical method to isolate preantral follicles from bovine ovaries. This method consists of the cut of the ovary using the tissue chopper adjusted at 500 μm. The procedure resulted in a great number of isolated preantral follicles, and later, Rodrigues et al. (1998) successfully used the same method for the isolation of caprine preantral follicles. However, this method was not tested in ovine ovaries. Besides, it is not known if the different cut intervals in the tissue chopper influence the number of isolated ovine preantral follicles.

The aim of the present study was to analyze the effect of the cut of the ovary using the tissue chopper adjusted at 500 μm. The procedure resulted in a great number of isolated preantral follicles, and later, Rodrigues et al. (1998) successfully used the same method for the isolation of caprine preantral follicles. However, this method was not tested in ovine ovaries. Besides, it is not known if the different cut intervals in the tissue chopper influence the number of isolated ovine preantral follicles.

The aim of the present study was to analyze the effect of the cut intervals in the tissue chopper on the number of isolated ovine preantral follicles. In addition, histological analysis was used to investigate the quality of the isolated preantral follicles.

2. Materials and methods

2.1. Source of ovaries

Ovaries (n=16) from adult undefined breed type ewes were collected at a local slaughterhouse. The ovaries were washed in 70% alcohol for approximately 10 s, and then twice in 0.9% saline solution. Finally, each ovary was transferred into 10 ml of saline solution and transported to the laboratory within 1 h in a thermostand filled with water at 4°C.

2.2. Isolation of ovarian preantral follicles from ovine ovaries

2.2.1. General protocol

In this study, the development of a specific mechanical method to isolate ovarian preantral follicles from the adult ewe was performed based on the mechanical procedure described by Figueiredo et al. (1993) to bovine ovaries. Briefly, the ovaries were cut into small fragments using a tissue chopper (The Mickle Laboratory Engineering, Gomshal, Surrey, England) adjusted at 500 μm. The ovarian fragments were suspended 40 times with a Pasteur pipette and filtered successively through 500 and 100 μm nylon mesh filters. The number of isolated preantral follicles in the suspension containing fragments <100 μm (suspension <100 μm) was counted and classified, using an inverted microscope.

2.2.2. Experimental protocol

This work tested the effect of the interval of serial sections in the tissue chopper on the number of isolated preantral follicles from ovine ovaries. For this, it was tested with eight treatments which differed to each other according to the cut interval adjusted in the tissue chopper: 12.5 (T1), 25.0 (T2), 37.5 (T3), 50.0 (T4), 62.5 (T5), 75.0 (T6), 87.5 (T7) and 100.0 μm (T8). To test simultaneously all the treatments, for each replica, both ovaries from the same animal were divided into four parts, resulting in eight pieces, which were distributed randomly to the eight tested treatments. For each treatment the ovarian piece was cut in the tissue chopper from the cortical to medullar side. Then, the obtained fragments were placed in phosphate buffered saline (PBS) plus 5% ovine serum, suspended 50 times with a large Pasteur pipette (diameter ~1600 μm) and 50 times with a smaller pipette (diameter ~600 μm). After that, the suspension was filtered successively through 500 and 100 μm nylon mesh filters (Fig. 1). The final volume of the suspension was 30 ml. To estimate the number of isolated follicles per treatment, the suspension was homogenized and then, two samples of 1 ml were taken and examined separately at the inverted microscope. The number of preantral follicles presented in the suspension was calculated as follows:

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\text{Number of follicles counted in the suspension 1} + \text{number of follicles in the suspension 2} \times \frac{\text{final volume}}{2}.
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The follicular diameters were measured with an ocular micrometer. The isolated preantral follicles were classified by their morphological appearance according to Hulshof (1995). Primordial follicles are oocytes surrounded by one layer of squamous or squamous-cuboidal granulosa cells; primary follicles have a single layer of cuboidal granulosa cells, and secondary follicles are oocytes surrounded by two or more layers of cuboidal granulosa cells.

Each treatment was repeated eight times and the time spent to perform each treatment was recorded. All the experiments were performed at room temperature (27°C).

2.3. Histological analysis of the isolated preantral follicles

Follicular quality was evaluated only in the treatment that yielded the largest number of isolated preantral follicles. To evaluate the quality of the isolated follicles, parameters as integrity of the basement membrane, cellular density, presence or absence of picnotic bodies and integrity of the oocyte were observed. The isolated preantral follicles were fixed in 2% para-formaldehyde, 2.5% glutaraldehyde and 0.1 M cacodylate buffer, during 3 h at room temperature. Subsequently, they were centrifuged at 4000 rpm for 15 min, and then, washed with 3 ml of 0.1 M cacodylate buffer solution.
The follicles were embedded in drops of 200 µl of 4% agar solution (agarose, Type VII, Sigma, St. Louis, MO, USA). The agar drops were submitted to the same fixation process described above and dehydrated in a graded series of ethanol. Finally, the drops were embedded in paraffin wax. Then, 7 µm sections were stained with periodic acid Schiff (PAS) and haematoxylin. It was used to analyze 50 follicles from each follicular class.

2.4. Statistical analysis

The Whitney–Mann test was used to compare the number of isolated preantral follicles among treatments. Values were considered statistically significant when \( P<0.05 \).

3. Results

3.1. Isolation of ovarian preantral follicles

Table 1 shows the effect of eight different intervals of serial sections in the tissue chopper on the number of isolated preantral follicles per ovary. Large numbers of isolated preantral follicles were recovered from the ovarian tissue in the mechanical treatments although large individual variation in the number of isolated follicles within the treatments was observed. The limits of this variation ranged from 28 to 2842 preantral follicles. Despite that, T7 (87.5 µm) showed a significantly higher number of isolated preantral follicles compared with the other treatments.

The time spent to isolate preantral follicles decreased as the cut interval increased and it varied from 12.7 min (T8: 100.0 µm) to 25.2 min (T1: 12.5 µm).

3.2. Morphology and distribution of fresh isolated preantral follicles

The suspensions from the mechanical procedure contained isolated primordial, primary and secondary follicles (Fig. 2), ovarian stroma cells, and large tissue parts. Freshly isolated preantral follicles appeared healthy under the inverted microscope. They were spherical, with one or more organized layers of granulosa cells around the oocyte, with no antrum.

Isolated primordial, primary and secondary follicles had a mean diameter of 18.0±4.5, 35.1±7.02, and 66.3±13.26 µm, respectively, and varied from 12.5 to 96.3 µm. Primordial follicles were characterized by an oocyte surrounded with one layer of flattened granulosa cells. In primary follicle, only a small part of the oocyte was visible; the remainder was covered by one layer of cuboidal granulosa cells. Secondary follicles had more than one layer of cuboidal granulosa cells. The oocyte was not always clearly visible because it was covered by granulosa cells.

Taking the treatments together, the percentages of isolated primordial, primary and secondary follicles were 92, 6, and 2%, respectively.

3.3. Histology of isolated preantral follicles

To evaluate follicular quality in the treatment that yielded the largest number of isolated preantral follicles (T7: 87.5 µm), histological examinations were performed. The morphological normal appearance of isolated preantral follicles at the inverted microscope was confirmed by histology. The T7 (87.5 µm) allowed the preservation of follicular integrity (Fig. 3). Basement membrane surrounding follicles was strongly stained PAS-positive. After mechanical isolation of the follicles, this basement membrane was still there, as revealed by histochemistry. The granulosa cells were well organized and without signs of necrotic bodies. The oocytes were slightly stained, without signs of degeneration or retraction.
Fig. 2. Isolated preantral follicles (arrow heads) from ovine ovary recovered after the mechanical procedure: morphologically normal (a) primordial, (b) primary and (c) secondary follicles (×300).
Fig. 2. (Continued).

Fig. 3. Histological section of preantral follicle stained with PAS and haematoxylin showing the normality of the oocyte and granulosa cells (×950).
4. Discussion

The results of this work show that a large number of ovine preantral follicles can be isolated using a simple and rapid mechanical procedure. The number of preantral follicles isolated by the treatments ranged from 28 to 2842 per treatment. The tissue chopper adjusted at 500 μm isolated a great number of preantral follicles from cow (Figueiredo et al., 1993) and goat (Rodrigues et al., 1998). In contrast, to isolate preantral follicles in our study, best results were obtained when the ovarian fragments were cut in the tissue chopper at an interval of 87.5 μm (1592 follicles per treatment). It seems that the use of smaller cut intervals may destroy preantral follicles, while larger intervals may reduce the efficiency of mechanical dissociation of the ovarian fragments with Pasteur pipette and in turn prevent preantral follicle release from the ovarian tissue.

Morphological examination using the inverted microscope has been shown to be very useful for the evaluation of isolated preantral follicles. However, histological analysis has been shown to be indispensable for the identification of an intact basement membrane and to judge the quality of the granulosa cells and oocyte. In addition to the large number of isolated preantral follicles, T7 (87.5 μm) allows the preservation of the oocyte and granulosa cells. The integrity of these cells is necessary for the in vitro development of the follicles. In our study, histochemical analysis demonstrated that mechanically isolated preantral follicles are surrounded by an intact basement membrane. Similar results were obtained by Figueiredo et al. (1994) and Hulshof (1995) using the tissue chopper to isolate bovine preantral follicles. Figueiredo et al. (1995) suggest that mechanical resistance of the basement membrane presumably protects the follicles from physical damage during isolation. In vitro, the culture of preantral follicles surrounded by a natural basement membrane may have many advantages including preservation of follicular morphology and maintenance of follicular adhesion to extracellular compounds (Figueiredo et al., 1995). Thus, the presence of a basement membrane around the isolated follicles may be important in further studies of in vitro culture of ovine preantral follicles.

Mechanical procedures for the isolation of preantral follicles are described in the literature. Some authors isolated a large number of preantral follicles from bovine ovaries using the tissue chopper (2142 follicles per ovary: Figueiredo et al., 1993), forceps (2918 follicles per ovary: Hulshof et al., 1994), mixer (90 follicles per ovary: Nuttinck et al., 1993), and ovarian scraper (335 follicles per ovary: Jewgenow and Pitra, 1991). Rodrigues et al. (1998) isolated 1067 follicles per ovary using the tissue chopper in caprine ovaries and the cell dissociation filters were used to isolate follicles from felids (300–24,650 follicles per domestic cat ovary: Jewgenow and Göritz, 1995, 1867 follicles per nondomestic felid ovary: Jewgenow and Stolte, 1996). With regard to the enzymatic procedures for the isolation of follicles, collagenase has been successfully used to isolate preantral follicles from hamster (125 follicles per ovary: Roy and Greenwald, 1985), pig (180,000 follicles per ovary: Greenwald and Moor, 1989), and woman (760 and 544 follicles per ovary, respectively, to 16- and 35-year-old women: Roy and Treacy, 1993). Other enzymes, such as pronase and trypsin were used to isolate preantral follicles from mouse (Grob, 1969) and pig (Morbeck et al., 1993). Although enzymatic treatments can also be used for the isolation of preantral follicles, some studies showed that enzymes may be harmful to the follicles, causing damages to the oocytes, especially in the smallest preantral follicles (Wandji et al., 1996), and in the basement membrane (Nicosia et al., 1975). Comparisons of results found in literature with our results are difficult to perform due to species-specific ovarian composition, variation in follicle classification, and types of follicles included in the final count.

Taking all the treatments together, the percentage of primordial follicles was higher than that of primary and secondary follicles. Similar proportions were reported in histological study by Driancourt et al. (1985), and Erickson (1966). Rodrigues et al. (1998) also showed similar results in the isolation of caprine preantral follicles. Jewgenow and Göritz (1995) showed that the population of isolated preantral follicles was composed of 80–90% primordial and primary follicles and 10–20% secondary follicles. However, Hulshof et al. (1994) isolated a higher percentage of primary (57.2%) than primordial follicles (12.4%) from bovine fetus ovaries. These authors suggest that the low number of isolated primordial follicles may be due to these follicles being tightly
embedded in the tunica albuginea, whereby a mild mechanical treatment is not sufficient to isolate primordial follicles.

The diameters of isolated primordial, primary and secondary follicles were similar to the diameters reported by Rodrigues et al. (1998) to caprine preantral follicles, but differ from those reported in preantral follicles from bovine fetuses (Hulshof et al., 1994). The primordial follicle diameter from ovine ovaries was similar to the diameter observed in primordial follicles from porcine (Greenwald and Moor, 1985), domestic cats (Jewgenow and Pitra, 1991), pigs (Morbeck et al., 1993) and bovine (Nuttinck et al., 1993). The smallest diameter of the ovine secondary follicle was smaller than in hamster (Roy and Greenwald, 1985), domestic cats (Jewgenow and Pitra, 1993), pigs (Morbeck et al., 1993) and bovine (Nuttinck et al., 1993). The smallest diameter of the ovine secondary follicles observed in our study may be due to the use of 100 μm filters. Such filters prevent the filtration of follicles with diameter >100 μm.

In conclusion, this work indicates that the cut intervals in the tissue chopper affect the number of isolated ovine preantral follicles. A higher number of intact preantral follicles was isolated when the ovarian fragments were cut in the tissue chopper at 87.5 μm interval. In the future, the use of a large number of preantral follicles will be essential to the recovery of oocytes necessary to in vitro maturation and fertilization. These preantral follicles will contribute to the multiplication of endangered or valuable animals.

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