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Effect of Oocyte Recovery Techniques on *in Vitro* Production of Swine Embryos

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Abstract

***In vitro* production of swine embryos is a valuable tool to generate clones and genetically modified pigs during a short period of time. However, the efficiency of the existing methods is extremely low and the oocyte quality and quantity represent important obstacles on the success of *in vitro* production of embryos. Therefore, the aim of this study was to compare the *in vitro* maturation, fertilization and subsequent embryo development rates of oocytes recovered by ovary slicing or follicular aspiration. The oocyte recovery rate (grade 1 COC/ovary) was higher (p = 0.0083) in the slicing group when compared to the aspiration group. No differences were observed between groups regarding *in vitro* maturation and early cleavage rates. A higher percentage of oocytes recovered by follicular aspiration reached the blastocyst stage after IVF when compared to the ovary slicing method (p = 0.0395). However, no difference on blastocyst cell number was observed. Although the recovery of oocytes using the slicing technique yielded more grade 1 oocytes per ovary than the aspiration method, the number of oocytes that reached the blastocyst stage after IVF by the slicing method was lower when compared with oocytes obtained by aspiration, as observed by lower blastocyst rates. In conclusion, the follicular aspiration is the method of choice for porcine *in vitro* embryo production.**

Keywords

Oocyte Recovery, Oocyte Maturation, HSP70, Embryo Development, Swine

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1. Introduction

The oocyte maturation is considered one of the most important steps of *in vitro* production of embryos, because in this period the oocyte needs to undergo nuclear (nuclear maturation) and biochemical, structural and cytoskeletal changes (cytoplasmic maturation) that are responsible for early embryonic development [1]. However, the percentage of embryos that become blastocysts after IVM and IVF is still substantially inferior to that observed *in vivo* [1].

The quality of oocytes and their developmental capabilities are acquired progressively throughout maturation, with major impacts on early embryo survival and the establishment and maintenance of pregnancy [2]. Successful production of high quality blastocysts *in vitro* depends on several factors, including the recovery technique of oocytes, which can affect oocyte quality and maturation [3]. Porcine oocytes require a 44-h maturation period *in vitro*. Since the porcine oocyte is exposed to an artificial environment for a long time prior to IVF, often cultured under normoxia conditions, metabolic and oxidative stresses are a recurrent concern.

Most of our knowledge of the early embryo development of livestock species often relies on using ovaries collected from slaughterhouses, which serve as an alternative source of oocytes for *in vitro* studies, such as *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT).

Several methods have been developed to obtain oocytes from slaughterhouse ovaries, being the follicular aspiration or ovary slicing the most used. The comparison between these two techniques has been performed on bovine [4] [5], equine [6] [7], goats [8], sheep [9] and camels [10], but not in swine.

Despite the wide availability of swine ovaries from slaughter plants, most of slaughtered females are prepubertal, which makes the recovery of oocytes by aspiration laborious, with low recovery rates when compared to other species such as bovine. Therefore, ovary slicing would be an alternative for faster recovery.

In this manner, the objective of this study was to compare the effects of two oocyte collection techniques, follicular aspiration and ovary slicing, on oocyte recovery efficiency, maturation rate, oxidative stress and subsequent *in vitro* embryonic development.

2. Materials and Methods

All experiments were approved by the Committee of Ethics and Animal Use of the College of Veterinary Medicine and Animal Sciences, University of Sao Paulo.

Unless otherwise stated, all reagents were purchased from Sigma (Sigma–Aldrich Corp., St Louis, MO, USA).

2.1. Oocyte Recovery and *in Vitro* Maturation

Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory in saline solution at 35˚C. Ovaries were then subjected to slicing or follicular aspiration techniques. In the slicing group, ovaries were held with a hemostatic forceps inside a 400 ml beaker containing 35 mL of Hepes-buffered Tyrode's media (HbT) and follicles (2 - 6 mm) were incised with a scalpel blade. Ovaries were immerged into the HbT right after being sliced. After every five sliced ovaries, the HbT containing follicular fluid was transferred to 50 mL centrifuge tubes. In the aspiration group, follicles (2 - 6 mm) were aspirated using 18-Gauge needle and 5 mL syringe. Follicular fluid of each ovary was transferred to 50 mL centrifuge tube containing 20 mL HbT. Tubes from both recovery techniques were placed in water-bath at 35˚C for 15 min for settling of cumulus-oocyte complexes (COCs). The supernatant was removed, the sediment resuspended in HbT and placed in water-bath at 35˚C for an extra 15 min incubation. Sediment was finally resuspended in 15 mL of HbT and COCs selected under a stereomicroscope (40× of magnification).

Only grade I COCs (homogenous cytoplasm with a compact, and more than four complete layers of the cumulus oophorus cells) were used in this study. Moreover, special attention was given to the oocyte diameter. Only oocytes with similar size (approximately 120 - 125 µm excluding zonapellucida) were selected under a stereomicroscope for maturation. To calculate the recovery rate of oocytes per ovary, 320 ovaries were used in 5 replicates, divided equally between the experimental groups: slicing or aspiration. The oocyte recovery rate of each technique was determined by the ratio between number of selected COCs and used ovaries.

The oocyte maturation was performed according to the protocol described by Marques *et al*. (2007). Briefly, COCs were *in vitro* matured for 44 hours in TCM199 (supplemented with 3.05 mM glucose, 0.91 mM sodium pyruvate, 50 IU/mL gentamycine, 0.57 mM cysteine, 50 µM of cysteamine) supplemented with 10% (v/v) porcine follicular fluid and hormones for the first 22 h (10 UI eCG/mL and 10 UI hCG/mL) at 38.5˚C, 5% CO_2 (v/v)

and high humidity.

2.2. Evaluation of the Oocyte HSP70 Content and Nuclear Maturation

When oocytes are exposed to stress, stored HSP70 is used to protect cellular processes [11]. As fully-grown oocytes are unable to induce HSP70 synthesis [11], its variation can be used as a parameter for measuring the stress caused by different systems of *in vitro* maturation.

In order to compare oocyte stress from both oocyte recovery techniques, HSP70 content was verified as described in Kawarsky and King (2001) [12]. Briefly, oocytes were fixed in 4% (w/v) paraformadehyde for 1 h at room temperature and then incubated in PBS without Ca^{2+} and Mg^{2+} with 0.1% (v/v) Triton X-100 and 2% (v/v) horse serum (PBS-HS) to permeabilize and block unwanted non-specific antibody binding. Oocytes were washed three times with PBS-HS and incubated for 1 h with rabbit anti-HSP70 polyclonal primary antibody (1:40 in PBS-HS; Santa Cruz Biotechnology, California, USA). After being washed with 0.05% (v/v) Tween-20 in PBS (Tween-PBS), oocytes were incubated for 1 h with FITC-conjugated monoclonal secondary antibody (1:100 in PBS-HS; Santa Cruz Biotechnology, California, USA). Oocytes were washed again with Tween-PBS and incubated for 30 min with 10 µg/mL RNase A in 1mg/mL polivinylpirrolidone in PBS (PBS-PVP). For negative control, a group of oocytes were incubated only with the secondary antibody.

For nuclear maturation stage evaluation, nuclei were stained with 10 µg/mL propidiumiodide in PBS-PVP for 10 min. Oocytes were placed on microscope slides with antifade DABCO medium. Slides were stored at 4˚C until Confocal microscopy evaluation (Zeiss LSM 510 Meta). HSP70 content was assessed with a 1.9 µm serial Z-section (40× of magnification). Image J 1.40 g® software (Integrated Density) was used for image processing, to measure the area and quantify pixels. Each image was normalized by the background. No immunofluorescence was observed in embryos exposed to the secondary anti- body only.

Five replicates were performed and included four experimental groups: slicing 0 hours, slicing after maturation period, aspiration 0 hours and aspiration after maturation period. A total of 20 - 23 oocytes per group were observed.

2.3. *In Vitro* Production of Embryos

After 44 hours of maturation, cumulus cells of the oocytes from all groups were removed and denuded oocytes were fertilized in Tris Buffered Medium supplemented with 2.0 mM caffeine, 0.57 mM cysteine and 1 mg/mL fatty acid free-bovine serum albumin (mTBM medium).

Crossbred adult boar semen (sperm-rich fraction) was collected with the gloved-hand methodand used for IVF. Five milliliters of semen were diluted in 10 mL of Beltsville Thawing Solution (BTS, IVP, Sao Paulo, Brazil) and stored between 15˚C and 18˚C for 24 hours. On the day of IVF, semen was heated to 37˚C in water-bath and centrifuged at 700 × g for 3 minutes. Semen sediment (200 µl) were gently deposited on the surface of a discontinuous Percoll density gradient (45% - 90%) and centrifuged for 4 minutes at 9000 × g to separate live sperm cells. After centrifugation, 100 µL of sediment were collected and centrifuged (3 min at 9000 × g) in 3 mL of mTBM medium to remove Percoll residues. Sperm motility and concentration were determined using light microscopy and a hemocytometer, respectively. The semen was diluted in mTBM medium to achieve the final concentration of 5×10^5 sperm cells/ml in the fertilization drop.

The oocytes and sperm were kept under 38.5˚C, 5% CO_2 in air and high humidity conditions during 30 minutes. After this period, oocytes from each group were gently washed in mTBM medium to remove sperm cells not adhered to the zonapellucida and then placed in a new drop of mTBM medium at 38.5˚C, 5% CO_2 in air and high humidity for five and a half hours.

After 6 hours of IVF, presumptive zygotes were cultured in Porcine Zygote Media-3 (culture medium) for 7 days under low oxygen tension atmosphere (38.5˚C, 5% CO_2 (v/v), 5% O_2 (v/v) and high humidity). Embryo development was evaluated at the third and seventh day of culture in order to determine the cleavage and blastocyst rates, respectively. Cleavage rate was given by the ratio of cleaved embryos and oocytes submitted to IVF. Blastocyst rate was calculated by the ratio of day 7 blastocysts and oocytes submitted to IVF. Five replicates were performed and a total of 415 oocytes from slicing group and 215 from aspiration group were *in vitro* fertilized.

2.4. Blastocyst Cell Counting

IVF embryos on the seventh day of development (20 - 16 embryos per treatment) were mounted on glass slides

with cover slips in a drop of glycerol containing 10 µg/ml Hoechst 33,342 and examined under epifluorescence microscopy (Axiorvert 100, Carl Zeiss, Oberkochen, Germany, filter of maximum excitation of 365 nm and maximum emission of 480 nm).

2.5. Statistical Analysis

Analysis of variance was performed using the SAS® MIXED procedure (SAS, 1997). To assess oocyte recovery rate; nuclear maturation rates, cleavage and blastocyst rates and blastocyst cell number the Unpaired t test was adopted with a significance level of 5%. To evaluate HSP70 content, a nested model was used; the treatment (recovery methods), the maturation period and the interaction (recovery methods * maturation period) were considered as sources of variation.

3. Results

3.1. Oocyte Recovery

After recovery using ovary slicing or follicular aspiration, grade I oocytes were morphologically undistinguishable between groups. By adjusting the number of oocytes per ovary we observed that the slicing method yielded more oocytes when compared to follicular aspiration (**Figure 1**, $p = 0.0083$).

3.2. Evaluation of Oocyte Nuclear Maturation and HSP70 Content

The oocytes recovered by the different techniques were stained with propidium iodide to assess nuclear status. No differences between oocyte recovery methods were observed on metaphase II (MII) rates at 44 hours (**Figure 2**, $p = 0.1664$).

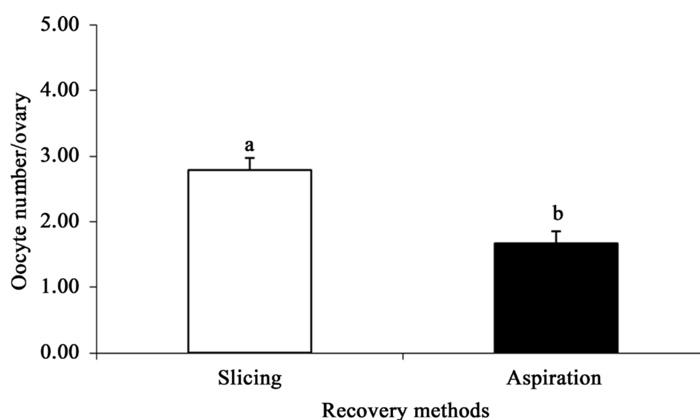

Figure 1. Number of grade I oocytes per ovary obtained from different recovery methods (slicing or aspiration). Different superscript letters in each bar represent significant statistical differences ($p < 0.05$). Data presented as least squares means ± SEM.

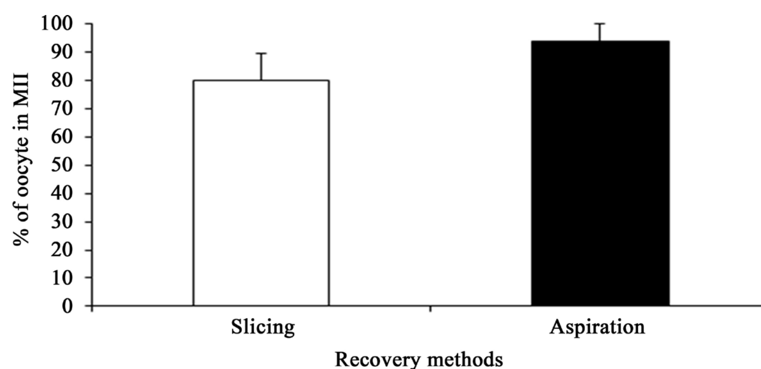

Figure 2. Percentage of oocytes in metaphase II (MII) after *in vitro* maturation (44 hours) in different oocyte recovery methods (slicing or aspiration). Data presented as least squares means ± SEM.

Oocytes were also evaluated for HSP70 content by immunocytochemistry. There was no interaction between the maturation period and the method of oocyte recovery regarding HSP70 protein quantification rates (p = 0.22). **Figure 3(a)** shows no effect of the oocyte recovery method on HSP70 protein quantification rates (p = 0.277). However, a decrease in the amount of HSP70 on oocytes during *in vitro* maturation was observed in both groups: HSP70 content was higher in oocytes before maturation period (0 h) when compared to 44 h after maturation (**Figure 3(b)**, p = 0.0002).

3.3. *In Vitro* Production of Embryos

Oocytes recovered by ovary slicing or follicular aspiration were *in vitro* fertilized and cultured until blastocyst stage. **Figure 4(a)** shows no effect of oocyte recovery method on cleaved embryo rates, at day 3 after IVF (p = 0.1211). However, IVF of aspirated oocytes yielded a greater number of blastocysts at day 7 when compared to the ovary slicing method (p = 0.0395) (**Figure 4(b)**).

3.4. Blastocyst Cell Counting

The method of oocyte recovery did not influence the embryo cell number of blastocysts obtained after seven days of IVF (**Figure 5**) (p = 0.0811).

4. Discussion

In this study, we compared the classic follicular aspiration to scalpel incision of follicles in order to obtain oocytes for porcine IVF. Aspiration of follicles resulted in a lower recovery rate of grade 1 oocytes than ovary slicing, which is consistent with data from studies in other species. In equine, a similar smaller number of viable

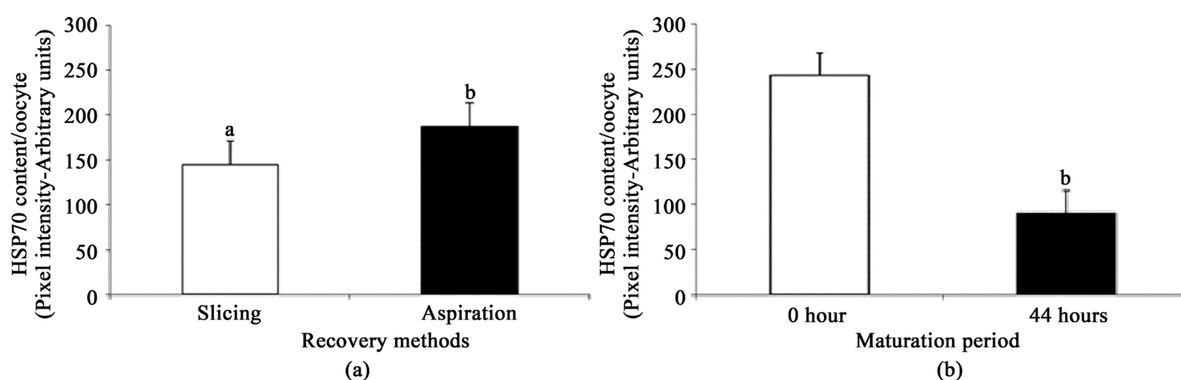

Figure 3. Effect of oocyte recovery method (slicing or aspiration) (a) and maturation period (oocytes before maturation - 0 hour and after maturation - 44 hours) (b) on the average concentrations of HSP70 (pixel intensity × 10^{-6}). Different superscript letters in each bar represent significant differences (P < 0.05). Data presented as least squares means ± SEM.

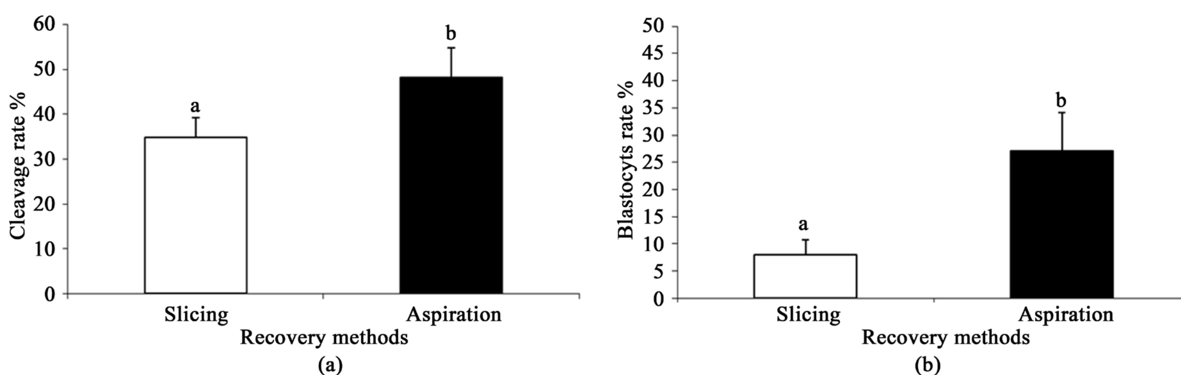

Figure 4. Effect of oocyte recovery method of (slicing or aspiration) on cleavage rates (3 days of development) (a) and blastocyst rates (7 days of development) (b). Different superscript letters in each bar represent significant differences (p < 0.05). Data presented as least squares means ± SEM.

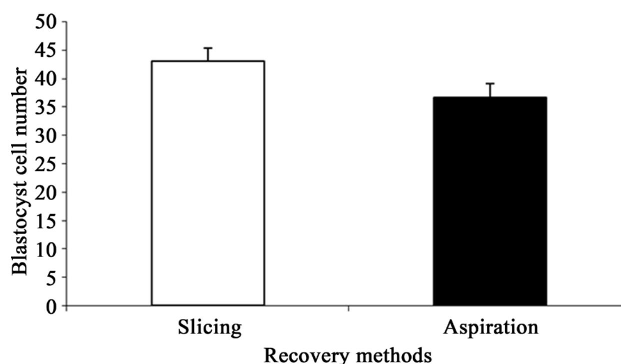

Figure 5. Effect of oocyte recovery method (slicing or aspiration) on blastocyst cell number. Data presented as least squares means ± SEM.

structures were recovered only on visible follicles at the ovary surface by the aspiration method when compared to the scraping method (follicles were opened with a scalpel blade and the granulosa cell layer scraped with a curette) [6] [7].

It has been shown that follicular aspiration is also less efficient when compared to whole ovary slicing. Wang *et al*., (2007) compared goat oocytes obtained from chopped ovaries, punctured follicles and aspirated follicles using an 18-g needle or a vacuum pump with constant pressure. They observed that chopping ovaries into small pieces with a surgical blade or puncturing the visible follicles with a scalpel yielded a higher number of oocytes per ovary as well as better grade oocytes compared to aspiration technique. Although they reported that less MII stage oocytes were observed after *in vitro* maturation in the ovary slicing group compared to the other three oocyte recovery techniques, no significant difference was observed between puncture and aspiration groups. These similar oocyte maturation rates were also observed in our study and agree with data obtained in equine [6].

Our quantification of protein levels of HSP70 to access cellular stress of oocytes during *in vitro* maturation matched the results presented by Lánská *et al*., (2006). These authors demonstrated high concentrations of HSP70 in pig oocytes from 2 - 5 mm follicles. After 6 hours of culture in maturation medium, the oocytes were depleted up to approximately two thirds of the initial concentration of HSP70. In our experiment, we observed that a decrease of HSP70 oocyte content during maturation was independent of the method used to obtain, suggesting both oocyte recovery procedures induced similar stress levels.

Our results indicate a similar fertilization capacity between groups since no effect of recovery method was observed on cleavage rates. It was shown in goat [13] and bovine [3], [4] that aspiration or surface dissection did not have a significant effect on cleavage rate or even blastocysts rate after IVP of embryos. However, we obtained higher blastocyst rates after *in vitro* fertilization of aspirated porcine oocytes when compared to oocytes obtained by ovary slicing. This data suggests that zygotes from the slicing group had the highest rate of developmental blockage after cleavage, which occurs at the 8-cell stage.

Gilt ovaries have large number of follicles in different stages of development. Due to the close proximity between these follicles, it is possible that smaller follicles were also incised at the same time of 2 - 6 mm tertiary follicles. It is not possible to separate these oocytes only by morphological evaluation and they can reach MII and develop to early cleavage stages. However, these oocytes are not able to develop to the blastocyst stage.

The early developmental program embedded in the oocyte is capable to sustain the initial embryonic development through the accumulation of messenger RNA and proteins. These accumulated molecules are also likely to be responsible for the proper execution of the embryonic genome activation [14]. Thus, the oocyte is influenced by the follicular status from which it is obtained. Since oocytes contained in smaller follicles have lower developmental ability than those of larger-diameter follicles [15], it can be inferred that not all oocytes recovered using the slicing technique have the same competence as the aspirated oocytes.

5. Conclusion

Despite the recovery of oocytes using the ovary slicing technique yielded more oocytes per ovary than follicular aspiration, more blastocysts after IVF were obtained with the latter technique. In conclusion, we propose that follicular aspiration is the method of choice for porcine *in vitro* embryo production.

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