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Effect of Low Oxygen Tension Atmosphere and Maturation Media Supplementation on Nuclear Maturation, Cortical Granules Migration and Sperm Penetration in Swine *In Vitro* Fertilization

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Contents

The aim of this study was to evaluate the efficiency of low oxygen tension (5% CO_2, 5% O_2 and 90% N_2) on *in vitro* oocyte maturation using defined media (0.1% polyvinyl alcohol – PVA) or 10% porcine follicular fluid (PFF)-supplemented media. To achieve this goal, oocytes were evaluated regarding cortical granules (GCs) migration, nuclear maturation and sperm penetration. Oocytes were *in vitro* matured under different conditions: 5% or 20% O_2 atmosphere and 0.1% PVA- or 10% PFF-supplemented media and evaluated at 0 and 44 h of maturation. To evaluate the migration of CGs and nuclear maturation, by confocal microscopy, oocytes were incubated with 100 μg of FITC-PNA/ml and 10 μg/ml of propidium iodide. To address sperm penetration, after maturation, *in vitro* fertilization for 6 h and *in vitro* culture for 18 h, zygotes were incubated with 10 mg/ml Hoechst 33342. Pronuclei and polar bodies were quantified using an epifluorescence microscope. Atmosphere conditions did not affect the CGs migration, but media supplementation did. Oocytes matured in 10% PFF media had a higher percentage of CGs in the oocyte periphery than oocytes matured in PVA-supplemented media. However, this fact did not have effect on *in vitro* sperm penetration levels. No effect of atmosphere conditions and media supplementation was observed on the rates of metaphase II oocytes. Therefore, the use of low oxygen tension in association with PVA maturation media does not improve the *in vitro* maturation system of porcine oocytes, because its use did not improve nuclear maturation, CGs migration and zygotes monospermic rates.

Introduction

The oocyte maturation is considered one of the most important stages of the *in vitro* production of embryos, because it is when oocyte undergoes nuclear (nuclear maturation) and biochemical, structural and cytoskeletal changes (cytoplasmic maturation) that are essential for early embryo development.

However, the percentage of embryos that develop from *in vitro*-matured and *in vitro*-fertilized oocytes is lower than those obtained *in vivo*, mainly owing to problems in the process of cytoplasmic maturation (Bertagnolli et al. 2004).

The cortical granules (CGs) are associated with a segment of smooth endoplasmic reticulum and remain close to the periphery of the cell. The cortical granules are vesicles filled with hydrolytic enzymes, proteases and peroxidases that are released into the perivitelline space after fertilization, aiming to prevent the penetration of more than one sperm into the oocyte (polyspermy) (Abeydeera 2002).

Wang et al. (1997) reported that soon after the germinal vesicle breakdown stage CGs of porcine oocytes were present in the cortex and they formed a halo nearby the outer oocyte membrane after maturation. *In vitro*-matured oocytes have low concentrations of glutathione, lower migration and content immature of CGs. These factors collaborate with high rates of polyspermy after *in vitro* fertilization (IVF) and the low quality of *in vitro*-produced embryos.

Supplementation of maturation medium with porcine follicular fluid (PFF) is routinely used in *in vitro* maturation (IVM) in pigs. Yoshiba et al. (1992) showed that PFF contains substances that enhance the expansion of cumulus cells, nuclear maturation and fertilization.

However, the use of PFF introduces a series of not known factors to the maturation medium, which hinders the standardization of the technique and the exact identification of substances, which are essential to regulate the process. In this way, the development of media without PFF allows the optimization of maturation conditions, in addition to collaborate for the decrease in the variability among laboratories.

As an alternative to the use of PFF, researchers have developed new maturation media with polyvinyl alcohol (PVA). However, to maintain the efficiency of the process, the PVA media need to be supplemented with growth factors such as EGF (Kishida et al. 2004) and/or essential and not essential amino acids (Hong et al. 2004). Furthermore, it is known that PFF contains antioxidants such as superoxide dismutase (Tatemoto et al. 2004), which are not present in the medium supplemented with PVA, possibly making the system more sensitive to oxidative stress.

The mean dissolved oxygen levels in human follicular fluid during the late antral and pre-ovulatory phases range between 11 and 51 mmHg (~1.5–6.7%) (Redding et al. 2008). Oocytes appear to be protected against oxidative stress by oxygen scavengers that are present *in vivo*. However, when oocytes are removed from their natural environment for assisted reproductive technologies, this natural defence is lost (Choi et al. 2007). In addition, it was observed that the use of supraphysiological oxygen tensions in protocols of *in vitro* oocyte maturation increased the formation of reactive oxygen species (ROS) (Soom et al. 2002). Thus, for the use of PVA in the maturation media, it was suggested to use a system that causes less oxidative stress, as the atmosphere with low oxygen tension. The use of low oxygen

tension in oocyte maturation is accompanied by a reduction in the concentration of ROS in cells, which increases the potential of developing oocytes (Luberda 2005). The increased accumulation of ROS results in depolymerization of microtubules. This may be one of the reasons of poor oocyte quality and subsequent low fertilization in human oocytes (Choi et al. 2007).

Microfilaments are involved in migration of CGs to the cortex and sperm penetration. Disruption of the normal organization of microfilaments coincides with a defect in sulfhydryl groups of actins after exposure of cells to ROS-generating agents (Kang et al. 2005).

The use of low oxygen tensions does not increase glutathione concentrations, which is an oxidative stress indicator in oocytes with cumulus cells (Bing et al. 2002). However, other effects as the improvement in embryo quality after IVF of oocytes matured in PFF-supplemented medium under low oxygen tension were verified by Kikuchi et al. (2002) and Iwamoto et al. (2005). This demonstrates the need for studies that evaluate which modifications may occur when oocytes are *in vitro* matured under low oxygen tension conditions. Bing et al. (2002) and Park et al. (2005) reported no effects of low oxygen tension atmosphere on polyspermy rates in oocytes matured in 10% PFF- and cysteine-supplemented medium in the presence of cumulus cells. However, these authors have no knowledge of studies about the efficiency of chemically defined media in quality of *in vitro*-matured oocytes when a 5% oxygen atmosphere is adopted.

Therefore, the use of chemically defined maturation media in association with low oxygen tension atmosphere (5% O_2, 5% CO_2 and 90% N_2) can be an alternative for the improvement in oocyte maturation, migration of CGs and normal oocyte fertilization.

Materials and Methods

Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). These experiments were approved by bioethics committee of the School of Veterinary Medicine and Animal Sciences, University of São Paulo.

Collection, selection and maturation of oocytes

Ovaries of pre-pubertal females were washed in saline at 28°C and subjected to slicing for oocyte recovery. For this, ovaries were held with a hemostatic forceps inside a beaker containing 35 ml of Hepes-buffered Tyrode's media (HbT) and follicles (2–6 mm) were incised with a scalpel. The HbT containing follicular fluid was transferred to 50-ml centrifuge tubes.

Tubes were placed in water bath at 35°C for 10 min for settling of cumulus–oocyte complexes (COCs). The supernatant was removed, and sediment was resuspended in HbT and placed in water bath at 35°C for extra 10 min. Sediment was finally resuspended in 15 ml of HbT, and COCs were selected under stereomicroscopy.

Oocytes with a thick and intact cumulus oophorus layer and cytoplasm with homogenous granules were selected for IVM and separated into five groups:

1. Group 0 h: Immature oocytes were evaluated before maturation;
2. Group PVA 5% O_2: Oocytes were incubated for 44 h at 38.5°C, under high humidity condition in low oxygen tension atmosphere (5% O_2, 5% CO_2 and 90% N_2) in maturation medium [TCM199 (Gibco, Carlsbad, CA, USA) with 5.3 mM glucose, 50 IU/ml gentamicin, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 50 μM cysteamine and 10 ng/ml EGF] supplemented with 0.1% PVA;
3. Group PVA 20% O_2: Oocytes were incubated for 44 h at 38.5°C, under high humidity condition in high oxygen tension atmosphere (5% CO_2 in air) in maturation medium supplemented with 0.1% PVA;
4. Group PFF 5% O_2: Oocytes were incubated for 44 h at 38.5°C, under high humidity condition in low oxygen tension atmosphere (5% O_2, 5% CO_2 and 90% N_2) in maturation medium supplemented with 10% PFF;
5. Group PFF 20% O_2: Oocytes were incubated for 44 h at 38.5°C, under high humidity condition in high oxygen tension atmosphere (5% CO_2 in air) in maturation medium supplemented with 10% PFF

The oocyte maturation was performed for 22 h in maturation medium supplemented with gonadotrophins, 10 IU hCG/ml and 10 UI eCG/ml (Chorulon and Folligon, respectively; Intervet International B.V., Boxmeer, the Netherlands) and 22 h without hormone supplementation.

At 0 (0 h group) and 44 h of maturation, the cumulus cells of the oocytes from all remaining groups were removed, and the oocytes were evaluated for the following: nuclear maturation, CGs migration and fertilized/polyspermic zygotes.

Evaluation of cortical granules migration and nuclear maturation

To evaluate the CGs migration, immature oocytes and *in vitro*-matured oocytes were fixed in 4% paraformaldehyde for 1 h, permeabilized in PBS/PVP with 0,1% Triton 100× and incubated in 100 μg of FITC-PNA/ml for 30 min. Oocytes were then incubated in 10 μg/ml of Rnase for 30 min and in 10 μg/ml of propidium iodide (for nuclear evaluation) for 10 min.

In each manipulation, a group of oocytes was fixed, permeabilized and incubated only with Rnase and propidium iodide, being used as a negative control.

To assess the exact equatorial plane, the oocytes were placed between slide and coverslip in DABCO mounting medium (23 mg/ml), surrounded by paraffin to maintain the three-dimensional structure of oocytes. The oocytes were evaluated by confocal microscopy (Zeiss LSM 510 Meta; FITC fluorescence detected by ion laser – argon adjusted to < 560 nm and propidium iodide fluorescence detected by laser helium–neon ion to more than 560 nm).

Images were obtained with a 40× objective and 1.9 μm Z serial sections of each oocyte. The Z serial sections were used to establish the equatorial plane of each oocyte. Images were analysed by the Image J 1.40 g®

software regarding the area and number of pixels (integrated density). For each oocyte, two concentric measures were made, considering the total area of the oocyte and 80% of this area. The pixel quantification of external halo (20%) was obtained by the subtraction of these measures.

One hundred and ten oocytes in five replicates, distributed in five groups: 0 h, PVA 5% O_2, PVA 20% O_2, PFF 5% O_2, PFF 20% O_2 (20–23 oocytes per group), were observed.

In vitro fertilization and evaluation of sperm penetration

After 44 h of maturation, cumulus cells of the oocytes from all groups were removed, and denuded oocytes were fertilized in mTBM medium. The oocytes and sperm were kept under 38.5°C, 5% CO_2 in air and high humidity conditions during 30 min. After this period, oocytes from each group were gently washed in mTBM medium to remove sperm not adhered to the zona pellucida (ZP) and placed in a new drop of mTBM medium being kept under 38.5°C, 5% CO_2 in air and high humidity for 5.5 h.

Boar semen (sperm-rich fraction), obtained by the gloved-hand method, was used for IVF. Five millilitres of semen was diluted in 10 ml of Beltsville Thawing Solution® (BTS) and stored between 15 and 18°C for 24 h. On the day of IVF, semen was heated to 37°C in water bath and centrifuged at $700 \times g$ for 3 min.

Two hundred microlitres of semen sediment was gently deposited on the surface of the Percoll gradient (90% and 45%) and centrifuged for 4 min at $9000 \times g$ to separate live sperm cells. After centrifugation, 100 μl of sediment was removed and centrifuged (3 min at $9000 \times g$) in 3 ml of Tris-buffered medium (mTBM – IVF medium) supplemented with 2.0 mM caffeine, 0.57 mM cysteine and 1 mg/ml fatty acid-free bovine serum albumin to remove Percoll residues.

For insemination of the oocytes, motility and sperm concentration were determined. The semen was diluted in mTBM medium to achieve the final concentration of 5×10^5 sperm/ml in the fertilization drop.

After 6 h of IVF, the presumptive zygotes were washed three times in PZM3 (culture medium) followed by incubation in this culture medium at 38.5°C, 5% CO_2 in air and high humidity conditions.

After 18 h of the *in vitro* culture, presumptive zygotes were removed from the ZP with pronase solution (8 mg/ml). This was necessary because of the large number of sperm adhered to the ZP that hinders the evaluation of sperm penetration. Oocytes without ZP were washed in TCM199 containing 10% foetal calf serum to neutralize the pronase, placed between slide and coverslip in a droplet with 10 mg/ml Hoechst 33342 in glycerol and analysed under an epifluorescence microscope (Zeiss) (filter excitation maximum of 365 nm and emission maximum 480 nm).

Only zygotes containing two pronuclei and polar bodies were considered monospermic. Zygotes showing more than two pronuclei were considered polyspermic and with less than two pronuclei were considered non-fertilized.

One hundred and ten oocytes in six replicates, distributed in four experimental groups: PVA 5% O_2, PVA 20% O_2, PFF 5% O_2, PFF 20% O_2 (23–27 oocytes per group), were observed.

Statistical analysis

All data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). ANOVA (PROC MIXED) was used to analyse nuclear maturation, CGs migration, fertilized/polyspermic oocytes and the interaction between supplementation of maturation medium (PFF or PVA) and atmosphere (high or low oxygen). Differences between least square means were determined using the Tukey test. A significance level of 5% was considered in all analyses.

Results

Evaluation of the migration of cortical granules and nuclear maturation

Figure 1 illustrates confocal microscopic images of the CGs in oocytes before maturation period (a) and after IVM (b), after staining with FITC-PNA and propidium iodide. We can observe the migration of granules to the periphery in the mature oocyte. It was not observed the staining in oocytes used as a negative control.

When comparing immature oocytes and oocytes from groups of IVM, PVA 5% O_2, PFF 20% O_2 and PFF 5% O_2 had higher rates of migration than oocytes at 0 h ($p = 0.0011$, 0.0002 and < 0.0001, respectively). In addition, the group PVA 20% of O_2 presented results similar to group 0 h (Fig. 2).

There was no interaction between the effect of the atmosphere (5% or 20% O_2) and supplementation of maturation medium (0.1% PVA and 10% PFF) in the rates of CGs migration ($p = 0.525$).

Figure 3 shows that there was no effect of the atmosphere in the CGs migration of *in vitro*-matured oocytes ($p = 0.219$). However, oocytes that were matured in medium containing 10% of PFF had a higher concentration of CGs in the oocyte periphery than oocytes matured in medium supplemented with PVA ($p = 0.0070$).

No interaction between the effect of the atmosphere and supplementation of the medium used for oocyte

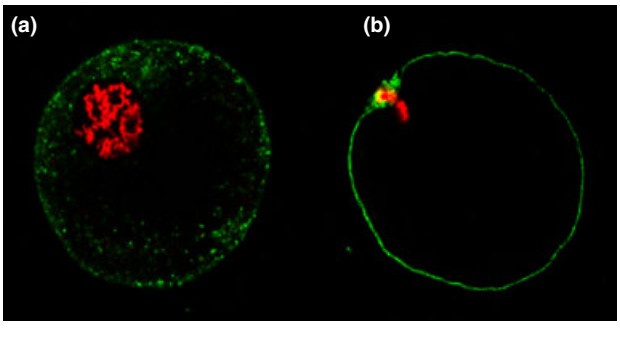

Fig. 1. Confocal microscopic images of the cortical granules (FITC – green) and chromatin (propidium iodide – red). In (a), oocytes with diffuse granules and chromatin in germinal vesicle. In (b), oocytes with granules in the periphery of the cell and chromatin in metaphase II

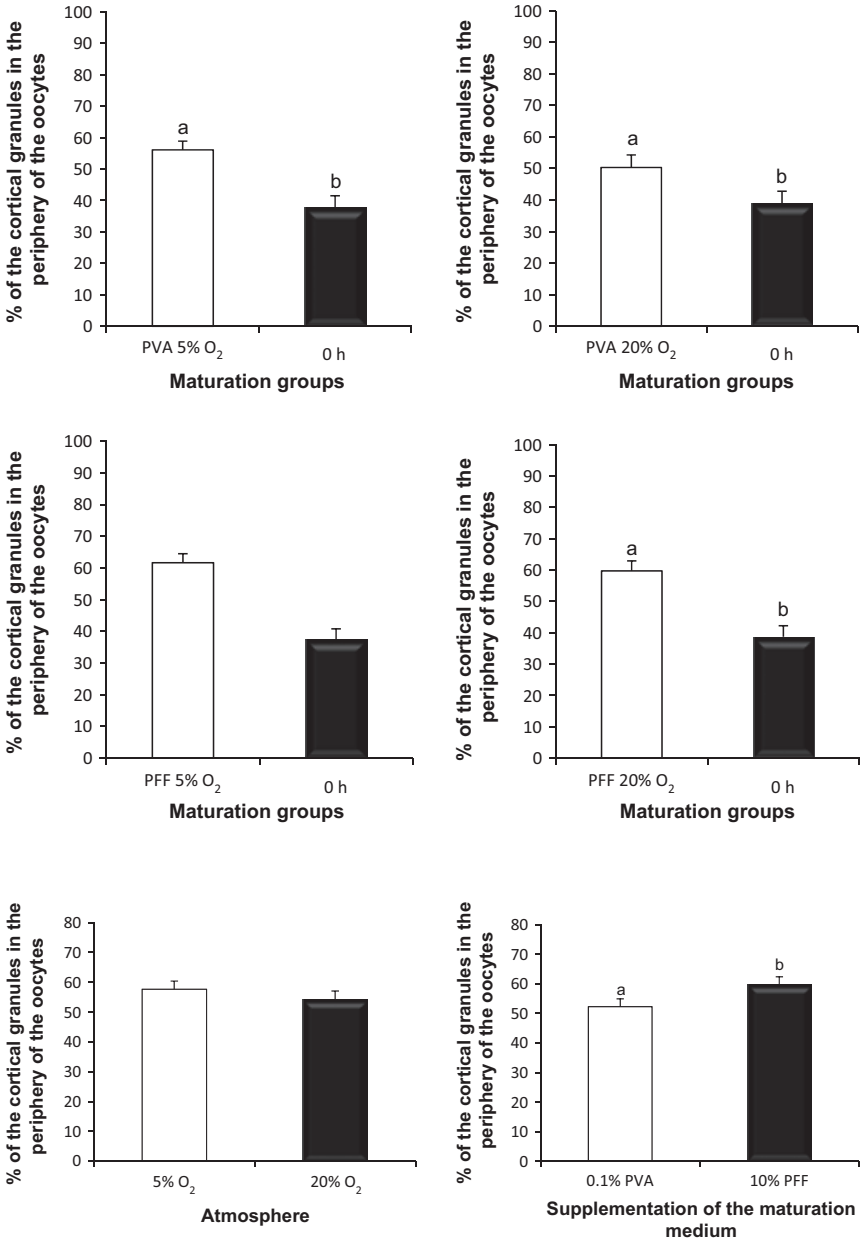

Fig. 2. Mean percentages of cortical granules present in the outer halo of 20% of the diameter of oocytes before (0 h) and after the *in vitro* maturation in the different treatments. Different superscript letters in each bar represent significant differences ($p < 0.05$). Data presented as least square means ± SE

Fig. 3. Effect of atmosphere and supplementation of maturation medium on the average percentages of cortical granules present in the outer halo of 20% of the diameter of *in vitro*-matured oocytes. Different superscript letters in each bar represent significant differences ($p < 0.05$). Data presented as least square means ± SE

maturation *in vitro* in the rates of oocytes in metaphase II ($p = 0.333$) was observed.

As described in Fig. 4, no effect of the atmosphere ($p = 0.087$) and supplementation of maturation medium ($p = 0.668$) were observed in the rates of oocytes at metaphase II.

Evaluation of *in vitro* fertilization

No interaction between the effect of the atmosphere and supplementation of oocyte maturation medium in the rates of zygotes monospermic, polyspermic and unfertilized oocytes ($p = 0.877$, 0.817 and 0.786, respectively) was observed.

The atmosphere and supplementation of the medium used during oocyte maturation had no effect on monospermic, polyspermic zygotes and unfertilized oocytes rates after IVF (Fig. 5).

Discussion

Many studies have investigated the effects of oxidative stress in oocyte maturation in pigs (Bing et al. 2002; Ozawa et al. 2006; Maedomari et al. 2007). However, more studies are necessary to understand the effect of this type stress on the maturation.

A series of factors contribute to the great variation in results found in studies of oocyte maturation in pigs compared with other animal species. This includes the variations between laboratories, age of donors, the quality of oocytes and methodologies for the oocyte maturation (cultivation systems and maturation media). In this way, the comparison with the literature presented for this species is very difficult.

This study is in agreement with results reported by Kikuchi et al. (2002) and Iwamoto et al. (2005) who have not found effect of a controlled atmosphere on nuclear maturation.

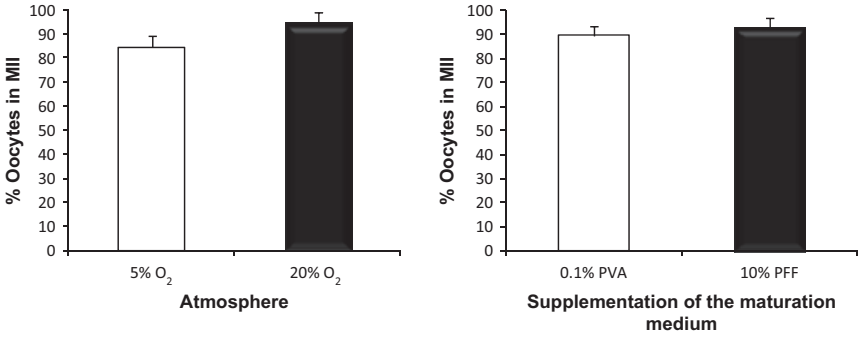

Fig. 4. Effect of atmosphere and supplementation of maturation medium on the average percentages of metaphase II *in vitro*-matured oocytes. Data presented as least square means ± SE

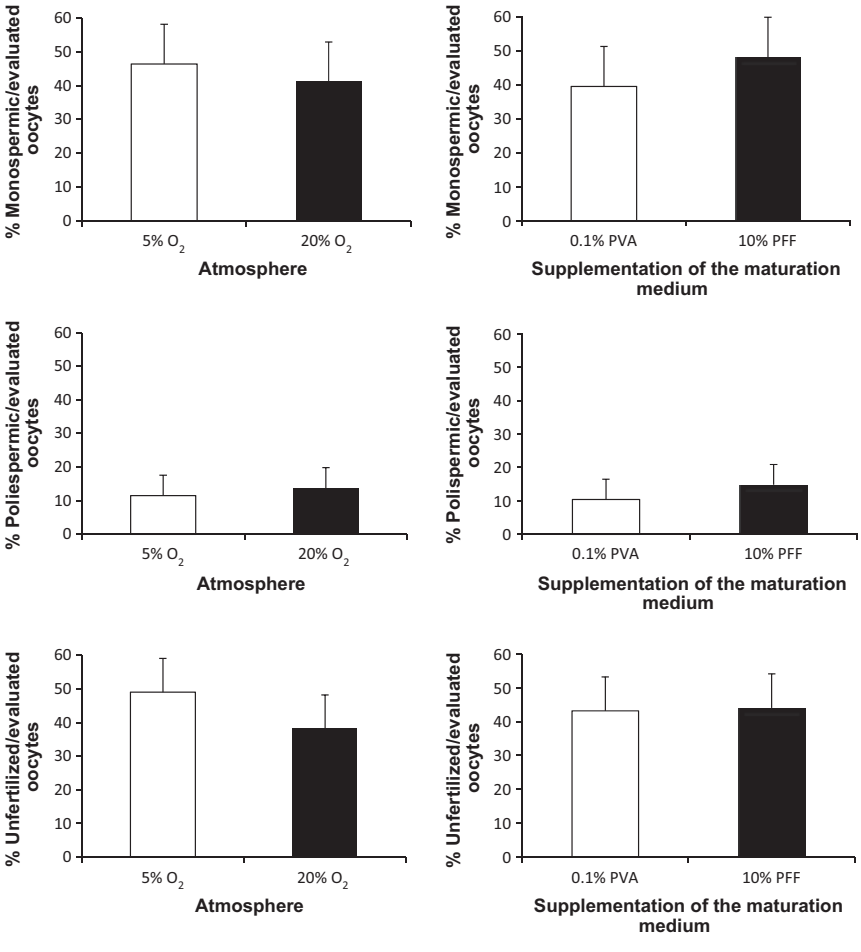

Fig. 5. Effect of the atmosphere and supplementation of maturation medium on the average percentages of monospermic, polismermic zygotes and unfertilized oocytes after *in vitro* fertilization of *in vitro*-matured oocytes. Data presented as least square means ± SE

In bovine oocytes, the presence of serum during maturation is important for embryo development under 20% O_2 only. Maturation with PVA and 5% O_2 provides a better environment for the oocyte, resulting in blastocysts with a lower apoptotic index, but it not found effect on nuclear maturation (Pereira et al. 2010).

However, it is known that in swine, several research laboratories have reached satisfactory levels of nuclear maturation but still fail to provide appropriate cytoplasmic maturation for the oocyte.

Results of the previous studies had demonstrated that medium supplemented with PVA was adequate for nuclear maturation and did not differ from media supplemented with PFF in an atmosphere of 20% O_2 (Marques et al. 2007). In this study, we were expected that it was occurred in a positive synergistic effect of the atmosphere of 5% O_2, supplemented with PVA, raising the rates of maturation, mainly cytoplasmic, but it was not verified.

The addition of antioxidants such as cysteine and cysteamine to maturation medium probably was already sufficient for the oocytes to be able to develop even in the absence of PFF and in the presence of high oxygen tensions. Thus, no changes were observed in nuclear maturation and migration of CGs, suggesting that the protocols used in this study provided microfilaments and microtubules to properly exercise their functions.

In this study, supplementation of maturation medium with PFF had higher rates of migration of CGs. This result could indicate that these oocytes would present

better response to IVF, because they would have been more able to block polyspermy. However, this was not verified because there was no effect of supplementation of maturation medium or the atmosphere in the polyspermy rates.

The IVF system used in this study showed that low polyspermy rates an average of 12.6%. The polyspermy rates presented in the literature are quite varied. Matás et al. (2003) showed 93% of polyspermy when sperm were separated by Percoll gradient. Suzuki et al. (2003) demonstrated that polyspermy rates after IVF vary according to breed of the male, presenting high rates of polyspermy: 21.5% for Duroc animals, 20.2% for Landrace animals and 54.1% for Large White animals.

However, in these studies, the incubation period of oocyte/sperm was 6 h. In the protocol used in our study, not adhered to the ZP, oocytes were separated from the spermatozoa after 30 min of incubation. Thus, we obtained low levels of polyspermy. On the other hand, the rates of unfertilized were high, 43.6% on average.

In the face of the fact that polyspermic embryos develop until the stage of blastocyst and based on what was described by Sonfai et al. (2008), who said that polyspermic embryos have a high frequency of fragmentation during the early embryonic development, in this study, we adopted a protocol that gave low rates of polyspermy, even with the disadvantage of having a greater number of non-fertilized. In this way, the embryos produced would be of better quality.

The problem of polyspermy after IVF in pigs still persists. Migration and exocytosis of CGs appear similar between oocytes matured *in vivo* and *in vitro*, but after fertilization, oocytes have an incomplete reaction of the ZP, decreasing inhibition of penetration of more than one sperm. This fact can be correlated with the immaturity of composition of these granules leading to high incidence of polyspermy *in vitro* (Funahashi 2003), evidencing the need for more studies on oocyte maturation in pigs.

Therefore, the use of low oxygen tension in association with PVA maturation media does not improve the IVM system of porcine oocytes, because its use did not improve nuclear maturation, CGs migration and zygotes monospermic rates.

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Conflict of interest

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

Author contributions

Flavia Regina Oliveira de Barros is an assistance in the IVM and fertilization. Marcelo Demarchi Goissis is an assistance in the IVM and analysed data. Paulo Varoni Cavalcanti is an assistance in the IVF. Carlos Henrique Cabral Viana is an assistance in the designed study. Mayra Elena Ortiz D'Ávila Assumpção is an assistance in the designed study and drafted paper. José Antonio Visintin is an assistance in the designed study and drafted paper.

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