LETÍZZIA RAPOSO ANDRADE

IMPACT OF FSH ON OOCYTE AND EMBRYO PRODUCTION IN PREPUBERTAL GILTS

Dissertation submitted to the Veterinary Medicine Graduate Program of the Universidade Federal of Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

Adviser: Simone Eliza Facioni Guimarães
Co-advisers: José Domingos Guimarães
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Simone Eliza Facioni Guimarães
Advisor

To my parents, Cláudio and Cidinha, for their confidence in my potential and for always encouraging me to keep going.

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To my parents, Cláudio and Cidinha, for always being by my side and for believing that I would be able to achieve all my dreams, even seeing how anxious I was during these two years.

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"All our dreams can come true if we have the courage to pursue them."

(Walt Disney)

ABSTRACT

ANDRADE, Letízzia Raposo, M. Sc, Universidade Federal de Viçosa, August, 2024. I**mpact of FSH on Oocyte and Embryo Production in Prepubertal Gilts.** Adviser: Simone Eliza Facioni Guimarães. Co-advisers: José Domingos Guimarães and Daniele Botelho Diniz Marques.

The aim of this study was to investigate the effects of the FSH treatment on the ovarian response of 140 days old gilts. The cumulus-oocytes complexes (COCs) were recovered from follicles of twenty-two prepubertal gilts, who were allotted to receive 100mg of FSH (treated group; n= 10) or saline solution (control group; n= 12). The percentage of small, medium and large follicles did not differ statistically (P>0.05). Concerning ovarian and uterine biometric data, the parameter "length of the left ovary" showed difference (P<0.05) (2.37 ± 0.26 for the control group and 2.49 ± 0.44 for the treated group (P= 0.02) and "thickness of the left ovary" too (1.31 ± 0.20 control group and 1.42 ± 0.41 treated group) (P=0.003). Besides that, the parameters related to the number and type of cells obtained on the last day of *in vitro* culture (D7) did not differ significantly (P>0.05). In terms of tendency, blastocyst conversion rate in relation to the number of morulas on day 7 (28.14 ± 13.10 – control group and 16.05 ± 14.16 – treated group) presented p-value between 0.05 and 0.10, since the control group presented higher numbers. Backfat presented tendency (P=0.09). In conclusion, due to the contradictory data regarding quantity and quality of follicles and embryos, more research is needed to fully understand the role of FSH in pre pubertal oocyte maturation in the pig, either on living gilts or being added to IVEP media.

Keywords: Cumulus-Oocytes Complexes. Hormone. *In Vitro* Fertilization. Morula. Ovary.

RESUMO

ANDRADE, Letízzia Raposo, M. Sc, Universidade Federal de Viçosa, agosto de 2024. **Impacto do FSH na Produção de Oócitos e de Embriões em Marrãs Pré-Púberes.** Orientadora: Simone Eliza Facioni Guimarães. Coorientadores: José Domingos Guimarães e Daniele Botelho Diniz Marques.

O objetivo deste estudo foi investigar os efeitos do tratamento com o Hormônio Folículo Estimulante na resposta ovariana de marrãs com 140 dias de idade. Os complexos cumulus-oócitos (COCs) foram recuperados de folículos de vinte e duas marrãs pré-púberes, que foram distribuídas para receber 100mg de FSH (grupo tratado; n= 10) ou solução salina (grupo de controlo; n= 12). A porcentagem de folículos pequenos, médios e grandes não diferiu estatisticamente (P>0,05). Em relação aos dados biométricos ovarianos e uterinos, o parâmetro "comprimento do ovário esquerdo" apresentou diferença (P<0,05) (2,37 ± 0,26 para o grupo controle e 2,49 ± 0,44 para o grupo tratado) (P= 0,02) e "espessura do ovário esquerdo" também (1,31 ± 0,20 grupo controle e 1,42 ± 0,41 grupo tratado) (P=0,003). Além disso, os parâmetros relacionados com o número e o tipo de células obtidas no último dia de cultura *in vitro* (D7) não diferiram significativamente (P>0,05). Em termos de tendência, a taxa de conversão de blastocistos em relação ao número de mórulas no 7º dia (28,14 ± 13,10 - grupo controle e 16,05 ± 14,16 - grupo tratado) apresentou p-valor entre 0,05 e 0,10, sendo que o grupo controle apresentou números maiores. A característica espessura de toucinho apresentou tendência (P=0,09). Em conclusão, devido aos dados contraditórios relativos à quantidade e à qualidade dos folículos e dos embriões, é necessária mais investigação para compreender plenamente o papel da FSH na maturação dos oócitos pré-púberes em suínos, quer em marrãs vivas, quer sendo adicionada aos meios de PIVE.

Palavras-chave: Complexos cúmulus-oócitos. Hormônio. Fertilização *in vitro*. Mórula. Ovário.

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ABBREVIATIONS

BCB	Brilliant cresyl blue (azul cresil brilhante)
BSA	Bovine serum albumina (albumina sérica bovina)
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO2	Dióxido de carbono
COCs	Cumulus-oocytes complexes (complexos cumulus-oócitos)
CG	Cortical granules
eCG	Gonadotrofina coriônica equina
FSH	Hormônio folículo estimulante
GnRH	Gonadotropin-Releasing Hormone
ICSI	Intracytoplasmic Sperm Injection
IVC	*In vitro* Culture
IVF	*In vitro* Fertilization
IVM	*In vitro* Maturation
LH	Luteinizing Hormone
mTBM	Trisbuffered medium
NCSU-23	North Carolina State University-23
O_2	Oxygen
OPU	Ovum Pick up
PVA	Polyvinyl alcohol
PZM	Porcine zygote medium
SAS	Statistical Analysis System
TALP	Tyrode's albumin lactate pyruvate
ZP	Zona Pellucida

SUMMARY

INTRODUCTION

The use of young females in embryo transfer programs offers considerable potential for accelerating genetic gain by reducing the interval between generations (PARAMIO; IZQUIERDO, 2014). Considering that the world's population is expected to grow to more than two billion by 2050, the demand for meat will also increase. Considering thus, a more sustainable way of producing livestock, increasing productivity and implementing methods that lead to faster genetic selection is of the utmost importance. In a holistic view, therefore, the establishment of routine and scaled production of *in vitro* embryos can be the solution to the problem (FOWLER et al, 2018).

In pigs, as in other species, immature oocytes released from follicles can complete maturation in culture. However, even if such mature oocytes can be penetrated *in vitro* by spermatozoa - under appropriate conditions - low rates of pro-nuclear formation and a high incidence of polyspermy are reported by many researchers (WANG et al, 1998). In this sense, studies such as Soriano-Úbeda et al. (2017) proposed an alternative protocol to mimic the periovulatory environment of the oviduct in vivo, combining media with physical barriers, with good efficiency in reducing polyspermy and increasing blastocyst rate formation. However, it introduces factors that have not yet been fully defined into the environment, which reduces the possibility of replicating the protocol.

Another complicating factor in the production of porcine embryos is the high endogenous lipid content, which makes the appearance of both oocytes and embryos darker under the microscope compared to rats and humans, and hides early factors of successful fertilization, such as pronucleus development and morphological assessment (FOWLER et al, 2018).

In vitro embryo production (IVEP) has become an alternative to *in vivo* production, since the oocytes used in this biotechnology can be obtained from infertile, sub fertile, pregnant, lactating, prepubertal or even recently deceased females (PARAMIO; IZQUIERDO, 2014). The possibility of using prepubertal females as oocyte donors is a major advantage of this biotechnology, as it allows for a reduction in the generation interval and increases genetic gain (PARAMIO; IZQUIERDO, 2014).

It is known that, on average, 114 days is the gestation period of a female pig and that at 220 days they are ready to enter the farm's reproduction program. However, it is also known that we can bring these 220 days forward to 140 days, starting with the collection of oocytes, as described in this work, thus also bringing forward the use of animals in the breeding program, reducing the generation interval and increasing genetic gain. In this way, it wouldn't be necessary to wait another 114 days until calving to evaluate the products obtained, but only the days of cultivation in the laboratory. In short, it saves time when evaluating the products obtained. Furthermore, for pigs, the literature suggests that *in vitro* fertilization (IVF) rates are approximately 45% and subsequent progression to blastocyst of 30% can be achieved (ABEYDEERA et al, 1999).

Some techniques, such as ICSI (Intracytoplasmic Sperm Injection), in swine, were already tested. Kim et. al (1999) demonstrated that the round spermatid nuclei of the pig can develop into a morphologically normal pronucleus in matured porcine oocytes that are competent to participate in syngamy with the ootid chromatin and compared fertilization rates and developmental ability following injection of round spermatid and round spermatid nuclei with different activation times. In their study, although a few injected oocytes also developed to the blastocyst stage, the incidence of development was very low and attainment of the blastocyst stage following injection was due to parthenogenetic activation. Blastocysts obtained by spermatid injection had a similar number of cells compared with those of *in vitro* developed embryos following collection from the oviduct, indicating good quality of the embryos.

The early induction of puberty in gilts and their subsequent use in reproductive activities are of great financial interest in the pig industry and are related to the formation of a stock of replacement gilts with females of high genetic merit (DO LAGO et al, 2005). It is important to say that the ovaries of gilts become responsive to gonadotropins around 60 days of age, when the development of tertiary follicles is observed (SCHWARZ et al, 2008).

Studies indicate that porcine oocytes have more than double the amount of lipids (135-156ng) when compared to bovine oocytes (58-59ng). Even though the role of these lipids is still not well understood, it is assumed that porcine oocytes use intracellular triglycerides as an energy source for maturation (MCEVOY et al, 2000). However, high lipid levels have been correlated with impaired development of oocyte competence and low cryopreservation survival rates, associated with temperature sensitivity (FOWLER et al, 2018). As for the ingredients used in embryo culture media, these can be very different from what is found in the *in vivo* environment. For example, the most used medium is NCSU-23, which contains glucose and is used as an energy source for embryonic development. It is interesting to observe that the concentration is approximately 32 times higher than *in vivo*. In addition, it has already been found that supplementing the embryo culture with pyruvate and lactate for the first two days and then glucose supplementation for the subsequent four days achieved the highest blastocyst formation rate (FOWLER et al, 2018).

In this study, the Follicle Stimulating Hormone (FSH), which had the function in folliculogenesis of stimulating the growth of antral follicles by proliferating granulosa cells and increasing the expression of anti-apoptotic factors, will be used as a precursor and trigger for the start of follicular development in prepubertal females. Therefore, it will help to ensure that these gilts succeed in obtaining considerable follicles at slaughter for analysis in this experiment.

In this way, the aim of this project is to make it possible to obtain embryos early in young (prepubertal) female pigs. In addition, this project aims to prove that it is possible to use the ovaries of females of high genetic value to use the oocytes obtained for cultivation and future export of embryos, bringing enhanced genetic gain. Therefore, the objectives of this project include hormonal stimulation using FSH to obtain an ovarian follicular response in prepubertal gilts. In addition, to recover good oocyte quantity and quality, with these oocytes being competent, capable of resuming meiosis after the *in vitro* maturation stage. The aim of this study is also for the matured oocytes to be capable of fertilization and cleavage, developing into blastocysts by the seventh day of culture. The embryos obtained must be of high quality and capable of generating pregnancy. This confirms the minimum age for using gilts in breeding programs as oocyte donors.

CHAPTER 1 – LITERATURE REVIEW

1. Ovaries, Follicular Dynamics and Oocytes in Gilts

The domestic pig is considered a continuous polyestrous species, that is, it has estrous cycles throughout the year. It lasts an average of 21 days (range 18-24 days) and has a follicular phase and a luteal phase. The follicular phase lasts four to five days and the luteal phase 15 to 17 days (DE FRIES et al, 2010).

Follicle development can be divided into two periods: gonadotrophin-independent and gonadotrophin-dependent. In the gonadotrophin-independent stage, the primordial follicles grow under the influence of local ovarian factors and can become atretic. The absence of adequate gonadotrophic stimulation is the primary cause of the impediment to continued follicular development. In pigs, there is no evidence of defined follicular waves as observed in other species (MADEJ et al, 2006). The expression of FSH receptors in the granulosa cells of bovine primary, secondary and antral follicles, as well as in oocytes from primordial follicles of laboratory animals, reinforces the idea of the action of FSH on the growth of preantral follicles (ROY et al, 1993 & Wandji et al, 1992).

In prepubertal, cyclic, pregnant and weaned females, groups of primordial follicles are continuously activated and begin to develop. The growth until the antrum is formed is very slow. The final growth from 1-4 mm to ovulatory size (6 to 10 mm) is very rapid and requires around four to six days, with proliferation followed by an increase in the volume of the antrum, differentiation of the follicular cells and an increase in the secretion of inhibin and estradiol, as well as the appearance of LH receptors on the granulosa cells (PRUNIER et al, 2000). During the development of antral follicles, the oocyte secretes factors that stimulate the proliferation and differentiation of porcine granulosa cells, promoting the suppression of progesterone production, which prevents premature luteinization (HUNTER et al, 2009).

The growth of follicles up to 2-3 mm does not require gonadotrophic support, as it is initially controlled by local ovarian growth factors. It is well known that the growth of follicles in the mammalian ovary can be regulated by gonadotrophins, somatotrophins and intra-ovarian factors. Numerous growth factors synthesized by follicular cells act by modulating the effect of hormones and regulating the development of ovarian follicles. Examples include Growth Differentiation Factor-9 (GDF-9), Insulin-like Growth Factor-1 (IGF-1), Kit Ligand (KL), Epidermal Growth Factor (EGF), Bone Morphogenetic Protein-15 (BMP-15), Fibroblast Growth Factor (FGF), Keratinocyte Growth Factor (KGF), Neurotrophins, Vasoactive Intestinal Peptide (VIP) and Activin. (MARTINS et al, 2008).

After luteolysis or weaning, around 15 to 25 healthy antral follicles with a diameter of 1-4 mm are recruited, selected and grow to the stage of preovulatory follicles. Then, after four to seven days, ovulation of the surviving follicles is observed.

It has been suggested that, in cyclic females, the decline in progesterone after luteolysis is the signal for recruitment and selection. However, experiments in the absence of gonadotrophins have shown that FSH is necessary to follicular growth above 2-3 mm and LH above 4 mm (PRUNIER et al, 2000).

Follicular survival factors include epidermal growth factor (EGF), insulin-like growth factor (IGF-1), gonadotrophins, activin and estrogen, while atresic factors include decreased estrogen, an increase in progesterone, testosterone, GnRH and interleukins. Follicular atresia is the result of a balance between survival and atresic factors in the porcine ovary and death by cell apoptosis is the mechanism by which it occurs (COX, 1986).

The follicular phase is the period that begins with the regression of the corpus luteum (CL) and lasts until ovulation. During this phase, there is a predominance of growing follicles, which can reach pre-ovulatory sizes, as well as estradiol production, and it can be subdivided into proestrus and estrus. Proestrus lasts from one to three days and during this phase there are not only anatomical changes (edema and vulvar hyperemia), but also behavioral changes, such as females trying to jump on each other. These changes are because during this period there is a high level of estrogen produced by the ovarian follicles, which acts on the central nervous system, causing a period of positive feedback on the gonadotropins (GORE-LANGTON et al, 1994). Estrus, on the other hand, has an average duration of 50-60 hours, but ranges from 24 to 96 hours (SOEDE et al, 1993; WEITZE et al, 1994). The female, in addition to still trying to mount the others, shows a tolerance reflex to the sexually mature male (DE FRIES et al, 2010).

The luteal phase is the period between ovulation and CL regression. In this phase, the main ovarian structure is the CL and progesterone is the predominant hormone. Even though this phase is characterized by the production of progesterone, the follicles continue to grow and regress, but do not produce high amounts of estradiol (SENGER, 2003). It is also divided into metestrus and diestrus, with each phase lasting two to three days and seven to 12 days, respectively. In metestrus, estrogen concentrations reach baseline levels and the hemorrhagic bodies (from the ovulatory follicles) begin to produce progesterone. Diestrus is characterized by the production of maximum levels of progesterone on days 12 to 14 of the cycle. Luteal regression begins on day 15 to 16 of the cycle with progesterone reaching baseline levels on days

17 to 18 of the cycle, characterizing the restart of the cycle (BORCHARDT NETO et al, 2005). During the luteal phase of the estrous cycle, there is a continuous growth of follicles without the appearance of dominant follicles or follicular waves (MADEJ et al, 2006).

Luteolysis or regression of the corpus luteum (CL) is characterized by the initial decline in progesterone secretion (functional luteolysis) followed by a change in the cellular structure of the physical space that comprised the CL (structural luteolysis). In other words, the cessation of progesterone production and loss of cellular components and integrity (functional and morphological regression), including reduced vascular supply, proliferation of connective tissue, increased cellular disorganization, degeneration and phagocytosis of luteal cells (MIYAMOTO, 1996).

Prostaglandins are local mediators produced by various tissues that play an important role in biological and pathological processes. Several studies have shown in vivo and in vitro the role of PGF2α in the mechanisms that lead to CL by: decreasing the synthesis of P4; regulating the receptors for luteotropic hormones; inhibition of cellular uptake of cholesterol; inhibition of cholesterol transport through the cell and/or mitochondrial membrane; inhibition of the expression of steroidogenic enzyme activities, required for P4 biogenesis and increased concentrations of free calcium ions (Waite et al. et. 2005).

The recruitment and selection processes allow the development of several ovulatory follicles, which are species-specific and gonadotrophin dependent (FOXCROFT et al, 1994). A significant increase in the growth of follicles selected for ovulation seems to occur between days 14-17 of the estrous cycle (FOXCROFT et al, 1985; Ryan et al, 1994), as this is the time that coincides with luteolysis and it is known that high levels of progesterone inhibit the release of LH, blocking the maturation of follicles. Thus, after luteolysis, low concentrations of progesterone cease to block the hypothalamic-pituitary axis, increasing LH levels and allowing follicle growth, maturation and ovulation to continue (FOXCROFT et al, 1982).

Therefore, in the case of the female pig, the onset of puberty begins with the intervention of the gonadotropins FSH and LH (follicle stimulating hormone and luteal hormone, respectively). This initiates the development of 15-30 or more primordial follicles located in the ovarian cortical (DA SILVA, 2020).

In the sow, the ovaries are two oval structures located in the abdominal cavity in the sub lumbar region on both sides. Generally, the right ovary is larger than the left and, as in the case of most female mammals, most of the ovarian surface is surrounded by the germinal epithelium, except for a small portion, the ilium-ovarian, through which the vessels and nerves penetrate (DA SILVA, 2020). The ovarian stroma is formed by a network of connective tissue in which there are numerous follicles containing germ cells or oocytes at various stages of development. These follicles appear on the surface of the sow's ovary in the form of a series of rounded eminences that give it a blackberry appearance. The sow is born with a certain number of primary follicles that originated during embryonic development, each follicle containing a first-order oocyte that has developed up to mitosis I. Secondary or polyestratified follicles originate from the primary follicles, reaching the level of tertiary Graaf follicles or mature follicles with the onset of puberty (DA SILVA, 2020).

Regarding oocytes, their quality is determined, among other characteristics, by the presence or absence of cumulus cells. Studies show that maturation does not occur or occurs to a lesser extent when the cumulus cells are removed before the oocytes are matured *in vitro* (GONÇALVES et al, 2002).

In pigs, the follicular antrum is fully differentiated into 0.5mm follicles, with the corresponding oocyte reaching only three-quarters (¾) of its final size (MOTLIK et al, 1984). These oocytes have a very limited ability to initiate nuclear maturation (MOTLIK et al, 1986). The ability of oocytes to continue and complete meiosis is not achieved all at once, at a certain point in their development, but is acquired gradually. Of the competent oocytes present in antral follicles, only those that have reached an adequate stage of development will respond to the maturation stimulus and begin morphological and functional changes, including the restart of meiosis (ABEYDEERA,2002).Yoon et al. (2000) reported that a greater number of oocytes from antral follicles(3 to 8mm) reached the MII stage (91 vs 58%), formed the pronucleus (90 vs 81%) anddeveloped to the blastocyst stage (10 vs 2%), compared to those from small follicles (<3mm).These results suggested that the full potential to complete meiosis and the subsequent competence to continue their development is acquired with follicular growth.

2. Follicle Stimulating Hormone

FSH is the main hormone controlling follicular growth and its secretion by the adenohypophysis is not only triggered by GnRH (hypothalamic origin) but is also partly controlled by the main products secreted by the developing follicles: estrogen and inhibin. Activin and TGF-beta (Transforming Growth Factor-beta) act as stimulators of FSH regulation (FOXCROFT et al, 1994).

LH acts together with FSH to induce the secretion of estrogen by ovarian follicles (HAFEZ, 1995). At first, high levels of estrogen block the release of LH by a negative feedback mechanism. This LH peak is responsible for ovulation and subsequent luteinization of the granulosa and internal theca cells (AINSWORTH et al, 1990). Although FSH stimulation is essential for the development of the group of pre-ovulatory follicles, the final part of follicular maturation and ovulation are dependent on pulsatile LH secretion (FOXCROFT et al, 1994).

That said, the effectiveness of FSH has been investigated in pigs for inducing estrus in prepubertal females by treating them hourly with high doses of the hormone (JACKSON et al, 2006). In this sense, Guthrie et al. (1997). reported that when gilts were treated with multiple injections of FSH, some LH activity was required, even in the form of hCG, to increase the ovulation rate, when compared to the ovulation rate of the group in which only hCG was applied. However, it has also been reported that FSH containing hCG, despite increasing ovulation rates, did not increase fertilization rates or recovered oocytes (GUTHRIE et al, 1997). The activity of FSH and LH seems to be critical according to Bolamba et al. (1996) who reported that when FSH was administered to prepubertal females, the number of quality oocytes increased and those with expanded cumulus and uniform and compact ooplasm, when compared to those animals where only eCG or saline was administered.

3. Brilliant Cresyl Blue Test (BCB)

There are many factors that can impinge on the efficiency of *in vitro* embryo production (IVEP) in mammalian species. However, the maturity and quality of oocytes arguably play an essential role in determining their developmental competence. While it is relatively easy to determine the meiotic maturity, there is no reliable method to estimate the cytoplasmic maturity of oocytes, which is predominantly responsible for the main outcome of IVEP – the blastocyst formation rate (OPIELA et al, 2013). As the oocytes after the BCB (Brilliant Cresyl Blue) staining can be processed further, there have been many attempts to verify if this method can be employed as a non-invasive, indirect predictor of the underlying features of oocyte quality and/or developmental competence.

The Brilliant Cresyl Blue (BCB) test determines the activity of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme synthesized in the growing oocytes but has low activity in the oocytes that have finished their growth phase. Therefore, the oocytes that have completed the growth phase are blue (BCB+), because the G6PDH activity is too low to reduce the staining, while the growing oocytes remain colorless (BCB-) (ALM et al, 2005).

Pawlak et al. (2014) assessed whether the BCB+ oocytes from the ovaries of pre-pubertal and cyclic gilts differed in terms of the incidence of numerical chromosomal aberrations. Only the BCB+ oocytes were chosen to mature *in vitro* and further subjected to fluorescence in situ hybridization (FISH) analysis using molecular probes for chromosome pairs 1 and 10. Interestingly, the proportion of aberrant oocytes was significantly lower (9.0%) in the non-BCB treated oocytes. The BCB+ and control oocytes collected from the ovaries of cyclic gilts did not differ in terms of the percentage of abnormal cells. A significant difference was, however, noticed in the oocytes derived from the ovaries of pre-pubertal gilts; the BCB+ oocytes showed a significant increase in the percentage of gametes compared with control oocytes. According to the authors, this phenomenon may be a result of subjecting the oocytes to extra 90-min incubation in BCB solution, which caused the first polar body extrusion oocytes from prepubertal gilts.

4. *In vitro* Maturation

Oocyte maturation is one of the most important stages for *in vitro* production of embryos. In addition to undergoing nuclear modifications (nuclear maturation) to develop fertilization capacity, oocytes store substances and undergo morphological alterations (cytoplasmic maturation), which promote and are essential for the early development of the embryo. Since *in vitro* maturation (IVM) is not as efficient as *in vivo* maturation, new studies are needed to clarify optimal culture media combinations needed to maximize maturations rates. Several media support *in vitro* nuclear maturation of pig oocytes but not cytoplasmic maturation. Problems in cytoplasmic maturation interfere with the formation of the pronuclei after penetration of the sperm, despite normal germinal vesicle (GV) breakdown and extrusion of the first polar body (ABEYDEERA, 2002).

In many laboratories, oocytes selected for routine IVM procedures generally contain >3 layers of surrounding cumulus cells. These cells are considered an important element of the cumulus oocyte complex (COC) that supports nuclear and cytoplasmic maturation necessary for successful pro nuclear formation and developmental competence. During the process of IVM, cumulus cells that surround each individual oocyte show varying degrees of expansion and may be functionally related to the nuclear or cytoplasmic maturation of the oocyte. To a certain extent, the degree of cumulus expansion could serve as an indicator of successful nuclear and cytoplasmic maturation. In most mammals, oocytes are enclosed in an expanded, mucitied mass of cumulus cells at ovulation (YAMAUCHI et al, 1990).

Epidermal growth factor can enhance nuclear maturation of COCs isolated from both small (< 4mm) and large (6 to 7 mm) size follicles with cumulus expansion induced only in COCs isolated from large follicles (YAMAUCHI et al, 1990). It was found that EGF stimulated both HA production in COCs from large follicles and its retention within the extracellular matrix of the expanding cumulus. The results indicated that the response of COCs to EGF induced hyaluronic acid synthesis and cumulus expansion occurred gradually during follicular growth. The presence of cumulus cells during maturation significantly influenced nuclear maturation (MII), intracellular glutathione (GSH) content, penetration rate, pro-nuclear formation, and histone H1 kinase activity (YAMAUCHI et al, 1990).

Initially, the *in vitro* conditions used to mature porcine oocytes were based on those used to successfully mature the oocytes of cattle and sheep. Although the early porcine embryo IVP achievements demonstrated that these IVM conditions could support the nuclear and cytoplasmic maturation of a proportion of immature oocytes, the developmental potential of porcine IVM oocytes was typically much poorer than that of cattle and sheep IVM oocytes. Obviously, the differences in oocyte quality between species may be attributed to differences in ovarian physiology and characteristics of the donor female at oocyte recovery (HUNTER et al, 2009; WEBB et al, 2007).

Pigs are generally slaughtered at 6 or 7 months of age for pork producers to meet market demands. Therefore, abattoir-sourced ovaries are usually collected from prepubertal gilts that have not yet experienced regular estrous cycles. Archibong et al. (1992), showed that the inherent quality of oocytes increases as gilts progress from their first to third estrous, because survival of embryos from first-estrous donor gilts after transfer to recipient gilts was lower than that of embryos from third-estrous donor gilts. The quality of immature oocytes selected for IVM is clearly associated with the follicular environment from which they were recovered.

Prepubertal porcine oocytes isolated from antral follicles 3, 4, and 5 to 8 mm in diameter formed blastocysts *in vitro* at rates of 17, 36 and 55%, respectively (BAGG et al, 2007). Although a blastocyst formation rate of 55% is comparable with that obtained from adult oocytes, the antral follicle distribution of prepubertal ovaries is such that there are relatively fewer large (5–8 mm) follicles and many smaller (3mm) follicles than is present on adult ovaries (BAGG et al, 2007).

Once isolated from antral follicles, the selection of each cumulus-oocyte complex for IVM is usually based on the number of compact cumulus cell layers and the appearance of the oocyte cytoplasm. A clear, positive association exists between the number of cumulus cells surrounding an oocyte and the capacity of that oocyte to complete meiosis and undergo fertilization events (NAGAI et al, 1993).

5. *In vitro* Fertilization

Successful *in vitro* penetration of IVM oocytes has been realized by using various types of fertilization media in conjunction with fresh or frozen-thawed spermatozoa. Nevertheless, large variations among boars as well as among different factions within the same ejaculate in terms of oocyte penetration and/or polyspermy have been observed (XU et al, 1996).

Cryopreservation of spermatozoa offers an effective solution for long-term storage of valuable genetic material and allows production of embryos with desirable genetic makeup through IVF in years to come. Development of successful techniques to freeze semen from a single ejaculate would minimize or eliminate the variability among trials. However, the same IVF protocol may not necessarily contain the optimal conditions for frozen semen from another boar. To realize desirable IVF parameters, it is necessary to optimize the IVF protocol for each individual batch of frozen semen. In addition, culture medium components, macromolecule type, sperm concentration, co-incubation interval, and presence or absence of caffeine have been shown to influence sperm penetration (ABEYDEERA, 2001).

Pig embryo production via IVM-IVF techniques has been hampered by two major problems, poor pro nuclear formation and polyspermy. Various refinements to IVM culture techniques, such as addition of follicular fluid, co-culture with follicular somatic cells, limited exposure to gonadotropins, and supplementation of EGF or cysteine, significantly improved cytoplasmic maturation as evidenced by higher pro nuclear formation after sperm penetration (ABENDEERA, 2001; DAY et al, 2000).

It is important to notice that in the *in vivo* setting, ovulated COCs meet oviductal secretions before being fertilized. These secretions induce changes at the zona pellucida (ZP) that affect further sperm-ZP binding and the role of the ZP in the control of polyspermy. Among these modifications is the hardening of the ZP, or an increase in its proteolytic resistance (BROERMANN et al, 1989).

In vitro, the ZP hardening that occurs prior to fertilization has been directly associated with a reduction in polyspermic fertilization in pigs and cows and it was described as a novel mechanism in preventing polyspermy (COY et al, 2008). Oviductal glycoprotein 1 (OVGP1) was identified as one of the main factors in oviductal secretions responsible for hardening the ZP together with heparin, and probably other sulphated glycosaminoglycans (GAGs) (COY et al, 2008).

Pig oocytes use prefertilization ZP hardening to control polyspermy but the concentration of the specific molecules responsible for the zona block is unknown and they are not commercially available. Moreover, since there are likely multiple factors in combination that contribute to the zona block, the preincubation of oocytes in ovarian fluid from the preovulatory phase of the estrous cycle represents the most efficient option to achieve full ZP maturation before coincubation with sperm. Routinely, *in vitro* matured porcine COCs are mechanically stripped of surrounding cumulus cells and the denuded oocytes are then inseminated. The effect of some molecules added to IVF media is opposite depending on whether cumulus-enclosed or cumulus free oocytes are inseminated (TATEMOTO et al, 2005).

A recent study showed best IVF and embryo development results with cumulus-enclosed, rather than with cumulus-free oocytes, together with other factors such as sperm concentration (LI et al, 2007). *In vivo* analyses using digital video microscopy showed that the external cumulus cells of ovulated bovine COCs attached to oviductal and cumulus cells detach slightly during migration of COCs down the oviduct to the fertilization site (KOLLE et al, 2009). In pigs, this feature is simulated by partially denuding the mature COCs before submitting them to insemination (GARCÍA-MARTINEZ et al, 2013).

The classical media used for porcine IVF include modified Tyrode's albumin lactate pyruvate (TALP), modified Tris-buffered medium (mTBM), modified tissue culture medium 199 (mTCM199) and porcine gamete medium (PGM) (ROMAR et al, 2016). Except for TCM-199, the stock media are not available commercially, so they are further modified in-house in different laboratories. Culture media share a common formulation containing various components including inorganic salts, nutrients, vitamins and growth factors (CHRONOPOULOU et al, 2015), and they are supplemented for gamete coculture. The number and type of molecules added to these media are wide and varied. Defined media are those in which all the chemical components are known, whereas in the semi-defined media there are one or more chemically undefined natural substances and in the undefined media the major components are mixtures of natural substances. Since the ideal concentration of each molecule necessary at the time of fertilization is unknown, in the laboratories the decision on choosing one medium over another is based on the type of semen employed (epididymal or ejaculated; fresh or frozen thawed), the sperm capacitation method, the desired IVF culture medium (defined, semi-defined or undefined), the IVF device (4- well dishes, climbing-over-a-wall, microfluidic devices), the external conditions of gamete coculture, the volume of culture medium for gamete coculture (microdroplet or well), and the final objective of the experiment.

Considering that live piglets have been obtained from different IVF media and systems, it is necessary to focus on the specific additives that markedly increase IVF efficiency in pigs. A thorough review of the different molecules that have been used during gamete coculture in different laboratories offers some clear conclusions. As single molecules, sildenafil, relaxin, casein phosphopeptides and methylcellulose are the additives with best results. However, the highest improvement is achieved when the IVF medium is supplemented with 1% Ovarian Fluid. In the future, the exosomes and macrovesicles contained in the Ovarian Fluid (VILELLA et al, 2014) could be explored as new additives. Considering that live piglets have been obtained from so many different IVF media and systems, it is necessary to focus on *in vivo* parameters such as fertility rate, number of piglets born, growing parameters, health status and productivity of animals born (VILELLA et al, 2014).

The use of new additives and devices for pig IVF must be accompanied by a review of the environmental conditions in which both gametes are cocultured, regarding pH, as well as temperature and gas atmosphere, since both parameters are easily adjustable in the incubator. The most widespread coculture conditions for porcine gametes are 38.5-39ºC under 5% CO_2, 20% O_2 and 75% N_2. In vivo, the average temperature in the oviductal ampulla ranges from 37.8ºC (in mated pigs) to 38.2ºC (in unmated pigs) (ROMAR et al, 2019).

As for gases, *in vivo* measurements of O_2 tension using minimally invasive methods within the oviduct and uterus of animals at different stages of the estrous cycle revealed higher O_2 in gilts compared to sows (10.0 vs. 7.6%) (GARCÍA-MARTINEZ et al, 2013). When the physiological O_2 tension in the female reproductive tract was mimicked in the laboratory, and gametes and embryos were cultured under 5% CO_2, 7% O_2 and 88% N_2, the final efficiency increased compared to traditional conditions (38.5ºC, 5% CO_2, 20% O_2 and 75% N_2) as did embryo development and quality (GARCÍA-MARTINEZ et al, 2013).

6. *In vitro* Culture

For swine and other farm domestic species, the development of culture systems capable of supporting the growth of immature follicles to a stage where they could be matured and the oocyte fertilized would ensure a large supply of oocytes for manipulation. These oocytes could potentially be used to shorten the generation interval of selected animals and, consequently, to increase the number of offspring born per animal. Development of a successful culture system for preantral follicles with immature oocytes is dependent upon efficient procedures to recover the follicles from the ovary and culture them as well (ARAÚJO et al, 2014).

Early attempts to culture porcine embryos *in vitro* throughout the pre-implantation stages were unsuccessful. *In vivo*-derived embryos could develop to the blastocyst stage in various media when cultured from the four-cell stage, but they arrested development at the four-cell stage when cultured from the one-cell stage

(DAVIS et al, 1985). To overcome this block in development, researchers cultured porcine embryos after IVF by transferring them to the oviducts of recipient pigs. Early-stage porcine embryos transferred to the ligated oviducts of anestrous sheep could also develop to the morula and blastocyst stages and give rise to offspring after subsequent transfer to recipient pigs (PRATHER et al, 1991).

It was therefore logical that embryo culture media should be modified to mimic the composition of oviductal fluid more closely. Supplementing medium with oviductal fluid, co-culturing with oviductal epithelial cells, and culturing in mouse oviduct in organ culture were other approaches that were found to be beneficial to *in vitro* porcine embryo development (PETTERS et al, 1993).

Studies that compared the effectiveness of different media consistently showed that NCSU-23 medium was superior in terms of supporting development to the blastocyst stage (PETTERS et al, 1993). Differences in the presence and abundance of glucose, pyruvate, and lactate were the primary cause for the observed variance between media. Findings suggested that porcine embryos did not require pyruvate or lactate, because NCSU23 medium only contained high levels of glucose, and that lactate inhibited the development of porcine embryos in the presence of glucose. It was subsequently shown that culture of porcine embryos in NCSU-37 medium lacking glucose and containing low concentrations of pyruvate (0.17 mmol/L) and lactate (2.73 mmol/L) for the first 48 hours, followed by NCSU-37 with glucose (5.55 mmol/L), improved blastocyst development (KIKUCHI K et al, 2002).

Besides that, similar alterations to the energy substrate concentrations ofNCSU-23 were found to be beneficial to the development of parthenogenetic porcine embryos (BEEBE et al, 2007). In the pig female reproductive tract, embryos traverse the oviducts to the uterine horns at the four- to eight-cell stage about 2 days after fertilization (HUNTER, 1974). Metabolite analyses of oviductal and uterine fluids have invariably found differences in the concentrations of energy substrates (GARDNER et al, 1996; LI et al, 2007).

Interestingly, the concentration of glucose in porcine oviductal fluid was found to decrease markedly from the pre- to postovulatory period via an unidentified systemic mechanism (NICHOL et al, 1992; NICHOL et al, 1998). Furthermore, analysis of IVP porcine embryo metabolism revealed that glucose utilization increases from the one-cell to the blastocyst stage (GANDHI et al, 2001). Therefore, changing the culture medium composition after 2 days to simulate the changing *in vivo* conditions seems a valid rationale. However, the effectiveness of a more recently described single-step culture medium, porcine zygote medium (PZM) (YOSHIOKA et al, 2002), the composition of which is based on the concentration of inorganic elements and energy substrates in porcine oviducts, challenges this reasoning.

The role of amino acids in porcine embryo development has received increasing attention over the last decade. This is unsurprising considering that oviductal and uterine fluids contain significant amounts of free amino acids, and the numerous demonstrations that amino acid supplementation stimulates the development of embryos in other species (GARDNER et al, 1996). The inclusion of hypotaurine and taurine in NCSU-23 medium was shown to improve the development of early-stage porcine embryos (PETTERS et al, 1993). Suzuki and Yoshioka (2002) reported the positive effects of adding glutamine and hypotaurine to PZM on blastocyst formation and found the effects of premixed solutions of amino acids to be concentration dependent. Because amino acids are known to degrade during culture to form ammonia, the beneficial effects of amino acids may be abolished at higher concentrations owing to ammonia buildup. The development of IVP porcine blastocysts was enhanced by adding glycine to glucose containing PZM at Day 5 of culture (MITO et al, 2012).

The results, until this date have already shown great progress, but it seems that there are still no defined methods for the three phases of PIVE, and that modifications to the protocols seem to be necessary to obtain better results, especially for biotechnological advances in the cryopreservation of the embryos that are produced through PIVE to be innovulated or exported.

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CHAPTER 2 - ARTICLE

IMPACT OF FSH ON OOCYTE AND EMBRYO PRODUCTION IN PREPUBERTAL GILTS

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ABSTRACT

The aim of this study was to investigate the effects of the FSH treatment on the ovarian response of 140 days old gilts. The cumulus-oocytes complexes (COCs) were recovered from follicles of twenty-two prepubertal gilts, who were allotted to receive 100mg of FSH (treated group; n= 10) or saline solution (control group; n= 12). The percentage of small, medium and large follicles did not differ statistically (P>0.05). Concerning ovarian and uterine biometric data, the parameter “length of the left ovary” showed difference (P<0.05) (2.37 ± 0.26 for the control group and 2.49 ± 0.44 for the treated group (P= 0.02) and “thickness of the left ovary” too (1.31 ± 0.20 control group and 1.42 ± 0.41 treated group) (P=0.003). Besides that, the parameters related to the number and type of cells obtained on the last day of *in vitro* culture (D7) did not differ significantly (P>0.05). In terms of tendency, blastocyst conversion rate in relation to the number of morulas on day 7 (28.14 ± 13.10 – control group and 16.05 ± 14.16 – treated group) presented p-value between 0.05 and 0.10, since the control group presented higher numbers. Backfat presented tendency (P=0.09). In conclusion, due to the contradictory data regarding quantity and quality of follicles and embryos, more research is needed to fully understand the role of FSH in pre pubertal oocyte maturation in the pig, either on living gilts or being added to IVEP media.

Keywords: Cumulus-Oocytes Complexes. Hormone. *In Vitro* Fertilization. Morula. Ovary.

1. INTRODUCTION

Oocyte competence refers to the ability of the oocyte to resume meiosis, cleave following fertilization and develop into a viable embryo (SIRARD et al, 2006). Changes in protein synthesis of cumulus-oocyte complex (COCs), oocyte energy metabolism and ultrastructural and cytochemical modifications of prepubertal oocytes have been related with the inability or failure to undergo nuclear and ooplasmic maturation and explain the reduced developmental potential of prepubertal oocyte (GANDOLFI et al, 1998; DE PAZ et al, 2001).

In pigs, puberty is regulated by multiple factors, such as age, breed, nutritional status, environment, body weight and backfat thickness. It is acceptable, however, to assume that gilts reach puberty around 150-180 days of age (EVANS and O'DOHERTY, 2001; MCGLONE et al, 2021). *In vivo* derived oocytes increase the developmental competence progressively after the occurrence of puberty (ARCHIBONG et al, 1987). However, the precise age in which oocytes obtained from prepuberal gilts become competent remains largely unknown. Strategies have been adopted to improve oocyte quality and/or developmental competence of prepubertal oocytes, some of these are supplementation of cytokines and other factors in *in vitro* maturation (IVM) medium (CÓRDOVA et al, 2010; HAMMAMI et al, 2014; TIAN et al, 2021), and hormonal treatment in the oocyte donor prior follicular aspiration (OROPEZA et al, 2004).

In this regard, gonadotropin stimulation (e.g. FSH, eCG) has been widely used in ruminant species (TECHAKUMPHU et al, 2000; LEONI et al, 2009; ZACARIAS et al, 2018). Studies showed that FSH treatment increase the number of viable oocytes (ZACARIAS et al, 2018) and the *in vitro* embryo development of oocytes recovered from prepubertal lambs (MORTON et al, 2005), this indicates that exogenous hormonal stimulation can be an alternative to improve oocyte competence in prepubertal oocyte donors. Recently, Yoshioka et al. (2020) reported the birth of piglets from *in vitro*-produced blastocyst from oocytes recovered by ultrasound-guided ovum pick-up (OPU). In this study, an increase in the efficiency of blastocyst production from oocytes collected from cyclic donor sows previously treated with FSH compared with oocytes obtained from gilts ovaries collected in slaughterhouse was demonstrated. This shows that FSH treatment prior to oocyte collection could improve the competence of the oocytes obtained from donor sows (YOSHIOKA et al, 2020).

Studies indicate that during the formation of blastocysts, some metabolic changes occur which increase the overall metabolic rate. There is an increase in the uptake and utilization of pyruvate and glucose and the production of lactate, denoting an increase in energy needs and, therefore, a faster rate of reactions for the synthesis of ATP and other important compounds for cellular functions (AMEISEN et al, 2002; LONERGAN et al, 2003; RIZOS et al, 2003).

In *In vitro* cultivation, there is an increase in the oxidative rate that occurs in the mitochondria, which leads to the generation of free radicals, damaging embryonic development (CHARPIGNY et al, 1997). For this reason, there is a need to study existing culture media to obtain the best medium, with the aim of improving them to obtain a higher viability rate after transfer (ROSSANT et al, 2001).

It is important to note that oocytes are highly specialized cells and the only ones dependent on the introduction of external deoxyribonucleic acid (DNA) from the sperm to carry out the following phases of development (GARCÍA, 2005). The diameter of the oocyte and the morphology of the cumulus-oocyte complex (COCs) are good indicators of the oocyte competence to produce embryos (ANGUITA et al, 2007).

It has been shown that *in vitro* maturation (IVM) of sow´s oocytes indefined media produces viable embryos (DUCOLOMB et al, 2005). To increase the efficiency and reproductivity in IVM and *in vitro* fertilization (IVF), as well as embryonic development, defined media have been created. These media are supplemented with polyvinyl alcohol (PVA), cysteine, glucose, sodium pyruvate, epidermal growth (EGF), LH and FSH (DUCOLOMB et al, 2005). In the sow, different modifications have been made in the techniques for embryo culture, the addition of hormones FSH and LH, as well as EGF in the culture media for oocyte maturation have been more efficient; with them quality embryos have been obtained and suitable for development (ABEYDEERA, 2002).

FSH induces cumulus cell expansion and nuclear maturation *in vitro* (SINGH et al, 1993). Mattioli et al. (1991) demonstrated that the mixture of FSH and LH accelerates and facilitates the mitotic progression of the oocyte. Stimulation with FSH has a positive effect on the quality of oocytes for IVM obtained by follicular puncture (SANCHÉZ et al, 2003). Paracrine interaction between the oocyte and surrounding granulosa cell factors is critical *in vivo* for its normal development and function (THOMAS et al, 2006). In IVM, special attention must be paid to the management of pH and temperature, to ensure protein synthesis and to maintain stable morphology of the meiotic axis, since alterations in these parameters cause a decrease in the maturation and fertilization capacity of the oocytes (JUREMA et al, 2006).

In this way, the aim of this project is to make it possible to obtain embryos early in young (prepubertal) female pigs. In addition, this project aims to prove that it is possible to use the ovaries of females of high genetic value to use the oocytes obtained for cultivation and future export of embryos, bringing enhanced genetic gain. Therefore, the objectives of this project include hormonal stimulation using FSH to obtain an ovarian follicular response in prepubertal gilts.In addition, to recover good oocyte quantity and quality, with these oocytes beingcompetent, capable of resuming meiosis after the *in vitro* maturation stage. The aim of this study is also for the matured oocytes to be capable of fertilization and cleavage, developing into blastocysts by the seventh day of culture. The embryos obtained must be of high quality and capable of generating pregnancy. This confirms the minimum age for using gilts in breeding programs as oocyte donors.

2. MATERIAL AND METHODS

2.1 Ethics, location and animal conditions

The Ethics Committee in the use of Farm Animals from Universidade Federal de Viçosa approved the current experimental design (CEUAP/UFV: 0104/2023). This experiment was conducted during October 2023 to February 2024 in the Pig Breeding Farm from the Department of Animal Science at Universidade Federal de Viçosa, Viçosa, MG, Brazil (Lat 20°46'32,60"S and Long 42°51'33,50"W). The animals were slaughtered at the slaughterhouse of the Universidade Federal de Viçosa (Municipal Inspection Seal: 040).

2.2 Media

Different media have been used for the *in vitro* production of pig embryos, all of which have been developed by Wang et al. (1998), Batista et al. (2016) and Yoshioka et al. (2002) for maturation, fertilization and culture media respectively. The washing medium used was TL-Hepes-PVA, composed of 114 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2$, 0.34 mM NaH_2PO_4, 10.0 mM sodium lactate, 2.0 mM $NaHCO_3$, 10.0 mM Hepes, 12.0 mM Sorbitol, 2.0 mM $CaCl_2 \cdot 2H_2O$, 0.2 mM sodium pyruvate, 0,1 mg/mL polyvinyl alcohol, 75 µg/mL penicillin G sodium and 50 µg/mL streptomycin sulfate.

Two specific media were used for *in vitro* maturation, consisting of TCM 199 medium supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 IU/mL hCG, 10 IU/mL eCG, 10 ng/mL epidermal growth factor, 75 µg/mL penicillin G sodium and 50 µg/mL streptomycin sulfate. The difference between the two-maturation media is the removal of hormones from medium 2 of IVM.

For fertilization, the medium used was composed of 113.1 NaCl, 3.0 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20.0 mM Tris, 11.0 mM glucose, 5.0 mM Na-pyruvate, 2.0 mM caffeine, 2.0 mg/mL fatty acid-free bovine serum albumin, 75 µg/mL penicillin G sodium and 50 µg/mL streptomycin sulfate.

Finally, the same formulation was used to produce two media, except that medium 2 contained glucose (5 mM) and glycine (10 mM). The composition of the IVC media (or PZM-3) was 108.0 mM NaCl, 10.0 mM KCl, 0.35 mM KH_2PO_4, 0.40 mM $MgSO_4 \cdot 7H_2O$, 25.07 mM $NaHCO_3$, 0.20 mM Na-pyruvate, 2.0 mM Ca-(lactate)$_2 \cdot 5H_2O$, 1.0 mM L-glutamine, 5.0 mM hypotaurine, 20 ml/L basal medium eagle amino acids, 10 ml/L minimum essential medium nonessential amino acids, 3.0 mg/mL fatty acid-free bovine serum albumin, 75 µg/mL penicillin G sodium and 50 µg/mL streptomycin sulfate.

2.3 Animals

Commercial line TN70 (Topigs Norsvin, Netherlands) gilts were housed in collective pens (2 animals/pen) and fed with soy/corn-based diet (Metabolizable Energy Weaning Phase: 3350,00 Kcal/Kg; Crude Protein Weaning Phase: 17,97%; Metabolizable Energy Nursery Phase: 3.350,00 Kcal/Kg; Crude Protein Nursery Phase: 15,69%; Metabolizable Energy Finishing Phase 1: 3350,00 Kcal/Kg; Crude Protein Finishing Phase 1: 13,72%; Metabolizable Energy Finishing Phase 2: 3350,00 Kcal/Kg; Crude Protein Finishing Phase 2: 12,00%) and *ad libitum* water. Gilts were selected based on following selection criteria (mean ± SD): birth weight (≥1.1 kg; 1.48 ± 0.25), weaning weight (6.69 ± 0.82 kg), 65 days weight (22.27 ± 3.87 kg), adequate body and vulva conformation, and absence of physical defects such as hernias or lameness. Gilts used in this study were at 143 or 144 days of age and had a mean body weight of 94.90±6.75kg (tested group) and 92.91±8.90 (control group) and backfat of 1.11±0.10 cm and 1.02±0.15 cm respectively for the control and treated group.

2.4. FSH Treatment

The animals in the tested group were treated with FSH, with equal doses of the hormone every 8 hours. Total dose of 100mg of FSH (Folltropin-V® 400mg; Vetoquinol Saúde Animal, São Paulo, Brazil) was divided into six similar doses (16,7mg/dose) administered by intramuscular injection 24h before slaughtering (BREEN and KNOX, 2012).

2.5 Follicular Aspiration and Obtaining of COC's

The reproductive tract was removed from each gilt after the animals had been slaughtered, then the ovaries were excised and placed in separate plastic bags containing pre-warmed (37ºC) 0.9% NaCl for transport to the laboratory. Small (<4 mm diameter), medium (4-6 mm diameter) and large (>6 mm diameter) follicles were counted and COCs were aspirated from medium follicles with an 18-gauge needle attached to a 3-mL disposable syringe, and placed in a petri dish containing manipulation media, which, is composed of 1.8262g sodium chloride, 0.0596g potassium chloride, 0.012g sodium phosphate, 0.042g sodium bicarbonate, 0.0735g calcium chloride dihydrate, 0.0254g magnesium chloride hexahydrate, 467μL sodium lactate (60%), 0.0025g phenol red, and 0.5975g hepes in a total of 250mL. In addition, 0,15g bovine serum albumin, 0,0011g sodium pyruvate and 50µL penicillin are added in the moment of use, for each 50mL of the first solution, cited above.

Then, the COCs recovered were placed in a petri dish containing TL Hepes-PVA media and next were morphologically classified according to Stringfellow and Givens (2010) as grade I (homogeneous cytoplasm oocyte and compact cumulus oophorus with more than three layers), grade II (homogeneous cytoplasm oocyte with small irregular pigmentation areas, and compact cumulus oophorus with 1-3 layers), grade III (oocyte with incomplete compact cumulus oophorus layer or irregular cytoplasm) or grade IV (naked oocytes or oocyte with heterogeneous cytoplasm or expanded cumulus cells). Finally, the picked ones (grades I and II) were placed in a petri dish containing phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) and BCB dye and incubated for an hour.

2.6 Experimental Design

Experimental design is illustrated in Fig. 1. Previous data from our laboratory led us to conclude that puberty occurs at 160 days of age in commercial maternal line TN70 (ALFRADIQUE et al, 2023). Twenty-two prepubertal gilts were redistributed into two experimental groups, gilts were allotted to receive FSH [treated; 100mg of FSH/total dose; G140+FSH (n = 10)] or saline [control; 0.9% sterile saline solution; G140+control (n = 12)] treatment. It is important to consider that two animals from the treated group and one from the control group were excluded from the statistics because they were considered outliers - they had 2 to 5 follicles on their ovaries. So, the final sample of treated group was n=8 and of the control group was n=11.

After 24 h of last FSH injection (coasting period; Day 3), gilts were slaughtered and ovaries collected. Medium follicles (4-6 mm diameter) and large follicles (> 6mm) were aspirated, recovered COCs were morphologically classified (grade I-IV) and COCs grades I-II were used brilliant cresyl blue staining, and *in vitro* oocyte maturation.

In this experiment, all the COCs graded I and II were washed twice in phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) and incubated in BCB medium (PBS supplemented with 0.4% BSA and 13 µM of BCB dye) for 60 min at 38.5ºC in a humidified atmosphere of 5% CO2, 10% O_2 and 85% N_2. Then, COCs were washed twice in PBS supplemented with0.4% BSA and classified under a stereomicroscope as: 1) BCB+ (fully grown and developmental competent) – COC with the ooplasma stained blue or 2) BCB- (growing and non-developmental competent) – COC with a colorless ooplasma (PAWLAK et al, 2014).

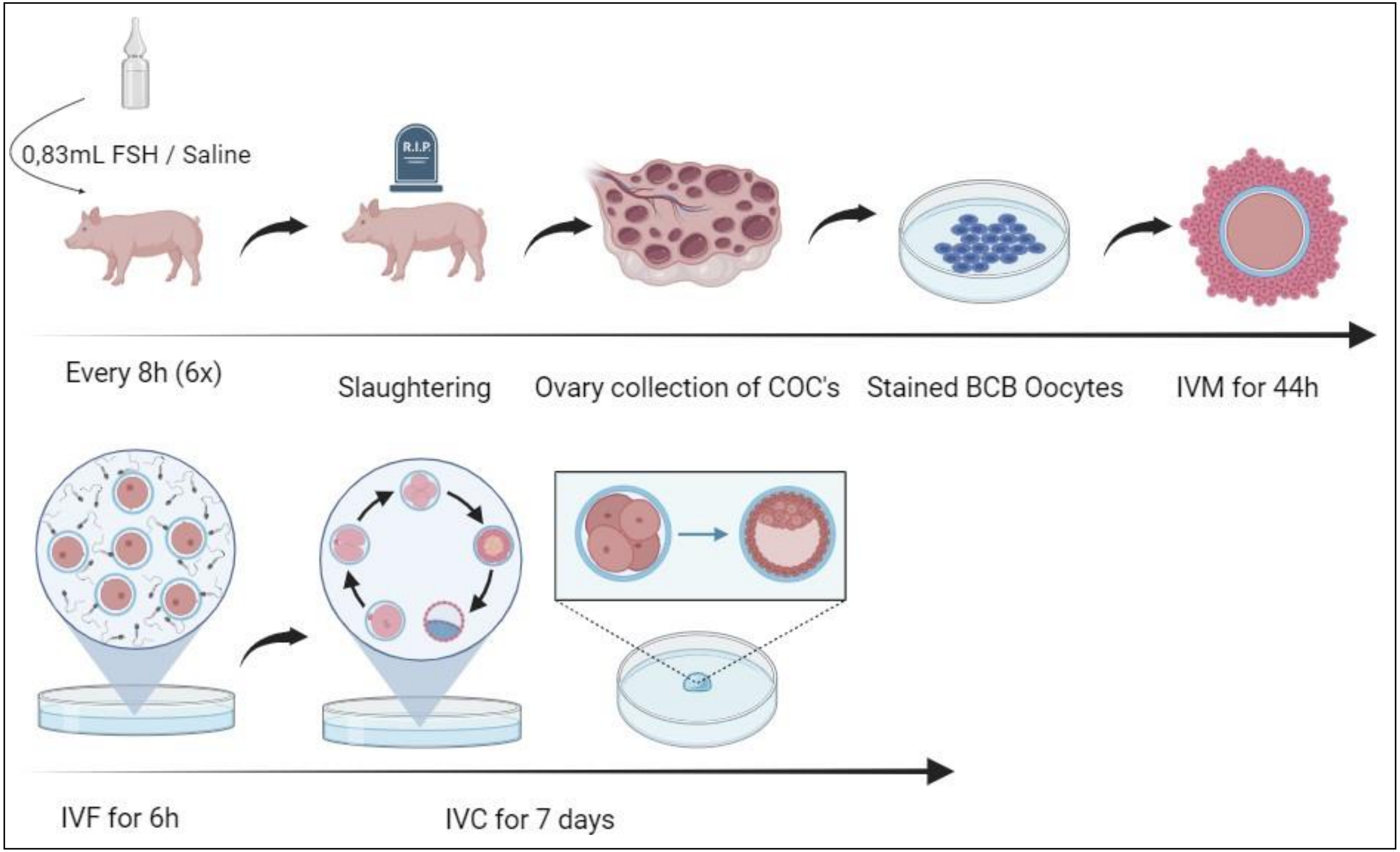

FIG. 1: Ovarian stimulation protocol and experimental design of the study: Effect of FSH stimulation on ovarian follicular response and oocyte, obtained from prepubertal gilts. COC’s (Cumulus Oocytes Complexes); BCB (Brilliant Cresyl Blue); IVM (In vitro Maturation); IVF (In vitro Fertilization); IVC (In vitro Culture).

2.7 *In vitro* Maturation

After BCB test, COCs were washed twice in TL-HEPES-PVA medium. Subsequently, COCs were washed twice in IVM medium. Groups of average 33 COCs were transferred into a drop (100µL) of IVM medium covered with mineral oil on a 35 mm dish, previously equilibrated in an incubator for at least 6h (at 38,5ºC), for 22 hours at 38.5ºC, in a humidified atmosphere of 5% CO_2, 10% O_2 and 85% N_2 (figure 2). COCs were then incubated for another 20-22h in IVM medium without hormones at 38,5ºC (figure 3) (FUNAHASHI and DAY, 1993).

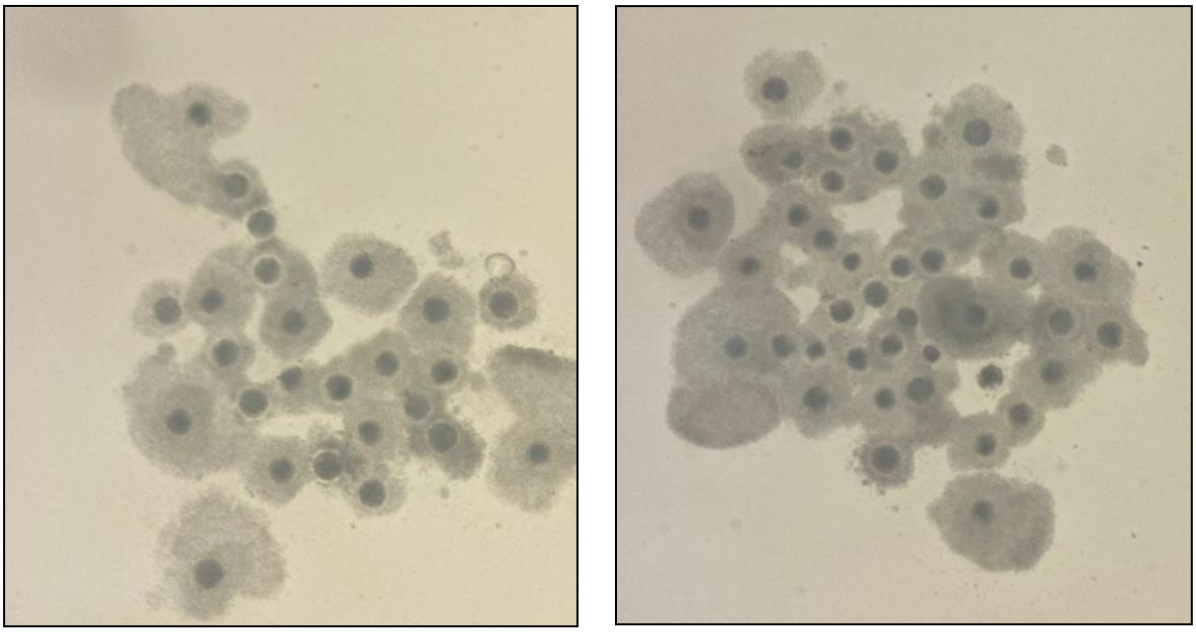

FIG. 2: COCs after 22h of *In vitro* maturation, from post-slaughter ovarian aspiration in 140-day-old gilts. Magnification: 120x.

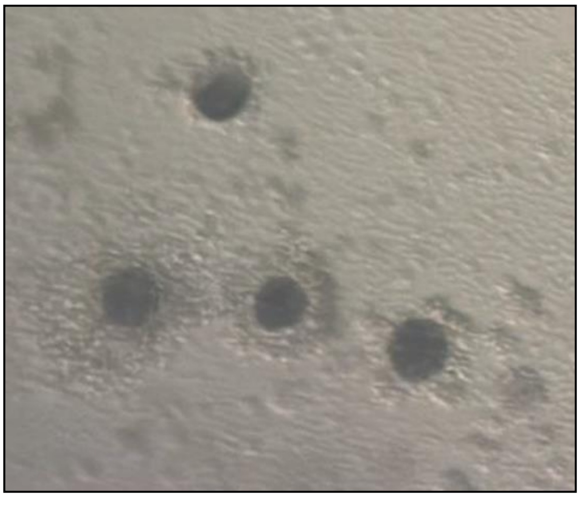

FIG. 3: COCs after 44h of *In vitro* maturation (cumulus cells expansion), from post-slaughter ovarian aspiration in 140-day-old gilts. Magnification: 300x.

2.8 Semen and *In vitro* Fertilization

For the sperm preparation technique sperm-rich fraction of ejaculate was use, and the following steps were done:

- Sperm washing centrifugation at 100 x g for 10 minutes in Beltsville thawing solution;
- Mini-Percoll discontinuous gradient (200 µL of 45% over 200 µL of 90%) for 30 minutes at 700 x g;
- Sperm washing: Centrifugation at 100 x g for 10 minutes in IVF medium.

After 44 hours of maturation, the oocytes were removed from maturation medium, washed three times in fertilization medium and then placed in petri dishes containing IVF medium (drops of 100µL) covered with mineral/paraffin oil, previously equilibrated in an incubator for at least 6h. Sperm cells were then added at a concentration of 3000 sperm cells per oocyte. The petri dishes were then placed in an incubator for 6 hours at 38.5ºC, in an humidified atmosphere of 5% CO_2, 10% O_2 and 85% N_2 for fertilization to take place (LI et al, 2007).

2.9 *In vitro* Culture

Putative zygotes were washed in PZM-3 medium (or IVC medium) three times. After 6 hours of *in vitro* fertilization, groups of average 33 putative zygotes were pippeted several times to remove the sperm cells attached to the zona pellucida and then transferred into a drop (100µL) of PZM-3 medium covered with paraffin oil on a 35mm dish. Half of the culture medium was renewed on day 6, with the PZM-3 medium added with 5mM glucose and 10mM glycine. Incubation conditions: 7 days, 38,5 °C in a humidified atmosphere of 5% CO_2, 10% O_2 and 85% N_2 (figure 4) (LI et al, 2007).

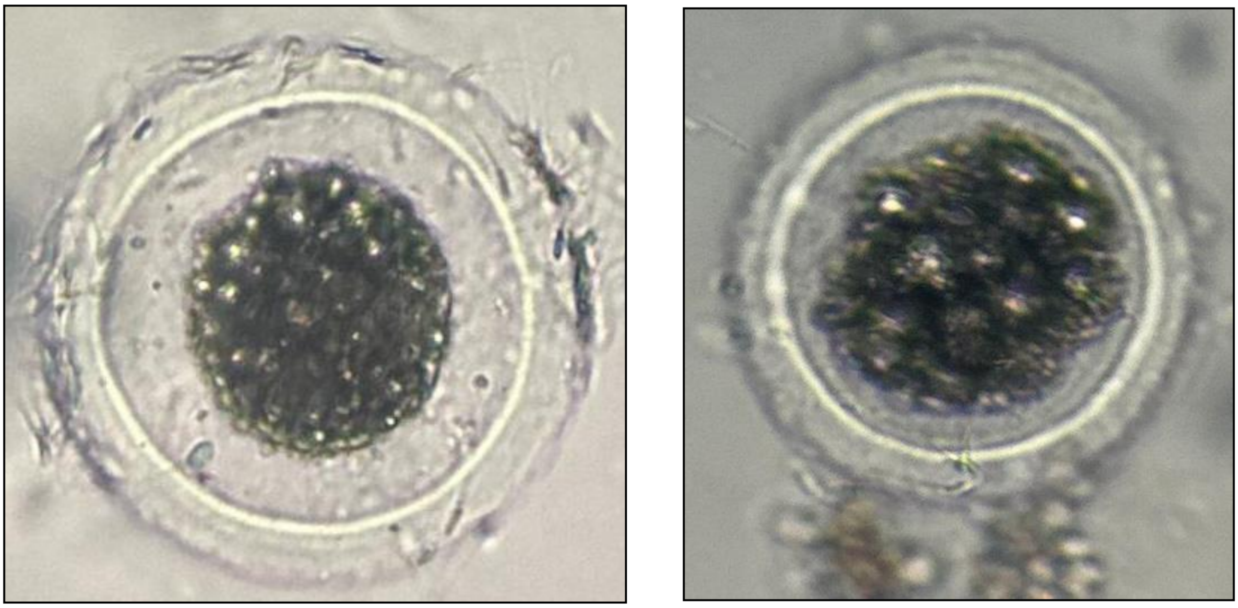

FIG. 4: Morulas after 5 days of culture, from post-slaughter ovarian aspiration in 140-day-old gilts. Magnification: 1000x.

2.10 Statistical Analysis

In this study, two animals from the treated group and one animal from the control group were excluded because they had ovaries with few follicles, which resulted in a very poor number of oocytes after aspiration (2 to 5 per animal).

The results of the variables analyzed were subjected to analysis of variance and the means were compared using the T test at a 5% probability level and tendency at a 10% probability level (SAS, 2000). For the statistical analysis of the resulting data, the SAS computer system proc GENMOD was used, considering the distribution of the variables to be gamma with an inverse link function. The treatment effect was considered by testing the inclusion against the control using a test of means.

3. RESULTS AND DISCUSSION

In vitro embryo production (IVEP) in pigs has been growing steadily over the last few decades following the increasing pork production chain. In this sense, IVEP has emerged for prepubertal females, from which oocytes are collected and subsequently cultured, with the aim of obtaining embryos. In addition, to improve results and increase the developmental competence of these oocytes, gonadotrophin stimulation (FSH) has been used (ALFRADIQUE et al, 2023). However, it is still unclear whether FSH treatment increases the quantitative and qualitative aspects of COCs or embryos obtained from prepubertal gilts, and whether the effects of FSH treatment are related to the donor age; to our knowledge, this is the first study reporting the use of FSH in pre pubertal gilts aiming the production of *in vitro* embryos.

The proportion of ovarian follicle population observed in the ovaries obtained from the prepubertal gilts at 140 days of age treated or not with FSH are shown in figure 5. The results showed difference (P=0.0038) considering the percentage of small follicles towards the control group and no effect (P=0.1462) of FSH regarding medium follicles population but there was tendency (P=0.0729) between groups considering the percentage of large follicles. Alfradique et al. (2023) found statistical difference between control and FSH treated groups just regarding small follicles (1-3mm). It is important to notice that the classification of the size of the follicles was different, as in this study the small ones were those <4mm, the medium ones between 4 and 6mm and the large ones >6mm and Alfradique et al. (2023) considered 1-3mm, 3-6.49mm and >6.5mm, respectively.

Concerning biometric uterine and ovarian parameters (tables 1 and 2), “length of the left ovary” and “thickness of the left ovary” presented differences (P=0.02 and P=0.003, respectively). This means that the majority morphology of the reproductive organs in the treated group was not affected by the FSH treatment, which corroborates with the study developed by Alfradique et al. (2023).

It is interesting that when it comes to number of small, medium, and large follicles, the control group had similar results (n=97,75±26.92) when compared to the treated group (n=81,81±42.53) (P=0.8702). In the same way when it comes to large follicles, the treated group had a similar number (n=7.81±10.51) versus n=3.75±8.65 (control group) (P=0.3953) (Table 3). In our study, FSH influenced the number of small follicles (P=0.0038), since in the control group this number was higher than in the treated group (figure 5), which means that the hormone affected the follicular waves in terms of stimulating the growth of medium ones in the tested group while the control group, without the hormone, had a bigger number of smaller ones.

Table 4 shows some absolute numbers in terms of the number of cells that were found in the last day of culture (d7). All the parameters that were evaluated presented practically the same result, as the values were very close. None of the parameters showed statistical significance (P>0.05).

The conversion rates on the seventh day (table 5) also showed no statistical difference (P>0.05), such as from morula to blastocyst (40,44% control group and 19,46% treated group) and percentage of morulas found (25,23% control group and 16,06% treated group). Regarding expected results of blastocyst formation and hatching rate on d7 of culture, some authors believe that a conversion of 30-45% and 15-25% can be reached, respectively, in bovines (HERNANDEZ-LEDEZMA et al, 1996). It is important to highlight though, that there was a tendency (0.05<P>0.10), towards the control group, to significancy regarding blastocyst conversion rate in relation to the number of morulas on d7 (P=0.0925).

Finally, it is important to emphasize some of our conversion rates obtained in the last day of culture: the conversions morula on d3 to blastocyst (d7) and blastocyst conversion rate in relation to the number of morulas on d7 had no statistical difference but presented tendency, respectively P=0.0773 and P=0.0518. Besides that, the conversion rate from oocyte to morula was 34,42% (control group) and 34,35% (treated group); the average fertilization rate from zygote, morula and blastocyst in relation to the number of cells on day 7 of culture was 48,06% for the treated group and the average fertilization rate from zygote, morula and blastocyst in relation to the number of cells on day 7 of culture was 55,03% for the control group, considering that neither parameters had statistical difference (P>0.05) nor tendency. It is important to mention that the coefficient of variance (CV) shown in all five tables and which can also be seen in the graphics, demonstrates that the big individual variation among animals, could have been responsible to affect directedly the significance of the evaluated parameters.

Considering the usage of FSH for embryo production in pre pubertal females, besides Alfradique et al. (2023), no other study in pigs was found in the literature. Regarding cattle, it was observed by Martins et al. (2013) in non-lactating cows that FSH treatment was able to increase the number of follicles as well as the number of structures suitable for maturation, but it was not effective in promoting the acquisition of oocyte competence as reflected in cleavage and blastocyst rates. Besides that, Snel-Oliveira et al. (2003) also found no statistical difference between groups of pre pubertal cows treated with FSH in three different doses and their conclusion was that the use of FSH and even FSH + eCG does not increase significantly the amount of follicles ≥ 3 mm at the OPU moment, as well as the total and viable oocytes by 10, 11 and 12 months of age.

There are some experiments in cattle that corroborate with the fact that it is possible to obtain a higher number of follicles in direct application of FSH to the cows, but it seemsto decrease quality and number of embryos obtained in the treated group. For example, a study carried out by Alvarenga et al. (2001) concluded that cows with a high response of follicular stimulation to FSH resulting in 20 or 30 ovulations have relatively low embryo recovery. Elsden and Seidel (1995) suggested that the fimbria may be unable to gather a high proportion of ova into the oviduct. A further possibility is that the high progesterone level produced may alter spermatozoa and ova transport. Also, according to these authors.

Regarding pigs, the study of Wu et al. (2007) showed that FSH or combined LH and FSH significantly enhanced follicular growth compared to LH alone or the controls. Combined LH and FSH treatment of preantral follicles significantly increased the percentage (59±5%) of competent oocytes to undergo cleavage to the two-cell stage after fertilization. Besides that, when FSH was added to the culture medium, preantral follicles grew rapidly to the antral stage, half of their oocytes matured, and subsequently developed to the blastocyst stage after IVF. In contrast, without FSH, pre-antral follicles failed to grow to the antral stage, and none became mature oocytes. The results indicated that FSH is essential for the *in vitro* growth of porcine preantral follicles, estradiol secretion, and for oocytes to acquire competence to resume meiosis and undergo fertilization and embryonic development. Moreover, FSH may indirectly drive the theca to make the androgen substrate that is converted to estrogen by the granulosa cell aromatase. Also, it is important to highlight that it is biologically and financially more viable to produce embryos by adding FSH to the media than applying directedly in the gilts, as the results obtained are more reliable, such as shown by the study of Wu et al, (2007).

A recent study from Lounas et al. (2024) who worked with COCs from slaughtered pre pubertal gilts and assessed the mitochondrial function in porcine cumulus cells, showed that the supplementation of FSH in the IVM medium induces important metabolic changes in cumulus cells, including steroid synthesis, glucose uptake, and glycogen consumption. Besides that, FSH significantly decreased the basal mitochondrial respiration of COCs compared to the control. These authors discussed that FSH plays a crucial role in oocyte maturation. The same authors show that FSH regulates mitochondrial structure in cumulus cells during IVM. Specifically, exposure of COCs to FSH caused a transient elongation of mitochondria, followed by mitochondrial shortening and fragmentation. These changes in mitochondrial morphology were accompanied by a rapid decrease in basal mitochondrial respiration and total ATP levels and an increase in glycolysis. The results indicate that FSH regulates mitochondrial dynamics and activity in cumulus cells during IVM, leading to a sustained metabolic switch driven by the FSH induced high glucose consumption as well as elevated lactate production, showing that FSH stimulates serum-derived glycogen consumption in swine COCs during IVM.

Despite the small number of animals in the current study, we can say that the use of FSH requires more studies, since we can state that FSH, as well as in Alfradique et al. (2023), might impact the number of ovarian follicles. Although its positive impact on follicular growth regarding small follicles (<3mm), in the current study the FSH treated group showed a tendency to decrease the total number of follicles and the conversion rate from morula to blastocysts at IVEP. Putting together these findings, we can suggest that the quality of the embryos generated under FSH treatment in pre pubertal gilts might not be enough to generate viable blastocysts.

As future perspectives, we suggest that a larger sample size and more repetitions would be necessary to confirm our results, especially considering commercial herds, instead of using experimental animals. In addition, a deeper understanding of the metabolic effects of FSH when injected in alive gilts before ovary collection must be evaluated. On top of that, more research about the use of FSH + LH on IVEP media, in the case of pre pubertal gilts, might also generate more accurate information about oocyte maturation and blastocyst production.

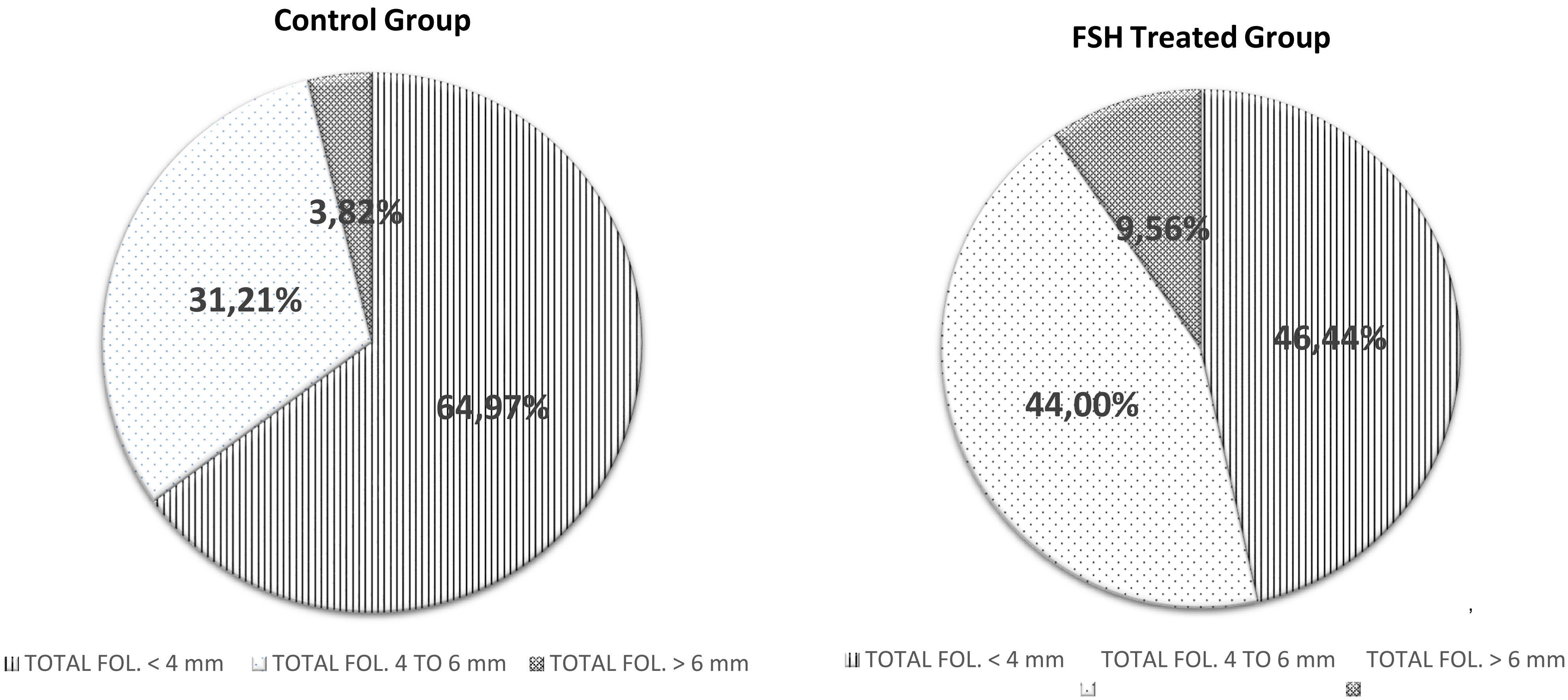

FIG. 5: Percentage of different antral follicles categories small (<4 mm), medium (4-6 mm) and large (>6 mm) follicles observed in ovaries obtained from prepubertal gilts at 140 days of age tested with FSH. (FSH treated and control groups).

Table 1: Uterine parameters evaluated from prepubertal gilts at 140 days of age in the control and FSH treated groups, where the differences between the organs of each animal were biometrically assessed.

	AVERAGE ± SD				
PARAMETERS	**CONTROL GROUP**	**TREATED GROUP**	**p-VALUE**	**CV (Control Group)**	**CV (Treated Group)**
Weight of the uterus (kg)	0.10 ± 0.07	0.09 ± 0.03	0.67	70.00%	33.33%
Length of the right horn (cm)	43.41 ± 7.12	44.72 ± 14.04	0.77	16.40%	31.39%
Length of the left horn (cm)	43.16 ± 8.52	44.18 ± 13.25	0.82	19.74%	29.99%
Width of right horn (cm)	1.37 ± 0.36	1.36 ± 0.30	0.99	26.27%	22.05%
Width of left horn (cm)	1.41 ± 0.36	1.43 ± 0.33	0.93	25.53%	23.07%
Thickness of the right horn (cm)	0.72 ± 0.13	0.77 ± 0.31	0.61	18.05%	40.25%
Thickness of the left horn (cm)	0.70 ± 0.15	0.80 ± 0.28	0.25	21.42%	35.00%

CV: Coefficient of Variation.

Table 2: Ovarian parameters evaluated from prepubertal gilts at 140 days of age in the control and FSH treated groups, where the differences between the organs of each animal were biometrically assessed.

	AVERAGE ± SD				
PARAMETERS	**CONTROL GROUP**	**TREATED GROUP**	**p-VALUE**	**CV (Control Group)**	**CV (Treated Group)**
Weight of the right ovary (g)	3.28 ± 0.79	3.38 ± 1.07	0.82	24.08%	31.65%
Weight of the left ovary (g)	3.48 ± 0.65	3.98 ± 0.62	0.22	18.67%	15.57%
Length of the right ovary (cm)	2.29 ± 0.43	2.28 ± 0.14	0.94	18.77%	6.14%
Length of the left ovary (cm)	2.37 ± 0.26	2.49 ± 0.44	0.02*****	10.97%	17.67%
Width of right ovary (cm)	1.66 ± 0.16	1.19 ± 0.32	0.49	9.63%	26.89%
Width of left ovary (cm)	1.75 ± 0.29	1.74 ± 0.50	0.69	16.57%	28.73%
Thickness of the right ovary (cm)	1.20 ± 0.21	1.67 ± 0.42	0.78	17.50%	25.14%
Thickness of the left ovary (cm)	1.31 ± 0.20	1.42 ± 0.41	0.003*****	15.26%	28.87%

CV: Coefficient of Variation and ***** = Statistically different ($p<0.05$).

Table 3: Follicular parameters evaluated from prepubertal gilts at 140 days of age in the control and FSH treated groups, in relation to the number of total, aspirable and non-aspirable follicles found in the individuals in each group.

	AVERAGE ± SD				
PARAMETERS*	**CONTROL GROUP**	**TREATED GROUP**	**p-VALUE**	**CV (Control Group)**	**CV (Treated Group)**
Total follicles	98.25 ± 26.29	81.81 ± 42.53	0.87	26.75%	51.98%
Small follicles (<4mm)	63.83 ± 25.85	38.00 ± 32.53	0.29	40.49%	85.60%
Medium follicles (4mm to 6mm)***	30.66 ± 26.92	36.00 ± 21.34	0.16	87.80%	59.27%
Large follicles (> 6mm)	3.75 ± 8.65	7.81 ± 10.51	0.39	230.66%	134.57%

*** Medium follicles were aspirated and BCB+ oocytes progressed to maturation and IVF.

CV: Coefficient of Variation.

Table 4: Parameters evaluated from prepubertal gilts at 140 days of age in relation to the number and type of cells obtained on the last day of *in vitro* culture (D7) in the control and FSH treated groups.

	AVERAGE ± SD				
PARAMETERS	**CONTROL GROUP**	**TREATED GROUP**	**p-VALUE**	**CV (Control Group)**	**CV (Treated Group)**
Total cells in d7 (ivc)	22.54 ± 5.64	22.75 ± 6.31	0.47	25.02%	27.73%
Total zygotes in d7 (ivc)	0.63 ± 1.02	0.50 ± 0.92	0.74	161.90%	184.00%
Total morulas in d7 (ivc)	10.81 ± 4.72	10.12 ± 4.58	0.39	43.66%	45.25%
Total blastocysts in d7 (ivc)	0.27 ±0.46	0.50 ± 0.75	0.12	170.37%	150.00%

CV: Coefficient of Variation.

Table 5: Parameters related to conversion rates found on days 3 and 7 of culture in the control and FSH treated groups from prepubertal gilts at 140 days of age.

PARAMETERS	**PERCENTAGE ± SD**				
	CONTROL GROUP	**TREATED GROUP**	**p-VALUE**	**CV (Control Group)**	**CV (Treated Group)**
Conversion from morula (d3) to blastocyst (d7)	40.44 ± 27.70	19.46 ± 16.40	0.39	68.49%	84.27%
Blastocyst conversion rate in relation to the number of morulas on d7	28.14 ± 13.10	16.05 ± 14.16	0.09**	46.55%	88.22%
Conversion from oocyte to morula (d7)	32.93 ± 13.48	35.15 ± 16.06	0.79	40.93%	45.68%
Fertilization rate from zygote, morula and blastocyst in relation to the number of cells (d7)	55.03 ± 16.91	48.06 ± 17.23	0.31	30.72%	35.85%

CV: Coefficient of Variation and ** = Tendency.

4. CONCLUSIONS

In conclusion, in this study, the effect of the administration of 100 mg of FSH equally divided into 6 injections before slaughter had no effect on increasing the percentage of follicles found in the treated group or the rates measured. The thickness and length of the left ovary had statistical difference. Besides that, a tendency was found considering conversions blastocyst conversion rate in relation to the number of morulas in the last day of culture, but it was towards an increase in the control group. Due to the contradictory data regarding quantity and quality of follicles and embryos, more research is needed to fully understand the role of FSH in pre pubertal oocyte maturation in the pig, either on living gilts or being added to IVEP media.

5. DECLARATION OF INTEREST

The authors have no conflict of interest to declare.

6. ACKNOWLEDGEMENTS

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