



**International Journal of Biology, Pharmacy  
and Allied Sciences (IJBPAS)**

*'A Bridge Between Laboratory and Reader'*

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**EFFECTS OF PMSG AND INSULIN IN OVARIAN ORGAN CULTURE AND STUDY  
ON DEVELOPMENT OF FOLLICLES AND EMBRYOS IN NMRI MICE**

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**ABSTRACT**

Follicle-stimulating hormone (FSH) plays an important role in the process of follicular development and maturation. Insulin is able to acts on many organs, including the ovary, and is routinely used as an important supplement for in vitro culture. The objective of this study was to determine the effect of different concentrations of insulin alone or in combination with PMSG on follicular development and embryos in ovarian organ culture and study morphology , diameter and number of follicles, oocyte and, granulosa cells. We also determined development of in vitro-matured (IVM) oocytes , fertilization and two-cells embryos .Ovaries from 5 weeks mouse were removed and cultured for 6 days in the absence or presence of insulin (0.2,1,5 and 10  $\mu\text{g/ml}$ ) alone or in combination with PMSG(10  $\text{ng/ml}$ ). The cumulus-oocyte complexes (COC) were obtained from antral follicles. Immature oocytes in the COCs were cultured in vitro to obtain metaphase II (MII) oocytes. Quality of the oocytes from IVM was tested by in vitro fertilization (IVF) and embryo development. The results showed that, after 6 days of culture, insulin at 0.2 and 1  $\mu\text{g/mL}$  plus PMSG was the best concentration to preserve and promote growth of follicles and oocytes and resulting the highest rates of MII oocytes and embryos. Diameter of follicles and granulosa cells in these groups was larger than other treatments. In addition , formation and size of preantral ,antral and Graafian follicles were significantly ( $P<0.01$ ) increased in these groups rather than to

other groups .However ,treatment with high concentration insulin 5 , 10  $\mu\text{g/ml}$  alone had fewer antral follicles in total compared other groups , but number of primordial and primary follicles was similar in all groups. In addition the rates of meiotic maturation ( $P<0.05$ ) and cleavage ( $P<0.05$ ) and embryo formation were increased in insulin at 0.2 and 1  $\mu\text{g/ml}$  plus PMSG groups.Thus this study shows, that addition of 0.2 and 1  $\mu\text{g/ml}$  of insulin plus PMSG(10 ng/ml) to the organ culture medium improved the survival , stimulated growth of follicles and activation. This suggests that the higher developmental competence is usually observed in low concentration of insulin (0.2 ,1  $\mu\text{g/ml}$  ) plus PMSG in ovarian culture .

**Key words: Insulin, PMSG, follicle development, Organ culture, granulosa cells, mice**

## INTRODUCTION

Many investigations have applied techniques for culturing ovarian tissue or isolated follicles in vitro. Whole ovaries from fetal or neonatal rodents have been incubated in organ culture systems.(1) Techniques for the culture of ovaries have been described since about 1930. Earlier in vitro techniques tended to use the culture of whole ovaries (2). Although this type of culture preserves normal tissue interactions, it is severely limited because long-term maintenance of organ explants is difficult. Although it is possible to maintain the cortical regions of the ovary to some extent, the inner medullary region is subject to anoxia,depletion of nutrients and accumulation of metabolites leading to necrosis. Because of these limitations, and due to the time required for full follicular development (especially in larger species), the culture of adult ovaries is probably has limited use and perhaps best confined to the study of a particular ovarian

event such as blood flow or ovulation. Ovarian fragments or tissue slices are sometimes used to overcome some of the problems associated with mass. In addition, a number of methods such as suspending or floating organ or tissue slices in culture have been developed, thus maximizing gas and nutrient diffusion and reducing necrosis. (2) Ovarian follicular development and growth are controlled by pituitary gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and by local factors, such as steroid hormones and growth factors(3). Pituitary gonadotropins are the most important survival factors for ovarian follicle cells by promotion of the expression of local survival factors in ovarian follicles. Specifically, gonadotropins promote cell proliferation and suppress ovarian cell apoptosis by activation of cAMP-dependent pathways and by increasing the production of paracrine and autocrine factors such as

estrogens, interleukin-1, nitric oxide, and insulin-like growth factor I (IGF-I). These factors promote cell survival and proliferation through activation of the nuclear estrogen receptor, the cGMP-dependent pathway, and protein tyrosine phosphorylation (4). It has been shown that FSH is a key regulator of ovarian function, in particular follicular growth and granulosa cell differentiation (3). Studies on rat, sheep and cattle showed that FSH alone or in synergy with other factors inhibited apoptosis and stimulated steroidogenesis of granulosa cells cultured in vitro (5). Since FSH has improved significantly the oocyte maturation and developmental competence, its receptor, FSHR, was assumed to be expressed in mural granulosa cells (MGC) and cumulus cell CCs starting at the secondary follicular stage in most mammals, including mice, pigs, sheep, cows and humans (6,7,8,9) FSH $\beta$ -deficient mice were unable to develop past the preantral stage (10). Thus these observations confirm that folliculogenesis is gonadotropin-dependent starting at the antral stage. The main functions triggered by FSH in the mammalian ovary are cell proliferation, apoptosis prevention, estradiol production, cell secretion, and regulation of several other genes (5). PMSG (pregnant mare's serum gonadotropin) and hCG

(human chorionic gonadotropin) are the two hormones that have been used to retrieve oocytes from laboratory mice usually with high yield (more than 20 per animal) (11,12,13,14). Insulin is a protein secreted by pancreatic  $\beta$ -cells, and it plays a central role in body metabolism and acts in the regulation of ovarian function. Many studies have identified the ovary as a target organ because its receptors are widely distributed in all of the ovarian compartments, including granulosa, theca, stroma and oocyte and due to its effects on follicle cells, from early developmental stages to oocyte maturation (15). Circulating insulin concentrations exhibit diurnal variation, but also change during the estrous cycle, with significantly increased concentrations during the preovulatory period (16). Insulin has been shown to facilitate FSH-dependent steroid production and/or luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor induction in cultured granulosa cells (GC) from the rat, pig, and human (17). oocytes synthesize glycosaminoglycans (GAGs) such as hyaluronic acid. GAGs are able to induce the acrosome reaction of bovine sperm. It is presumed that the addition of insulin to the maturation medium stimulates GAGs secretion from cumulus cells and improves

the fertilization rate as a result of the promotion of cumulus-induced sperm capacitation. It has been shown that insulin has broad effects on preimplantation embryos (18).

Thus, according to the stimulatory effect of FSH on folliculogenesis and positive effect of insulin on ovarian and since most medical centers are used of FSH to stimulate the follicles and increase oocytes and also considering that the influence of this hormones on development of ovarian follicles in humans in vivo do not accept due to legal and ethical barriers , we decided to investigate the effect of these hormones on follicular development in the ovary cultured in vitro .

Therefore, a simple and efficient method should be developed to culture contact mouse ovaries using a three-dimensional culture system with serum-free medium. Serum provides numerous known and unknown proteins that could interact with the other components added in the medium. Furthermore, serum should be avoided from in vitro follicular culture medium regarding further clinical application of the procedure (19). Furthermore, the actual concentrations of insulin and FSH inside normal mouse ovarian tissue are 0.379 ng/ml and 0.1 mIU/ml, respectively (20). In the present

experimental design, the concentrations of insulin and PMSG in the medium were 0.2–10 µg/ml and 0.1 mIU/ml, respectively. We conclude that PMSG plus insulin (0.2 ,1 µg/ml ) can contribute to maintain and progress folliculogenesis in mice . Moreover, in vitro addition of PMSG and insulin may facilitate follicle formation and their quality. We subsequently evaluated the development to 2-cell stages of the oocytes after in vitro fertilization (IVF) to determine the quality of the oocytes.

## **MATERIAL AND METHODS**

### **Culture of mouse ovaries**

Mouse ovaries of 4 weeks old were selected and cultured overnight at 37°C with 5% CO<sub>2</sub> in 500 µl tissue-specific medium on 16-well plates (21). The next day, 500 µl of fresh medium was added into each well, and half of the total medium (500 µl) was replaced with fresh medium every other day. Following day 4, the depleted medium was replaced by 500 µl of fresh medium every other day. The day when isolated ovaries were placed in culture was marked as day 0. The basic medium for fetal ovarian culture consists of Dulbecco's modified Eagle's medium (DMEM)/F12 plus a-minimal essential medium (a-MEM) (1:1) (Gibco-BRL, Carlsbad, CA, USA) with 3% (w/v) BSA, 1 mg/ml of Fetuin (Sigma, St. Louis,

MO, USA), 0.23 mmol/l pyruvic acid, 100 IU/ml of penicillin G, and 100 mg/ml of streptomycin sulphate (Gibco-BRL) (Shen et al., 2006a,b) in the absence or presence of insulin (0.2, 1, 5 and 10 µg/ml) alone or in combination with PMSG (10 ng/ml) (20). Fresh control and cultured tissues were processed for histology and IVF and analyzed in regard to development parameters.

### **Follicle growth and survival**

Follicles were considered to be degenerating if (i) the oocyte was no longer surrounded by granulosa cells (the oocyte is separated from and no longer inside the follicular wall), (ii) the oocyte became dark, (iii) the granulosa cells became dark, or (iv) the diameter of the follicle decreased. For each follicle, photographs were taken, and diameters measured using Image software. The mean of 5 measurements per follicle was then calculated and documented as the follicle diameter (22).

### **Isolation of COCs from Antral Follicles and IVM of the Follicular Oocytes**

After 5 days cultured ovaries were dissected and oocytes were extracted. COCs were isolated from antral follicles mechanically by carefully and gently puncturing the follicles using a pair of 30 gauge needles attached to disposable syringes under a

stereomicroscope. The COCs containing immature oocytes in the antral follicles from both the outer (ovarian surface) and inner (deep ovarian tissue) layer of ovarian cortex were collected. IVM of oocytes in the COCs were done by culturing them for 17, 18, 19 or 20 h in 500 ml of minimum essential medium-a containing Earle's salts (Invitrogen) and supplemented with 10 mg/ml streptomycin sulfate, 75 mg/ml penicillin G and 5% (v/v) heat inactivated FBS covered with 250 ml of mineral oil in a 4-well plate at 37°C in 5% CO<sub>2</sub> air [12]. After IVM, COCs were collected and incubated in M2 medium containing 200 IU/ml hyaluronidase at 37°C for up to 3 min to remove cumulus cells, and further washed twice in fresh M2 medium to obtain clean oocytes. Matured oocytes at the metaphase II stage were judged by the appearance of the first polar body (23).

### **In Vitro Fertilization (IVF) and Embryo Culture**

To obtain sperm for IVF of oocytes, male mice of 12 to 14-week old were euthanized by cervical dislocation and epididymides were collected by dissection. The sperm suspension (1×10<sup>6</sup> motile spermatozoa/ml) was capacitated for 1.5-2 hours in 400 µl of T6 media supplemented with 16 mg/ml BSA. In vitro matured (MII stage) oocytes from

each treatment group were placed in 0.9 ml T6 and 0.1 ml capacitated spermatozoa was added. After 4-6 hours incubation, the oocytes were washed through three droplets of T6 medium. The oocytes were then cultured in a droplet of T6 (100 µl) under mineral oil. They were assessed for cleavage to the 2-cell stage 24 after fertilization (24).

### **Microscopic Study**

After 6 days in culture, four ovaries from each treatment were fixed in 4% paraformaldehyde for 4 h and then embedded in paraffin. Serial sections (thickness, 5 µm) were cut and stained with hematoxylin-eosine. Each nucleus of oocyte from each follicles were chosen for follicle/oocyte counts, and the average was used as the follicle/oocyte number of one ovary. To assess the progression of follicle formation, we counted four different categories of follicles/oocytes (primordial, primary, preantral, antral, graaf) in ovarian sections. Follicles containing a single layer of squamous follicular cells were considered as primordial. A primary follicle contains an oocyte surrounded by a single layer of cuboidal follicular cells; the secondary follicle contains more than one layer of follicular cells around the oocyte. Follicles containing scattered spaces or a distinct antrum were considered as antral. All the

follicles were classified as either healthy or atretic, respectively, according to the absence or presence of signs of oocyte and/or granular degeneration such as pyknosis of the nucleus, infolding of cell membrane in oocyte, ingression of granulosa cells within the antral cavity, pulling away of granulosa cells from the basement membrane, infolding or thickening of basement membrane. For measuring the diameter of ovarian follicle in each developmental stage, 45 microscopic fields were randomly chosen in each mice. Then, using the ocular micrometer of a light microscope (Olympus EH, America Inc.), at a magnification of 40×, the diameter of each ovarian follicle, oocyte, granulosa cells, interna and externa theca cells were measured. To avoid recounting the same follicle, only individual follicles, containing an oocyte with a nucleus, were evaluated.

**Statistical Analysis:** The data were analyzed using SPSS, version 16 for Windows. The number and diameter of follicles and number of MII oocytes and embryos in the control and cultured groups were compared by one-way analysis of variance (ANOVA) and Tukey's post hoc test. The differences were considered to be significant when  $P < 0.05$ .

### **RESULTS**

In the first series of experiment ovaries were cultured for 5 days in Dulbecco's modified

Eagle's medium (DMEM)/F12 plus a-minimal essential medium (a-MEM) (1:1) with 3% (w/v) BSA, 1 mg/ml of Fetuin, 0.23 mmol/l pyruvic acid, 100 IU/ml of penicillin G, and 100 mg/ml of streptomycin sulphate in the absence or presence of insulin (0.2, 1, 5 and 10  $\mu\text{g/ml}$ ) alone or in combination with PMSG (10 ng/ml).

### The histological analysis of the follicles

The sections from ovarian treated by different concentration of insulin alone or combination plus PMSG and PMSG alone and fresh controls were analyzed for morphological assessment of the follicles. Follicular morphology was examined in primordial, primary, pre-antral, antral and graaf follicles. Follicles in fresh tissue showed intact morphology with minimal cell-to-cell space between the oocyte and surrounding granulosa cells as well as between neighboring granulosa cells (Fig. 1A, B, C). There was difference in morphology of preantral and antral and graafian follicles in insulin 0.2, 1  $\mu\text{g/ml}$  groups plus PMSG 10 ng/ml (Fig. 1D, E, F) with PMSG alone 10 ng/ml (Fig. 1G, H, I) and insulin groups (5, 10  $\mu\text{g/ml}$ ) plus PMSG 10 ng/ml (J, K, L) tissue compared with those in fresh tissue.

Morphology of preantral and antral and graaf follicles, however, was consistently better

preserved in insulin groups (0.2, 1  $\mu\text{g/ml}$ ) plus PMSG (10 ng/ml) tissue (Fig. 1D, E, F) compared with PMSG alone tissue (Fig. G, H, I). The majority of the follicles in these groups were preserved in a good morphology. A homogeneous and vacuole-free distribution of cytoplasm in oocytes was observed in follicles from PMSG (Fig. 1G, H) and insulin alone tissue (Fig. 2A-F). These observed mostly in insulin alone tissue and less in PMSG alone tissue. Induced damage in preantral and antral follicles included shrunken oocytes (Fig. 2B, E, F), vacuoles in oocytes (Fig. 2A, C) and granulosa cells (Fig. 2B, E, F), granulosa cells with enlarged space between the oocyte and surrounding granulosa cells as well as between neighboring granulosa cells, resulting in the apparent loss of cell density (Fig. 2B, C, D, E, F).

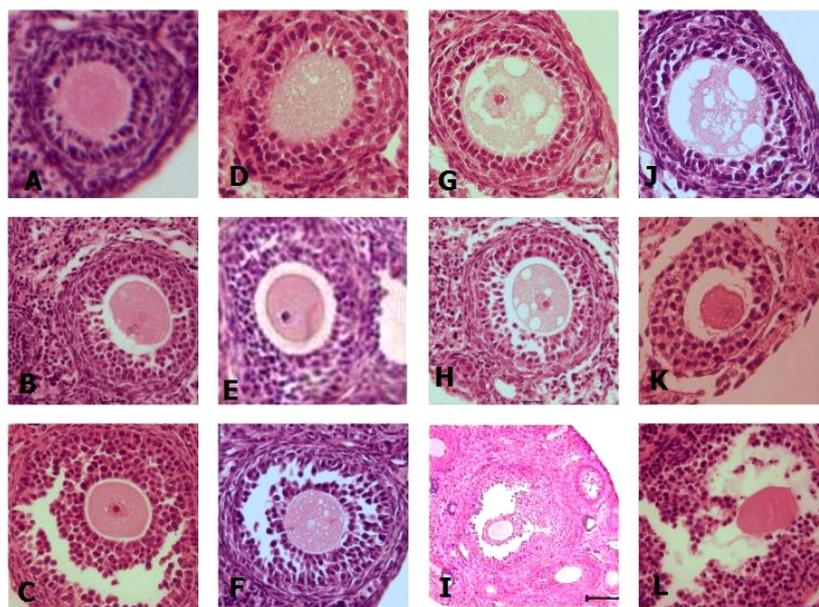
Follicles showing damaged oocytes (Fig. 2B, E, F), granulosa cells (Fig. 2B-F) or both (Fig. 2E) and quantified. Follicles with abnormal granulosa cells displayed vacuoles and dying cells with pyknotic nuclei resulting in abnormal space between neighboring granulosa cells. Total number of follicles in H&E stained sections in fresh and treatment tissues are presented in Table I. Damaged tissue were observed in insulin alone groups. Specifically, damage observed in tissue

seemed to be more prominent for absence of PMSG when compared with insulin plus PMSG.

### Follicular diameter, growth rate

The addition of insulin in combination with PMSG affect follicular diameter and growth rate, with significant differences between treatments (Table 1). The mean numbers of preantral, antral and graaf follicles were higher in the low concentration of insulin (0.2, 1  $\mu\text{g/ml}$ ) plus PMSG (10  $\text{ng/ml}$ ) groups than other groups ( $p < 0.05$ ). There

was no difference in the average number of primordial and primary follicles in the treatment groups; however, the average number of preantral follicles and antral follicles was decreased in high concentration insulin (5, 10  $\mu\text{g/ml}$ ) alone and combination with PMSG ( $p < 0.05$ ) (Table 1). There were significant ( $p < 0.05$ ) increases in the mean number of graaf follicles in insulin 0.2, 1  $\mu\text{g/ml}$  plus PMSG treatment groups in comparison with the controls.



**Figure 1:** The photomicrographs and histological appearance by HE staining of cultured mouse ovarian tissue was affected by insulin and PMSG. Compared with (A,B,C) control, (D,E,F) insulin 0.2  $\mu\text{g/ml}$ +PMSG(10  $\text{ng/ml}$ ), (G,H,I) PMSG(10  $\text{ng/ml}$ ) and (J,K,L) insulin 10  $\mu\text{g/ml}$  groups+ PMSG (10  $\text{ng/ml}$ ). In fresh mouse ovarian tissue, the morphologically normal preantral follicles (A) and the antral follicles (B) and graaf follicles (C) consisted of intact oocytes and compact granulosa cells. In the ovarian tissues cultured by insulin 0.2  $\mu\text{g/ml}$  plus PMSG(10  $\text{ng/ml}$ ), the primary follicles (D) and the antral follicles (E) and graaf follicles (F) were all morphologically normal. In the ovarian tissues cultured by PMSG alone, the pre-antral, antral and graaf follicles have normal morphology but with some vacuoles in the oocyte compare to the control group. (G,H,I) The ovarian tissues cultured by insulin in high concentration (10  $\mu\text{g/ml}$ ) plus PMSG, abnormal preantral follicles with the retracted cytoplasm in oocytes were observed and have abnormal antral and graaf follicles (J,K,L) (magnification  $\times 400$ , Bar = 10  $\mu\text{m}$ ).

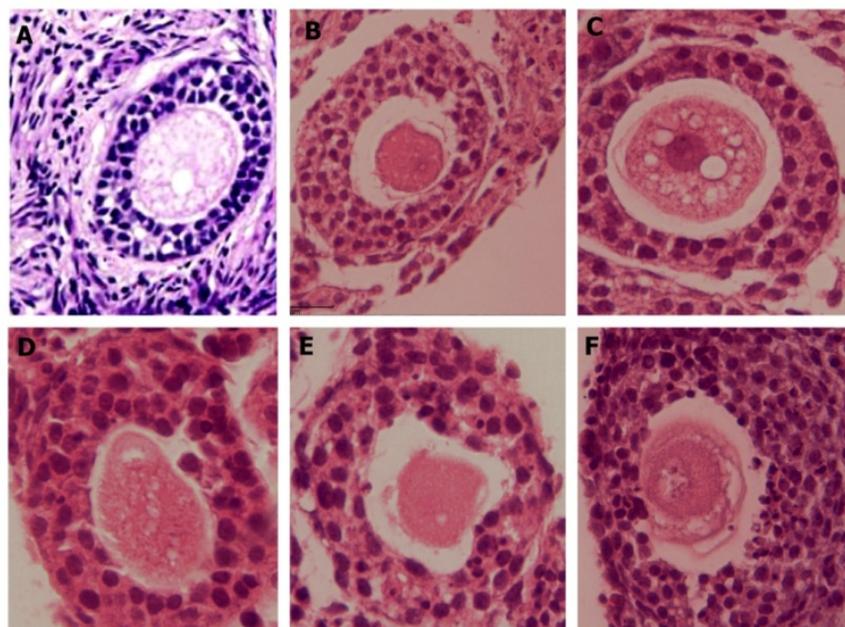


Figure 2 : Representative photomicrographs of preantral (A,D) and antral (B-F) follicles in insulin 0.2µg/ml groups (A,B) , insulin 1 µg/ml groups(C) , insulin 5 µg/ml groups(D), insulin 10 µg/ml groups (E,F) .Abnormal morphology observed in follicles included vacuoles in the oocyte (A, C) and granulosa cells (B,E), shrunken oocytes (B,E,F), as well as degenerating granulosa cells (B-F) and oocytes (A,B,F). (magnification ×400, Bar = 10 µm).

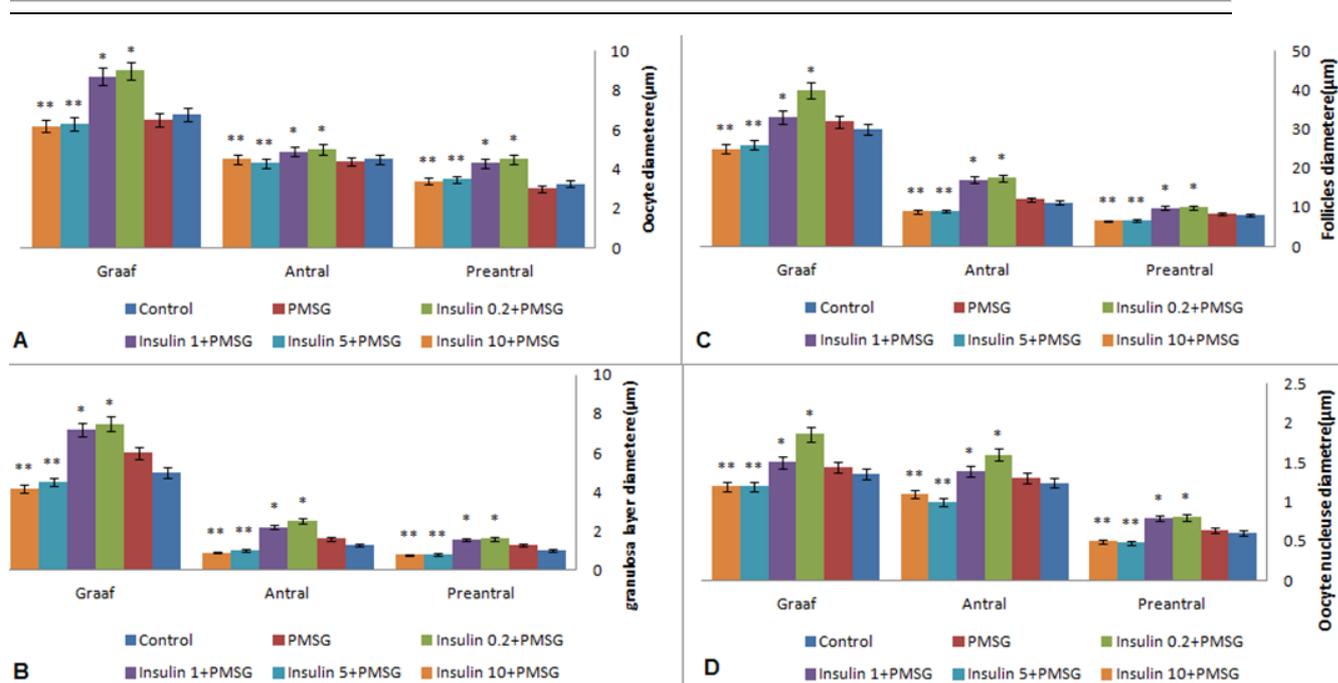
Table 1: The mean number of ovarian follicles in the control and treatment groups (Mean±SEM).

| Groups                 | Primordial F. | Primary F. | Preantral F.            | Antral F.              | Graaf F.               |
|------------------------|---------------|------------|-------------------------|------------------------|------------------------|
| Control                | 48.59±5.29    | 24±0.44    | 12.8±0.37               | 6.2±0.34               | 5.6±0.24               |
| PMSG                   | 45.7±4.27     | 18.4±0.41  | 13.8±0.37               | 7.6±0.34               | 6.6±0.4                |
| Insulin 0.2 µg/ml+PMSG | 44.57±4.12    | 20.8±0.37  | 15±0.32 <sup>*a</sup>   | 8.8±0.32 <sup>*a</sup> | 8.8±0.58 <sup>*a</sup> |
| Insulin 1 µg/ml+PMSG   | 40.4±3.98     | 22±0.31    | 15.8±0.35 <sup>*a</sup> | 8±0.37 <sup>*a</sup>   | 9±0.31 <sup>*a</sup>   |
| Insulin 5 µg/ml+PMSG   | 41.2±4.1      | 18.4±0.5   | 9.6±0.32 <sup>*b</sup>  | 4±0.32 <sup>*b</sup>   | 2.6±0.37 <sup>*b</sup> |
| Insulin 10 µg/ml+PMSG  | 40.5±3.67     | 19±0.44    | 9.4±0.32 <sup>*b</sup>  | 3.8±0.31 <sup>*b</sup> | 1.9±0.31 <sup>*b</sup> |
| Insulin 0.2 µg/ml      | 43.4±4.15     | 20±0.7     | 11.8±0.8                | 5.8±0.22               | 0                      |
| Insulin 1 µg/ml        | 41.6±4.32     | 20±0.71    | 7.2±0.76                | 5.6±0.25               | 0                      |
| Insulin 5 µg/ml        | 41.7±4.5      | 18.2±0.73  | 7.4±0.59 <sup>*b</sup>  | 2.9±0.23 <sup>*b</sup> | 0                      |
| Insulin 10 µg/ml       | 40.9±3.54     | 18.8±0.81  | 6.2±0.67 <sup>*b</sup>  | 3.2±0.25 <sup>*b</sup> | 0                      |

\*a The mean number of primordial, preantral and follicles increased significantly (P<0.01) in 0.2 and 1 µg/ml plus PMSG(10 ng/ml) groups comparison with other groups . \*b The mean number of preantral follicles and antral follicles was decreased in high concentration insulin (5 ,10 µg/ml) alone and combination with PMSG (p<0.05).

Statistical differences were also observed in the mean increase of diameter of preantral, antral and graaf oocyte(Fig.3 A), the granulosa layer (Fig.3B), follicles (Fig.3 C), and the oocyte nucleus (Fig. 3D) during the culture . Significant (p<0.01) increases were observed in the mean follicles diametere of

preantral and antral follicles in insulin 0.2, 1 µg/ml plus PMSG treatment groups in comparison with the other groups. In addition, on these groups showed a higher percentage of antrum formation compared to the control and other groups (Table 1).



**FIG 3.** The diameter(µm) of ovarian oocyte(A), the granulosa layer (B), follicles (C), and the oocyte nucleus (D) with normal morphology after in vitro culture in medium in the presence of different concentrations of insulin (0.2,1, 5, and 10 µg/ml ) plus PMSG (0.1 mIU/ml),PMSG alone and control. \* There were significant increase diameter between 0.2,1µg/ml insulin plus PMSG culture with other groups(P<0.01).\*\* There were significant decrease diameter between 5,10µg/ml insulin plus PMSG culture with other groups(P<0.01)

There were no significant differences between mean follicles diameter of preantral and antral follicles in the controls and PMSG group (Fig.3C).Antral follicles were not seen in insulin 5,10 experiment groups. There were no significant differences between the means of primordial and primary follicles in diameter in the control and treatment groups . The mean for preantral and antral follicles diameter decreased significantly (P<0.05) in high dose insulin groups(5,10 µg/ml) plus PMSG in comparison with the controls. Moreover, significant (P<0.05) decreases were seen between the mean of graaf follicle

diameter in the between 0.2,1µg/ml insulin plus PMSG culture and other group (Fig.3C).

**In vitro maturation of mouse oocytes**

Fig 4 shows the number of oocytes that attained the MII stage after 24 hours of culture. The maturation rate of oocytes in groups treated with 0.2 and 1 µg/ml insulin plus PMSG were significantly higher than the control and other groups (P<0.05). The degeneration rates in 5 and 10 µg/ml insulin groups were significantly higher than other groups (P<0.05).

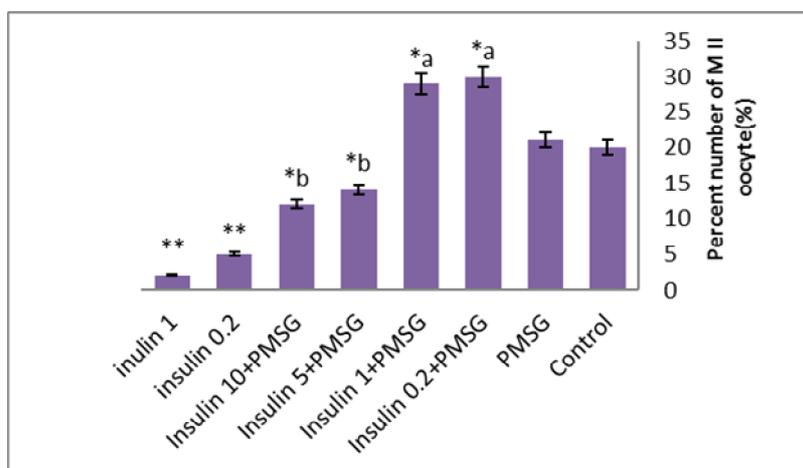


Fig. 4: Maturation rate of oocyte after in vitro culture in medium in the presence of different concentrations of insulin (0.2, 1, 5, and 10 µg/ml) plus PMSG (10 ng/ml PMSG), PMSG alone and control. \*a There were significant increase between 0.2, 1 µg/ml insulin plus PMSG culture with other groups ( $P < 0.05$ ). \*b There were significant decrease between 0.2, 1 µg/ml insulin plus PMSG culture with other groups ( $P < 0.05$ ). \*\* There were significant decrease between 0.2, 1 µg/ml insulin alone culture with other groups ( $P < 0.01$ )

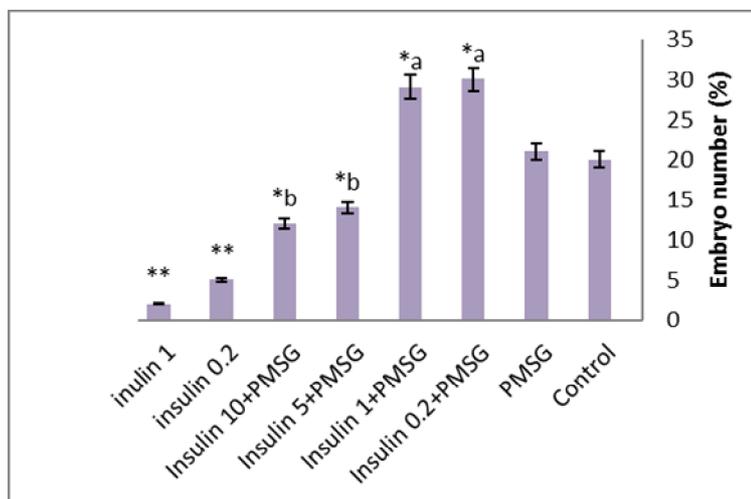
#### IVF and development of mouse oocytes

As shown in Fig 5, the rate of fertilization in oocytes treated with 0.2 and 1 µg/ml insulin plus PMSG were significantly higher than other group ( $p < 0.05$ ). However, the difference between the percent of fertilized oocytes which developed to the 2-cell stage (Fig. 5) was significant ( $p < 0.05$ ) between 0.2 and 1 µg/ml insulin plus PMSG groups and other groups. In contrast, increasing concentrations of insulin during oocyte development in vitro had surprising deleterious effects on oocyte developmental competence. While insulin treatment alone significantly reduced the percentage of oocytes competent to undergo fertilization and cleavage to the 2-cell stage. When ovaries were cultured in medium insulin (0.2, 1 µg/ml) with PMSG (10 ng/ml PMSG),

35% of the metaphase II (MII) oocytes developed to the 2-cell stage. This percentage was reduced to approximately 15% when the complexes were cultured with only 10 ng/ml PMSG (Fig. 4). Since the concentration of insulin in 0.2, 1 µg/ml acts synergistically with PMSG in promoting fertilization and cleavage to the 2-cell stage. The effect of insulin on the acquisition of oocyte developmental competence was assessed when ovaries were cultured in the absence of PMSG. In this case, addition of insulin alone to cultures of ovaries had significant ( $p < 0.01$ ), deleterious effects on the acquisition of competence to undergo fertilization and cleavage to the 2-cell stage (Fig. 5). However, PMSG in the absence of insulin did not have a deleterious effect on the acquisition of competence to undergo

fertilization and cleavage to the 2-cell stage . A fewer percentage of oocytes grown in medium without either insulin or PMSG

were competent to undergo fertilization and cleavage to the 2-cell stage.



**Fig. 5:** Percent number of embryo after in vitro culture in medium in the presence of different concentrations of insulin (0.2,1, 5, and 10 µg/ml ) plus PMSG10 ng/ml ,PMSG alone and control. \*a There were significant increase between 0.2,1µg/ml insulin plus PMSG culture with other groups (P<0.05).\*b There were significant decrease between 0.2,1µg/ml insulin plus PMSG culture with other groups (P<0.05).\*\* There were significant decrease between 0.2,1µg/ml insulin alone culture with other groups (P<0.01)

## DISCUSSION

The present study evaluated the effect of different concentrations of insulin alone or in association with PMSG on the ovarian organ culture and in vitro embryo development . In this study, the addition of low insulin concentration (0.2,1 µg/ml), in the presence of PMSG (10 ng/ml), increase follicular survival and maintaining morphologically normal follicles and cleavage rates of mouse oocytes compared with the control . Insulin is commonly used in cultured cells and tissues to increase cell viability due to its ability to remove pro-apoptotic molecules and phosphatidylinositol-3 kinase activation (19, 20). In addition, insulin regulates important

intracellular processes, such as amino acid transport, glucose and lipid metabolism, gene transcription and protein synthesis (21).

Insulin can maintain survival indirectly by stimulating the action of other substances such as FSH and insulin-like growth factor I (IGF-I), which are known to promote follicular viability in mice , bovine and goats (12,22,23). Our results were corroborated by studies that showed an increase in the percentage of follicular atresia, and the consequent reduction in survival, in media without insulin or at high doses of this hormone (24).

However, at high concentrations, insulin appears to have a toxic effect on follicle

cells. This hypothesis was corroborated by recent studies showing that basic culture medium containing 10 µg/ml insulin (ITS) have not been able to maintain ultrastructural integrity (25, 26).

Amsterdam et al. (1988) showed that a combined treatment with insulin and FSH markedly increased gap junction and microvilli formation and enhanced the development of the smooth endoplasmic reticulum and the Golgi complex relative to treatment with either hormone alone (17) . However, it has been reported that mammalian oocyte or follicle growth, in the presence of prolonged elevated insulin levels, has a negative impact on oocyte developmental competence (27). This fact confirms our results where the presence of PMSG with or without high insulin concentrations (5,10 µg/ml) was not satisfactory to promote meiotic resumption in oocytes from antral follicles. This suggests that high insulin levels in vitro may induce a delay in meiotic resumption.

Although the FSH played an important role in the follicular diameter increase in our study, it had no additional effect in antrum formation, which is confirmed by a previous study reporting that FSH is not required in vivo until antrum formation (24).

In relation to follicular growth, the present study found a higher follicular diameter after using insulin (0.2 and 1 µg/ml) in the presence of PMSG. It is known that insulin has specific effects on granulosa and theca cell function (28) and on several growth factors such as insulin-like growth factor I (IGF-I). Insulin stimulates granulosa and thecal cell proliferation and mitogenesis and synergizes with gonadotropins to stimulate granulosa and thecal cell steroidogenesis (29). Several studies in different species have shown that the association between insulin and FSH favors follicular growth (cattle: 30, murine: 31, primates: 32, buffalo: 33, goats: 34 and canine: 35). The obtained data from this study probably shows an interaction between insulin and FSH on the mice COCs.

Our results are in agreement with previous reports in which addition of FSH and insulin to the culture medium showed a positive effect on in vitro development of bovine , goat and mice oocytes (36). Makoto (1996) showed that FSH and IGF-I synergistically stimulate DNA synthesis in granulosa cells under certain conditions while each factor has no effect by itself. Coordination of the action of FSH and IGF-I is required for the promotion of DNA synthesis in granulosa cells obtained from DES-primed rats. The growth promoting effect of IGF-I is exerted

in a dose-dependent manner(37). Our results extend their study by showing that low concentration insulin plus PMSG improve follicular and oocyte quality and fertilization rate.

The size of the follicle seems to be an important factor in the selection of potential oocytes (reviewed by Sirard et al., 2006), involving RNA or protein stores as factors involved in oocyte competence. Increased developmental competence of oocytes has been associated with increased follicular diameter as reported in several studies in various species. The comparison of the oocyte diameter is often used as a marker for oocyte maturity or meiotic competence, able to attain their full developmental competence to blastocysts in vitro, as there is an intensive synthesis of RNA during this phase that causes an increase in size. According to Breviui et al., (2007), during the oocytes growth, messenger RNAs and proteins of maternal origin are accumulated into the oocyte throughout its growth in the ovary, upon fertilization, several mechanisms are activated that control the appropriate use of such material and prepare for the synthesis of new products supporting fertilization and initiating embryo development. The association between the oocyte diameter and its ability to resume and complete meiotic

maturation in vitro has been described in several farm animals. (38)

Our results agree with previous reports in which increase follicular diameter , increased developmental competence of oocytes. In ovary cultured with 0.2 and 1 µg/mL of insulin plus PMSG ,increase size of follicles and oocyte was observed compared to all other treatments . In conclusion, low concentrations of insulin (0.2 and 1 µg/ml ) with PMSG were more efficient in promoting oocyte meiosis resumption in mice follicles. This combination of hormones also stimulated follicular development and maintained follicular survival .The results of this study highlight how prolonged exposure to elevated levels of insulin during follicular growth may compromise oocyte maturation. Thus, we have revealed how a new basic medium, combining PMSG and low concentration of insulin, can influence the development of ovary organ culture. For the first time, this study shows how these hormones influence follicle growth, oocyte maturation and rate of fertilization.

## **REFERENCES**

- 1- PJ Devine, KS Rajapaksa, and Patricia B Hoyer .in vitro ovarian tissue and organ culture: a review. *Frontiers in Bioscience*. 1989; 7, d1979

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- 2-David K. Gardner, Michelle Lane, Andrew J. Watson. Follicles Development in Vitro. Oxford University Press. 2004; 2-21
- 3-Mourad A, François J, Richard I and Marc-André Sirard. FSH in vitro versus LH in vivo: similar genomic effects on the cumulus. *Journal of Ovarian Research*. 2013; 6:68
- 4-Alison Y. Ting, Richard R. Yeoman, Maralee S. Lawson, and Mary B. Zelinski. In vitro development of secondary follicles from cryopreserved rhesus macaque ovarian tissue after slow-rate freeze or vitrification. *Human Reproduction* .2011; Vol.26, No.9 pp. 2461–2472.
- 5-Yuansong Yu<sup>1</sup>, Wei Li, Zhengbin Han, Mingjiu Luo, Zhongle Chang, Jinghe Tan. The effect of follicle-stimulating hormone on follicular development, granulosa cell apoptosis and steroidogenesis and its mediation by insulin-like growth factor-I in the goat ovary. *Theriogenology* 60 . 2003; 1691–1704
- 6- Accardo C, Dattena M, Pilichi S, Mara L, Chessa B, Cappai P: Effect of recombinant human FSH and LH on in vitro maturation of sheep oocytes; embryo development and viability. *Anim Reprod Sci* .2004; 81:77–86.
- 7- Ye J, Campbell KH, Craigon J, Luck MR: Dynamic changes in meiotic progression and improvement of developmental competence of pig oocytes in vitro by follicle-stimulating hormone and cycloheximide. *Biol Reprod*. 2005; 72:399–406.
- 8- Merriman JA, Whittingham DG, Carroll J: The effect of follicle stimulating hormone and epidermal growth factor on the developmental capacity of in-vitro matured mouse oocytes. *Hum Reprod*. 1998, 13:690–695.
- 9- Anderiesz C, Ferraretti A, Magli C, Fiorentino A, Fortini D, Gianaroli L, Jones GM, Trounson AO: Effect of recombinant human gonadotrophins on human, bovine and murine oocyte meiosis, fertilization and embryonic development in vitro. *Hum Reprod* .2000; 15:1140–1148.
- 10- Kumar TR, Wang Y, Lu N, Matzuk MM: Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility.
11. Byers SL, Payson SJ, Taft RA .Performance of ten inbred mouse strains following assisted reproductive technologies (ARTs). *Theriogenology*.2006; 65: 1716–1726.
12. Martin-Coello J, Gonzalez R, Crespo C, Gomendio M, Roldan ER .Superovulation and in vitro oocyte maturation in three species of mice . *Theriogenology* .2008;70: 1004–1013.
-

13- Vergara GJ, Irwin MH, Moffatt RJ, Pinkert CA . In vitro fertilization in mice: Strain differences in response to superovulation protocols and effect of cumulus cell removal. *Theriogenology*.1997; 47: 1245–1252.

14-Choi JK1, He X. In vitro maturation of cumulus-oocyte complexes for efficient isolation of oocytes from outbred deer mice. *journal.pone*. 2013;8(2):e56158

15-Chaves RN1, Alves AM, Faustino LR, Oliveira KP, Campello CC, Lopes CA, Bão SN, Figueiredo JR.

How the concentration of insulin affects the development of preantral follicles in goats . *Cell Tissue Res*. 2011;346(3):451-6.

16-Shimizu T1, Murayama C, Sudo N, Kawashima C, Tetsuka M, Miyamoto A. Involvement of insulin and growth hormone (GH) during follicular development in the bovine ovary. 2008 Jun;106(1-2):143-52.

17-Amsterdam A1, May JV, Schomberg DW. Synergistic effect of insulin and follicle-stimulating hormone on biochemical and morphological differentiation of porcine granulosa cells in vitro. 1988;39(2):379-90.

18- Cheatham B, Kahn CR .Insulin action and the insulin signaling network. *Endocr Rev*.1995

16:117-142.

19- LeRoith D, Werner H, Beitner-Johnson D & Roberts Jr CT .Molecular and cellular aspects of the insulin-like growth factor I receptor.1995; *Endocrinology Review* 16 143-163.

20- Louhio H, Hovatta O, Sjoberg J & Tuuri T .The effects of insulin and insulin-like growth factors I and II on human ovarian follicles in long-term culture.2000; *Molecular Human Reproduction* 6 694-698.

21- Cheatham B & Chan CR .Insulin action and the insulin signaling network.1995; *Endocrinology Review* 16 117-142.

22-Martins FS, Saraiva MVA, Celestino JJH, Bruno JB, Almeida AP, Cunha RMS, Silva JRV, Campello CC, Lucci CM, Matos MHT, Figueiredo JR.Expression of protein and mRNA encoding Insulin Growth Factor-I (IGF-I) in goat ovarian follicles and the influence of IGF-I on in vitro development and survival of goat preantral follicles. *Anim Reprod* .2010;7:349-361

23-Matos MHT, Lima-Verde IB, Luque MCA, Maia JE, Jr Silva JRV, Celestino JJH, Martins FS,

Bão SN, Lucci CM, Figueiredo JR.Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability in vitro. *Zygote* .2007;15:173–182.

24- Wright CS, Hovatta O, Margara R, Trew G, Winston RM, Franks S, Hardy K . Effects

of follicle-stimulating hormone and serum substitution on the in-vitro growth of human ovarian

follicles. *Hum Reprod* .1999;14:1555-1562.

25- Chaves RN, Alves AMCV, Duarte ABG, Araújo VR, Celestino JJH, Matos MHT, Lopes CAP, Campello CC, Name KPO, Bão SN et al. Nerve Growth Factor Promotes the Survival of Goat Preantral Follicles Cultured in vitro.2010; *Cells Tissues Organs* 192 272-282.

26- Faustino LR, Rossetto R, Lima IM, Silva CM, Saraiva MV, Lima LF, Silva AW, Donato MA, Campello CC, Peixoto CA et al. Expression of Keratinocyte Growth Factor in Goat Ovaries and Its Effects on Preantral Follicles Within Cultured Ovarian Cortex. *Reproductive Science* 'in press'.2011.

27- Dumesic D, Schramm R, Peterson E, Paprocki A, Zhou R & Abbott D . Impaired developmental competence of oocytes in adult prenatally androgenized female rhesus monkeys undergoing gonadotropin stimulation for in vitro fertilization. *The Journal of Clinical Endocrinology & Metabolism* .2002; 87 1111-1119.

28- Yen HW, Jakimiuk AJ, Munir I & Magoffin DA . Selective alterations in insulin receptor substrates-1, -2 and -4 in theca but not granulosa cells from polycystic ovaries.

*Molecular Human Reproduction* .2004;10 473-479.

29- Silva JM & Price CA .Effect of FSH on steroid secretion and messenger ribonucleic acid encoding cytochromes P450 aromatase and cholesterol side chain cleavage in bovine granulosa cells in vitro. *Biology of Reproduction* .2000;62 186-191.

30- Machado R, Da Silva JCB, Barbosa RT, Bisinotto RS, Siqueira AFP & Binelli M .Remoção farmacológica ou mecânica do folículo dominante como estratégia anti-luteolítica em bovinos. *Acta Scientiae Veterinariae*.2006; 34 344-349.

31- Liu HC, He Z & Rosenwaks Z . In vitro culture and in vitro maturation of mouse preantral follicles with recombinant gonadotropins. *Fertility and Sterility* .2002;77 373-383.

32- Xu J, Lawson MS, Yeoman RR, Pau KY, Barrett SL, Zelinski MB & Stouffer RL .Secondary follicle growth and oocyte maturation during encapsulated three-dimensional culture in rhesus monkeys: effects of gonadotrophins, oxygen and fetuin.*Human Reproduction* .2011; 261061-1072.

33- Gupta PSP, Nandi S, Ravindranatha BM & Sarma PV .In vitro culture of buffalo (*Bubalus bubalis*) preantral follicles. *Theriogenology* .2002; 57 1839-1854.

34- Saraiva MVA, Celestino JJH, Araújo VR, Chaves RN, Almeida AP, Lima-Verde IB, Duarte ABG, Silva GM, Martins FS, Bruno JB et al. Expression of follicle-stimulating hormone receptor (FSHR) in goat ovarian follicles and the impact of sequential culture medium on in vitro development of caprine preantral follicles. *Zygote* .2011; 19 205-214.

35- Serafim MK, Araújo VR, Silva GM, Duarte AB, Almeida AP, Chaves RN, Campello CC, Lopes CA, Figueiredo JR & da Silva LD . Canine preantral follicles cultured with various concentrations of follicle-stimulating hormone (FSH). *Theriogenology* .2010;74 749-755.

36- Dieleman, S.J., P.J.M. Hendriksen, D. Viuff, P.D.Thomsen and P. Hyttel, et al. Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of preimplantation embryos. *Theriogenology*.2002; 57: 5-20. DOI: 10.1016/S0093-691X(01)00655-0,

37-Makotok A, Masa A. Growth or Differentiation: Determination by FSH of the Action of Insulin-Like Factor-I in Cultured Rat Granulosa Cells. *Endocrine Journal* .1996; 43(1), 15-23.