An *in vitro* comparative study of growth media, sera and FSH effects on the growth and maturation of Syrian mice preantral follicles and enclosed-oocytes

Javed, A.[1, 2]; Ghani, M. J.[3]; Soufian, S.[4]; Rezaei-Zarchi, S.[5*] and Kalantar, S. M.[1, 6]

[1]Animal Biotechnology Lab: Research and Clinical Center for Infertility, School of Animal Biology, Shahid Sadoughi University of Medical Sciences, Yazd, Iran; [2]Molecular Biochemistry Lab, Department of Biochemistry, School of Agriculture, Faisalabad University of Agriculture, Faisalabad, Pakistan; [3]Department of Bioinformatics, School of Bioinformatics, Government University of Faisalabad, Faisalabad, Pakistan; [4]Department of Biology, School of Biological Sciences, Payame Noor University, Arak, Iran; [5]Department of Biology, School of Biological Sciences, Payame Noor University, Yazd, Iran; [6]Yazd Medical Biotechnology and Genetic Engineering Incubator, Yazd, Iran

[*]**Correspondence:** S. Rezaei-Zarchi, Department of Biology, School of Biological Sciences, Payame Noor University, Yazd, Iran. E-mail: srezaei@ibb.ut.ac.ir

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Summary

Developing a culture system for preantral follicles has important biotechnological implications due to the potential to produce a large number of oocytes for embryo production and transfer. To accomplish this goal, the present study was aimed to culture preantral follicles in the presence of different media, sera and FSH concentrations. Six-week-old preantral follicles (95 ± 5 μm) were cultured in North Carolina State University medium 23 (NCSU23), tissue culture medium 199 (TCM199) and leibovitz-15 medium (L-15) for 6 days. Tissue culture medium 199 showed a significant increase in the follicle diameter (115 μm), survival (39%), oocyte maturation (32%) and germinal vesicle breakdown (GVBD) (29%) rates as compared to L-15 and NCSU23 ($P<0.05$). A 6-day culture showed increased follicular growth as compared to 2, 4 and 8-days ($P<0.05$). When the experiment was run with 1, 2, 5 and 10% fetal calf serum (FCS), prepubertal gilt serum (PGS), embryonic stem cell fetal calf serum (ESFCS) and hypogonadal mouse serum (hpgMS), the 5% FCS showed increased follicle diameter (134 μm), survival (52%), oocyte maturation (49%) and GVBD (45%) as compared to control and other types of sera used ($P<0.05$). While 100 mIU/ml FSH + 5% FCS in TCM199 showed a significant increase in follicle diameter (197 μm), survival (96%), oocyte maturation (91%) and GVBD (67%: $P<0.0001$). So, it is concluded that the TCM199 medium, with the addition of 100 mIU/ml FSH and 5% FCS, is appropriate for the optimal *in vitro* growth of Syrian mice preantral follicles and enclosed oocytes.

Key words: Serum, FSH, Germinal vesicle breakdown, Oocyte maturation, Follicle maturation

Introduction

In vitro culture of mice preantral oocyte can afford a model to study the regulation of hormone and growth factor during early follicle development. *In vitro* cultures include all the processes of follicle development, maturation and ovulation *in vitro*. Ancient studies are mostly based on morphology and chemical criterion (secretion of estradiol and progesterone), but the evaluation of the final development status is decided by a survivable offspring (Gao *et al*., 2007). The normal result of follicle development is to generate a meiotically competent and fertilizable oocyte. This process depends on physical incretory regulation and a series of complicated interactions between cells inside the follicle. Follicle *in vitro* culture simulates the transform *in vivo*, and the culture condition is changed in different stages to promote follicle development (Bao *et al*., 2002; Demeestere *et al*., 2005).

Choosing an artificial culture system will always imply a compromise in the *in*

vitro maturation (IVM) of oocytes. For culturing oocytes, the environment should be supportive of the growth of this large type of cell and should supply the essential nutrients. This support is feasible even with a non-spherical "open" structure. The accessibility of nutrients, hormones and gases is much better in an open structure and leads to an improved oocyte survival rate. Open culture systems can be refined by adapting the composition of the medium to preserve the normal differentiation status of the cells surrounding the oocyte (Smitz and Cortvrindt, 2002). The cross-talk between the oocyte and the cumulus cells is of major importance in determining oocyte developmental competence. Fine tuning of the culture components, including the types of cell, gonadotrophin concentration and ratio, growth factors, protein source (serum supplements) and concentration, can lead to optimization of the culture system. The culture media used in different studies were simple media supplemented with a large variety of additives, such as serum or protein supplements, growth factors, and more or less purified hormone preparations. It is important to recognize the balanced interrelationships between the biochemical and physical components of the system when we have to optimize the culture conditions. It has been demonstrated that there is close interaction between the oxygen requirements of a particular system, the hormonal and growth factor composition and the protein source (Smitz and Cortvrindt, 2002).

The role of FSH in the acquisition of developmental competence is primarily associated with its effect on follicular growth as several days of treatment are required to obtain oocytes of higher competence. Early stages of follicles, present in the ovary, are a potential source for the supplies of oocytes. However, the ability to bring primary or early preantral follicular stages with immature oocytes to maturity *in vitro* is a prerequisite. To accomplish this, isolation of ovarian follicles and insights into the requirements, metabolism and differentiation processes of *in vitro* maturation of ovarian follicles have already been studied by many researchers, using rodents, especially rats (Spears *et al*., 2002) and also using other mammals, e.g. cows, pigs and even humans. Most authors used a short culture period (4-6 days) for the follicles cultured either singly or in groups (Zhang *et al*., 2001).

The present study was carried out to better characterize the nature and impact of different culture media and sera on the growth and maintenance of Syrian mice follicles and the enclosed oocytes.

Materials and Methods

Chemicals and hormones

North Carolina State University medium (NCSU), Leibovitz-15 medium (L-15), tissue culture medium 199 (TCM199), fetal calf serum (FCS), prepubertal gilt serum (PGS), embryonic stem cell tested fetal calf serum (ESFCS) and hypogonadal mouse serum (hpgMS) (GIBCO BRL, Tokyo, Japan) were used to evaluate their effects on the growth and maturation of Syrian mice preantral follicles. Human recombinant (rh) FSH (HP Metrodin: Serono, Welwyn Garden city, UK) was prepared in un-supplemented culture medium and stored at -20°C until used to produce the final concentrations of 100 mIU/ml. All other chemicals were of analytical grade or the highest quality commercially available.

Animal model for follicle recruitment

Female Syrian mice were housed and bred in Central Animal House of Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Animals were kept under controlled conditions with 12 h light: 12 h dark photoperiod, and fed with water and food pellets ad libitum. Six-week-old mice were used for the isolation of cumulus enclosed oocytes (Mahmoudi *et al*., 2005). The animals were killed by cervical dislocation after 44-48 h of stimulation by an i.p. injection of 7.5 IU per mouse PMSG (pregnant mare's serum gonadotrophin).

In vitro maturation of preantral follicles and enclosed oocytes

For preantral follicles, the ovaries were removed aseptically and placed in Falcon plastic Petri dishes (Falcon 3037, Becton

Dickinson and Co., Rutherford, NJ) filled at room temperature with α-MEM (GIBCO BRL, Tokyo, Japan). After removing the surrounding tissue, the ovaries were micro-dissected using two 27-gauge needles attached to 1-ml syringes under the stereomicroscope and preantral follicles (95 ± 5 μm in diameter) with one or two layers of granulosa cells around the oocyte and an intact basal lamina with theca cells mechanically isolated. Thirty preantral follicles were transferred into a Falcon plastic Petri dish filled with 1 ml serum-free growth media: NCSU, L-15 and TCM199 (15 mM HEPES buffered) supplemented with 6.25 mg/ml insulin, 6.25 mg/ml transferrin, 6.25 ng/ml selenious acid, 5.35 mg/ml linoleic acid, 0.15% BSA, 45 mg/ml penicillin G, 350 mg/ml streptomycin and 1.75 mg/ml amphotericin (Alak *et al*., 1998; Bishonga *et al*., 2001). The follicles were then cultured in a humidified chamber with 5% CO_2 in the air at 37°C for 6 days. Each experiment was repeated 5-6 times (Bishonga *et al*., 2001). The medium was refreshed by changing half of the quantity every other day.

Experimental design

All studies were approved by the Institutional Laboratory Animal Care and Use Committee at the Shahid Sadoughi University of Medical Sciences, Yazd, Iran, and were in accordance with the National Institutes of Health guide for the care and use of laboratory animals. The present experiment was accomplished in the following steps: 1) to examine the effect of simple and complex culture media, three groups of preantral follicles (n = 30) were cultured in 5 ml of a simple medium (NCSU23) and complex media such as; TCM199 and L-15 (Leibovitz). 2) During the second experiment, the follicles were grown for 2, 4, 6 and 8 days under the above-stated conditions. The effect of incubation time was then evaluated on the follicle- and oocyte-growth and maturation. 3) The effect of FCS, PGS, ESFCS and hpgMS was also evaluated. Different concentrations of the sera used were 1, 2, 5 and 10% for 30 follicles in each group (Wu *et al*., 2001; Thomas *et al*., 2003). 4) Growth changes in preantral follicles were studied during a serum-free culture of 6 days in the presence of 10, 25, 50, 75, 100, 150 and 200 mIU/ml FSH and then 5) the experiment was repeated with the appropriate serum type and concentration with the addition of the most-effective dose of FSH. All of the experiments contained 30 follicles and each of them was repeated 5 times.

Histological measurements

Histological measurements and observations were made under the inverted microscope with a crossed micrometer (IMT-2, Olympus Corp., Tokyo, Japan). Maximum and minimum lengths of each follicle were observed microscopically and morphological changes in the nucleus or the extrusion of first polar body (MII) were used as the criterion for nuclear maturation of GV-stage oocytes. The mean diameter of the follicle was calculated by averaging these two measurements. Follicles with maintained basement membrane integrity were considered as survived and used for further analysis (Alak *et al*., 1996; Liu *et al*., 1999; Thomas *et al*., 2003).

Statistical analysis

The influence of different concentrations of sera and FSH on the extent of follicle and enclosed-oocyte maturation was determined by comparing percentages using one-way analysis of variance (ANOVA) (Wu *et al*., 2001), and $p<0.05$ was considered significant.

Results

Culture media had significant effects on the follicular survival rate (Fig. 1). The follicles grown with L-15 and NCSU23 showed low survival rates (25 and 15%), follicle diameters (109 and 103 μm), oocyte maturation (25 and 17%) and GVBD rates (20 and 14%) as compared to those grown in TCM199 with 39% survival, 115 μm diameter, 32% oocyte maturation and 29% GVBD ($P<0.05$). So, these results revealed that TCM199 medium was appropriate for the growth and maintenance of Syrian mice preantral follicles and enclosed-oocytes. So, TCM199 was used for the subsequent studies.

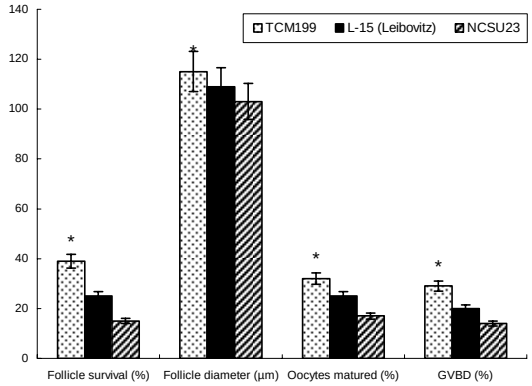


Fig. 1: Comparison of different culture media on the follicle and oocyte maturation parameters during 6 days culture. Preantral follicles with a mean diameter of 95 ± 5 µm were cultured for 6 days in TCM199, L-15 and NCSU23 media. Follicle and oocyte maturation rates were checked every day and degenerated follicles were removed from the medium. (n = 30; total number of follicles in each experiment). The esterics (*) are showing the most significant results conducted using one-way ANOVA (P<0.05)

Figure 2 shows the observations recorded for an 8-day culture of preantral follicles in TCM199 medium to evaluate the optimum culture period for follicle growth and maturation. Follicles cultured for 6 days had an increased survival rate (39%) and diameter (115 µm) than those grown for day 2 and 4, while the day-8 group showed a constant growth rate of the follicles, as was seen for the day-6 group. The comparison of the follicular diameter between culture days showed that the growth rate increased up to day-6 but on day-8, it was constant. Figure 2 also shows the increased oocyte maturation (32%) and GVBD (29%) rates (P<0.05) after a 6-day culture period as compared to days 2 and 4 (P<0.05). Regarding the primary objective for developing a preantral follicular culture system, for the *in vitro* studies of Syrian mice, to increase the percentage of surviving preantral follicles and to recover more COCs, the 6-day culture system was thought to be the most appropriate for subsequent experiments.

As shown in Figs. 3a-d, the effect of serum type was at maximum when the follicles were grown in the presence of 5% FCS (Fig. 3c) as compared to the same concentration of other types of sera (Figs. 3a, b and d). Fetal calf serum (5%) showed 52% follicle survival rate, 134 µm diameter, 49% oocyte maturation and 45% GVBD (Fig. 3c) as compared to the control and other types of sera used (P<0.05).

As shown in Fig. 4, preantral follicles harvested with 10, 25, 50, 75, 150 and 200 mIU/ml of FSH showed no significant changes in diameters and survival rates. On the contrary, as a result of stimulation by 100 mIU/ml of FSH, a significant increase in follicular diameter (190 µm), survival (91%), oocyte maturation (81%) and GVBD (59%) was recorded as compared to the control and the groups cultured in the presence of other FSH concentrations (P<0.0001).

As shown in Fig. 5, 5% FCS + 100 mIU/ml FSH-containing medium showed a significant increase in follicular growth, while FSH- and FCS-containing media showed a relatively decreased growth of the

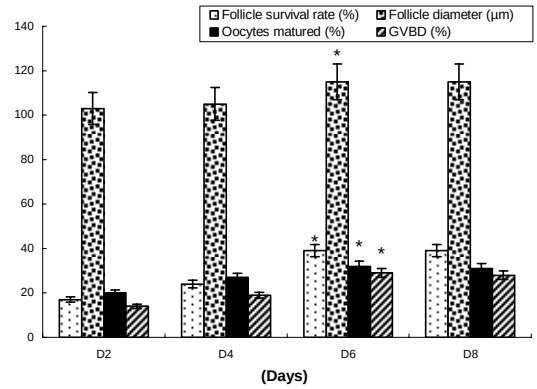
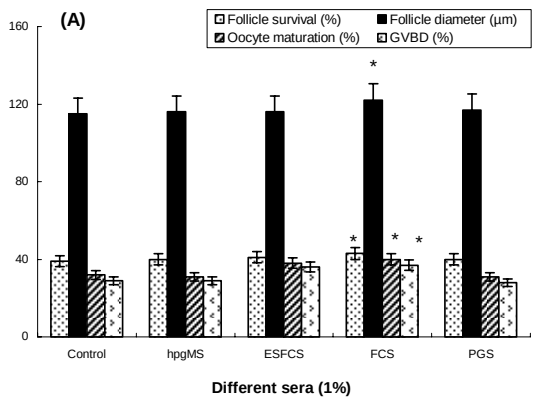


Fig. 2: Effect of TCM199 on follicle and oocyte maturation during different culture days. Follicular and oocyte maturation was evaluated after 2, 4, 6 and 8 days after incubation in TCM199 (n = 30; total number of follicles in each experiment). The esterics (*) are showing the most significant results conducted using one-way ANOVA (P<0.05)



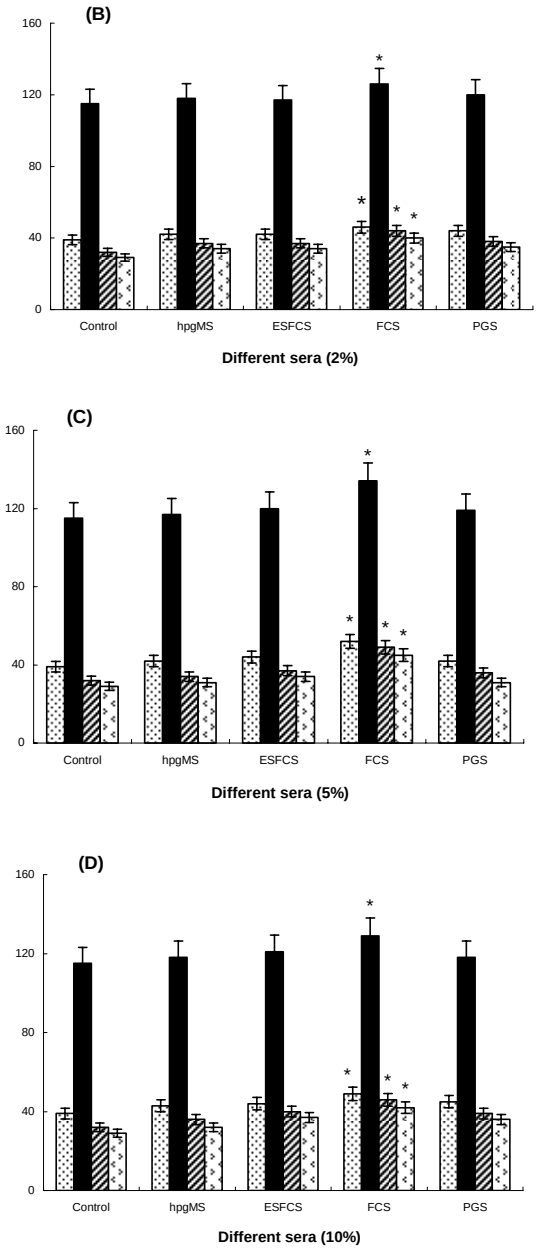


Fig. 3: Combined and comparative effects of different sera on the follicle and oocyte maturation. Preantral follicles with a mean diameter of 95 ± 5 µm were cultured for 6 days in TCM199 alone (control) and in the presence of A) 1%, B) 2%, C) 5% and D) 10% of FCS, PGS, ESFCS and hpgMS (n = 30; total number of follicles in each experiment). The esterics (*) are showing the most significant results conducted using one-way ANOVA (P<0.05)

follicles and enclosed oocytes. As shown in Fig. 5, 197 µm follicle diameter, 96% survival, 91% oocyte maturation and 67% GVBD rates were recorded in FCS + FSH-containing medium, as compared to the control group (P<0.0001).

Histological evaluation of the cultured follicles was done on serial semi-thin 1 mm plastic sections after Toluidine blue staining. Figure 6 demonstrates the following: (A)

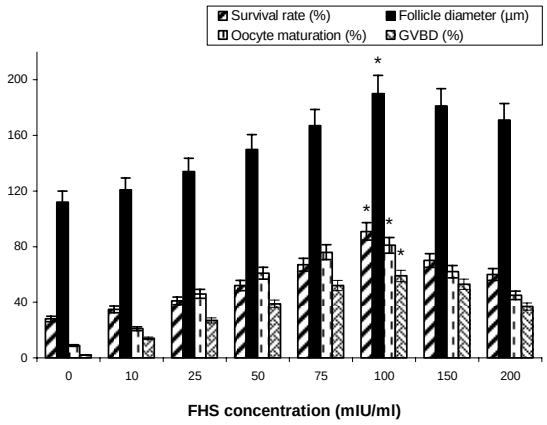


Fig. 4: Effect of different concentrations of FSH on the growth of follicles and enclosed oocytes. Preantral follicles were cultured for 6 days in TCM199 alone (control) and in the presence of 10, 25, 50, 75, 100, 125, 150 and 200 mIU/ml of FSH. Follicle and oocyte maturation rates were checked and degenerated follicles were removed from the medium (n = 30; total number of follicles in each experiment). The esterics (*) are showing the most significant results conducted using one-way ANOVA (P<0.05)

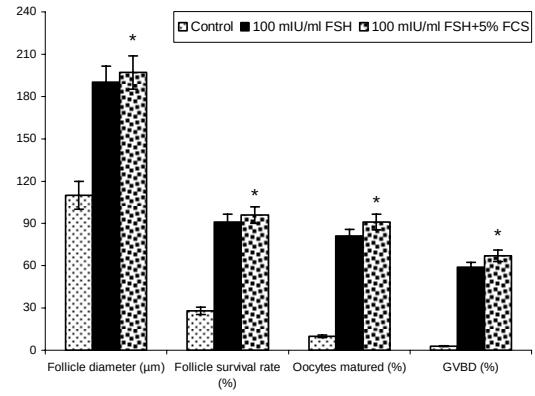


Fig. 5: Combined and comparative effect of FSH and 5% FCS on the follicles and enclosed oocytes. Preantral follicles were cultured for 6 days in 1) TCM199 alone (control) and in the presence of 2) 100 mIU/ml of FSH and 3) 100 mIU/ml FSH + 5% FCS. Follicle diameter was checked every day and degenerated follicles were removed from the medium (n = 30; total number of follicles in each experiment). The esterics (*) are showing the most significant results conducted using one-way ANOVA (P<0.05)

early preantral follicle in which the compact theca externa is defined around the follicle, (B) early preantral follicle cultured for 1 day. Central germinal vesicle (GV)-stage oocyte with a thin zona pellucida can be seen, surrounded by two layers of granulosa cells, a basal membrane and a single layer of thecal cells. Some cells have started to colonize the culture dish, attaching the follicle to the plate formation of a monolayer around the follicle and outgrowth of granulosa cells through the basal membrane, spreading over the monolayer (scale bar = 50 µm), (C) early preantral follicle cultured for 3 days. A well-developed monolayer around the follicular structure can be seen, while the oocyte is centrally located with the germinal vesicle preparing to enter the MII (meiosis II), (D) early preantral follicle cultured for 4 days and the outgrowth of the granulosa cell layers can be seen through the basal membrane, spreading over the monolayer, (E and F) the follicles cultured for 5 and 6 days and the development of a defined and compact granulosa cell-layer can be seen around the follicle, which is the onset of antrum formation around the oocyte covered by tightly packed corona cells. The mural granulosa cells are more loosely interconnected in Fig. 6 (F).

Discussion

A variety of complete media, including TCM199, have been used in attempts to develop culture systems for preantral follicles in a number of species. Alternatively, NCSU23, a simple medium, was used by Wu *et al*. (2001) in a porcine preantral follicular culture system and a small number of the oocytes recovered from

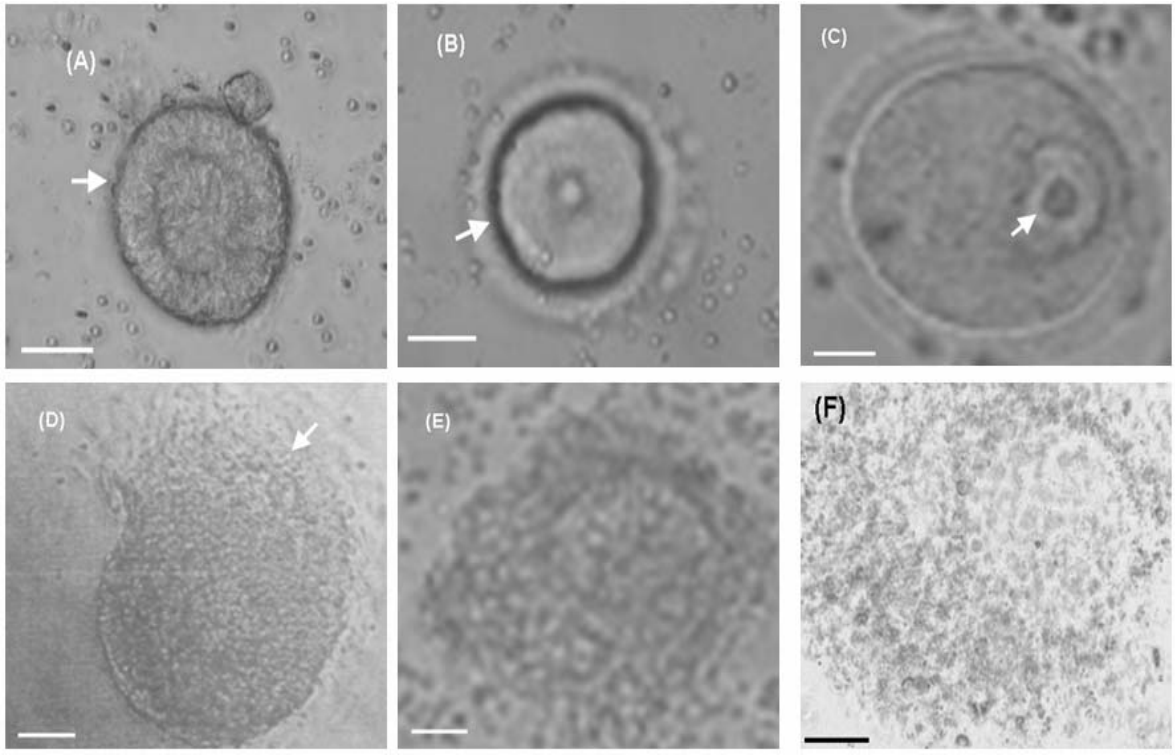


Fig. 6: *In vitro* growth and differentiation of preantral follicles from 6-week-old mice (A) Early preantral follicle with compact theca externa, as directed by arrow-head (scale bar = 50 µm). (B) Early preantral follicle cultured for 1 day: central germinal vesicle-stage oocyte with a thin zona pellucida, as directed by the arrow (scale bar = 50 µm). (C) Early preantral follicle cultured for 3 days: well-developed monolayer around the follicular structure (scale bar = 60 µm). (D) Early preantral follicle cultured for 4 days: outgrowth of the granulosa cell layers through the basal membrane (directed by the arrow-head), spreading over the monolayer (scale bar = 80 µm). (E and F) Follicles cultured for 5 and 6 days: development of a defined and compact granulosa cell-layer around the follicle, (F) more loosely interconnected mural granulosa cells (scale bar = 120 µm). For more complete details, refer to the text

these follicles were able to be fertilized after *in vitro* maturation. To the best of our knowledge, no report has been published comparing the effects of a simple medium (NCSU23) versus complete media (TCM199 and L-15) on the *in vitro* growth and development of preantral follicles in Syrian mice species. TCM199 and L-15 are complex media containing amino acids, vitamins, ribonucleosides and deoxy-ribonucleosides in addition to the usual inorganic salts and energy sources (glucose) of a simple medium such as NCSU23 (Mao *et al*., 2002). However, when follicles were cultured in NCSU23 or L-15 (Leibovitz) medium, a lower growth and survival rate of cultured follicles was seen.

It is supposed that the oocyte continues to grow and it is speculated that mouse preantral follicles require all the ingredients found in the complete medium for oocyte RNA and protein synthesis and for follicle growth and maturation. Culture systems for domestic animals and rodents are at an early stage of development and are at present being used to define the characteristics of preantral follicular growth and development (Saha *et al*., 2000).

Comparison of different sera on follicular and oocyte maturation showed that 5% FCS proved to be appropriate for the growth and maturation of the follicles and enclosed oocytes. So, compared with PGS, ESFCS and hpgMS, the FCS provided a superior serum supplement for preantral follicular development *in vitro* (Mao *et al*., 2002). When serum is a component of the culture medium, it improves follicular growth and survival in rodents, human and pig follicle cultures (Michele *et al*., 2005). Fetal calf serum is often used in the culture of bovine (Saha *et al*., 2000), mouse and porcine preantral follicles (Telfer *et al*., 2000).

Our results demonstrated that fetal calf serum provided the superior serum supplement for preantral follicular culture and a higher number of intact follicles could be recovered. From the experimental results mentioned above, it was revealed that the follicular growth rate increased linearly up to day-6, but after this, it became almost constant (day-8), as shown in Fig. 2. These observations imply that these culture conditions alone were not ideal to sustain a long-term follicular culture and follicles needed some growth enhancers, gonadotropins and some dietary factors.

The above experiment clearly indicates the essential role of FSH on early preantral stage follicles for further growth and differentiation. FSH also regulates the transzonal connection between the oocytes and the surrounding GCs (Wang *et al*., 2003). From a biochemical perspective, as follicles develop 2-3 layers of granulosa cells and the theca interna and externa cells begin to differentiate, FSH receptors appear on granulosa cells and follicles become gonadotropin dependent (Wang *et al*., 2003). Cortvrindt *et al*. (1997) showed that a minimal concentration of 10 mUI/ml of FSH is essential during *in vitro* culture of intact preantral follicles. In the absence of FSH, only 17% of the follicles survived (Cortvrindt *et al*., 1997). In this culture system, full differentiation of the preantral follicles *in vitro* can be achieved only in the presence of FSH, at least from the late preantral stage (Adriaens *et al*., 2004). Using the same culture model, Mitchell *et al*. (2002) also reported a follicular survival rate of only 10% in the absence of FSH during *in vitro* growth (Mitchell *et al*., 2002).

A dose-response curve for the effect of FSH during intact preantral follicular culture showed an increase in the follicular mean growth rate with increasing concentrations of FSH to a maximum of 100 mUI/ml (Nayudu and Osborn, 1992). When the follicles were cultured in the presence of 1000 mUI/ml FSH, the proportion of follicles ovulating was significantly lower than that in the presence of 100 mUI/ml FSH (Mitchell *et al*., 2002; Michele *et al*., 2005). Excessive exposure to FSH could result in FSH receptor down-regulation, leading to a suboptimal follicular response. However, in the same culture system but in the presence of serum, the follicular diameter increased after culture in medium supplemented with FSH was compared with culture without FSH or serum (Nayudu and Osborn, 1992). Our results demonstrate that at 100 mIU/ml FSH, the maximum follicles survived but with the increasing concentrations of FSH, the follicle survival rate decreased with a decreased percentage

of germinal vesicle breakdown and mature oocytes for ovulation. These results are in agreement with those reported by Nayudu and Osborn (1992). Our results also revealed that the oocyte maturation and germinal vesicle breakdown increased remarkably by the addition of FSH. These observations are also described by Cortvrindt *et al*. (1997).

So, the present experiments have demonstrated that a carefully selected culture system can yield the required *in vitro* growth of the follicles. This study was carried out with the hope of generating a natural and healthy environment by using mouse species, which proved to be an efficient model to assess the effects of the different *in vitro* components on follicular development and to improve our knowledge regarding *in vitro* folliculogenesis. However, further work is required to improve *in vitro* maturation methods and to increase the yield of viable follicles that can undergo *in vitro* fertilization and embryogenesis, which is the basic purpose of establishing and modifying these culture systems.

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