IJAS Iranian Journal of Applied Animal Science

Role of Follicle Stimulating Hormone in the Survival, Activation and Further Growth of *in vitro* Cultured Sheep Primordial Follicles

Research Article

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Received on: 1 May 2012
Revised on: 1 Sep 2012
Accepted on: 27 Sep 2012
Online Published on: Dec 2013

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Online version is available on: www.ijas.ir

ABSTRACT

The aim of the present study was to investigate the effect of follicle stimulating hormone (FSH) on survival, activation and growth of ovine primordial follicles using histological studies. Pieces of ovine ovarian cortex were obtained and cultured for 6 days in Minimum Essential Medium supplemented with or without FSH (50 ng/mL). These fragments were then process to be used for histology compared to each other and non-cultured group. The results showed that the lowest percentage of normal follicles in control medium was observed after 6 days of culture. A higher percentage of growing follicles was observed in presence vs. absence of FSH. Moreover, an increase in diameter of both oocyte and follicle was observed in those fragments that have been cultured in presence of FSH. This study demonstrated that FSH not only maintains the morphological integrity of ovine preantral follicles cultured for 6 days *in vitro*, but also stimulates the activation of primordial follicles and the growth of activated follicles.

KEY WORDS FSH, *in vitro* culture, ovine, primordial follicles.

INTRODUCTION

Culture systems for primordial follicles are important tools for studying the mechanism of oocyte development, and as a potential source of oocytes that can be used for *in vitro* embryo production. The factors that control primordial follicle activation and further growth of primary follicles are not well understood (Picton *et al*. 2008). Endocrine hormones, like as follicle stimulating hormone (FSH), are known to regulate the production of several growth factors that play a critical role in primordial follicle activation and growth.

FSH acts via binding to its receptor expressed on granulosa cells (O'Shaughnessy *et al*. 1996) and more recent reports indicate its presence in oocytes, suggesting additional sites of action in the ovary (Meduri *et al*. 2002). Although FSH receptors are expressed from the primary follicles onward (Oktay *et al*. 1997), FSH may play an indirect effect on very early follicle development via factors released by larger follicles or ovarian stromal cells. For example, FSH promotes proliferation of granulosa cells via paracrine factors such as IGF-1 and activin (van den Hurk and Zhao, 2005). In addition, FSH regulates expression of kit ligand (KL), growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) in murine follicles (Thomas *et al*. 2005). These factors have been implicated in the activation of primordial follicles (van den Hurk and Zhao, 2005).

It has been shown that addition of FSH to culture media promotes preantral follicular growth and antrum formation in many species (Zhou and Zhang, 2005; Matos *et al*. 2007 a; Matos *et al*. 2007b). Moreover, it is known that FSH inhibits apoptosis in preantral follicles cultured *in vitro* (McGee *et al*. 1997). In goats, Silva *et al*. (2004) demonstrated that FSH, at concentration of 100 ng/mL, increased follicle and oocyte diameters after 5 days culture, but no effect of FSH on both primordial to primary follicle transition and viability was observed. However, despite numerous studies on regulatory importance of FSH on follicle development *in vivo* and *in vitro*, the specific role of FSH on promoting primordial to primary follicle transition and growth in some species have not been tested. Therefore, the aim of the present study was to investigate whether FSH has a beneficial role in the survival, activation and further growth of *in vitro* cultured sheep primordial follicles enclosed in ovarian cortex.

MATERIALS AND METHODS

Unless otherwise specified, all chemicals and media were obtained from Sigma Chemical Company (St. Louis, MO, USA) and Gibco (BRL, Grand Island, NY, USA) companies, respectively.

Source of ovaries

Ovaries (n=10) from five native ewe lambs (10-12 months old) were collected at a local slaughterhouse of Isfahan, and transported to the laboratory on ice within 2 h. All of sheep, which used for current study, had a nice body condition and normal estrus.

Experimental protocol

At the laboratory, ovaries were washed three times and then soaked for five to 10 seconds in 70% alcohol under a flow hood and washed in phosphate buffer solution (PBS) supplemented with antibiotic (100 IU/mL penicillin and 100 µg/mL streptomycin). After removing the medulla, large antral follicles and corpora lutea , the cortexes of ovaries were isolated using scalpel blades and minced into 3 × 3 × 1 mm^3 fragments in phosphate buffer solution (PBS) supplemented with antibiotic (100 IU/mL penicillin and 100 µg/ml streptomycin), and 3 mg/ml bovine serum albumin (BSA) (Peng *et al*. 2010). Five fragments of each ovary were immediately fixed for classic histological studies (non-cultured control), whereas the other fragments were cultured in four-well culture plates (250 µL of culture media, 5 fragments per well and total of 10 fragments per treatment) for 6 days at 38 ˚C, 5% CO_2, 5% O_2, 90% N_2 and maximum humidity (Peng *et al*. 2010; O'Brien *et al*. 2003). The basic culture medium was Alfa-Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 5 µg/mL insulin, 5 µg/mL transferin, 5 ng/mL selenium (ITS), L-glutamine (2 mM), penicillin G (75 µg/mL), streptomycin (50 µg/mL) and amphotricine (50 µg/mL) (O'Brien *et al*. 2003; Peng *et al*. 2010). For treatment group, basic medium was supplemented with constant concentrations of recombinant human FSH (0.5 IU/mL, Gonal-F, Serono, Solna, Sweden) (50 ng/mL, Matos *et al*. 2007a; Matos *et al*. 2007b). Every 3 days, the culture medium was replaced with fresh medium.

Histological assessments

Histological assessment of ovarian tissues was performed as described by Peng *et al*. (2010). Before [un-cultured (control) group] and after 6 days of organ culture, five slices from each group were removed and fixed with 10% formaldehyde for 24 h, dehydrated in ethanol, clarified with xylene and embedded in paraffin wax. Next, 5 µm sections were prepared using a microtome (Leitz, Wetzlar, Germany), with 60 µm intervals to avoid counting a section twice. The sections were stained with haematoxylin and eosin and then were analyzed by light microscopy at every tenth section using 400 × magnifications (Olympus BX51, Japan).

As described by Durlinger *et al*. (2002) and Silva *et al*. (2004), follicles were categorized to 1) primordial (one layer of squamous granulosa cells around the oocyte) or growing follicles initially. Growing (or developing) follicles were further classified to 2) primary follicles (a single layer of cuboidal granulosa cells around the oocyte), 3) secondary follicles (two or multi layers of cuboidal granulosa cells around the oocyte without antrum formation), and 4) antral follicles (oocyte surrounded by multi-layer of granulosa cells and a formed antrum). In this study the intermediate follicles between primordial and primary follicle were considered as primordial follicle, whereas follicles from groups 2, 3 and 4 were collectively called developing follicle (Oktay *et al*. 1998). Therefore, the percentages of various types of intact follicles were evaluated at day 0 (non-cultured control) and after 6 days of culture in the different media tested.

The follicular activation and growth was determined based on the criteria of normal follicles including spherical shape, spherical oocytes surrounded by regular granulosa cells with a circular distribution, and normal theca. Degenerated follicles were defined as those with a retracted oocyte, which have a pyknotic nucleus, and/or are surrounded by disorganized granulosa cells, which are detached from the basement membrane (Yu-bin *et al*. 2007; Matos *et al*. 2007a; Matos *et al*. 2007b). For each group, 100 follicles were randomly recorded in each replicate. Oocyte and follicular diameters were also evaluated in two directions, and

then averaged using an inverted microscope (Olympus BX51, Japan) adapted on a computer equipped with Image J 1 software (National Institute of Mental Health, Bethesda, MD, USA, 1.34S program).

Statistical analysis

The different types of normal follicle percentages (for evaluating of surviving and growing follicles) and the diameter of follicles and oocytes were analyzed by one-way ANOVA, followed using post-hoc testing (Tukey's test). Analysis of variance was done, when normality and homogeneity of data variance were proved. Values were observed significant at P-Values of ≤0.05. SPSS 18 (2011) for Windows (SPSS Science, Chicago, IL, USA) was used to compute.

RESULTS AND DISCUSSION

Effect of FSH on follicle survival

Figure 1 shows the effect of constant concentration of FSH (50 ng/mL) on follicle survival, i.e. the percentage of histological normal follicles in ovarian tissues after 6 days of culture. Compared to non-cultured follicles, a significant decrease in the percentages of histologically normal follicles (Figure 1 and Figure 2) was observed after 6 days culture (P<0.05). The decrease of normal follicles percentage in control medium was significantly greater than treatment group (Figure 1).

Effect of FSH on follicle activation and growth

The percentages of primordial, primary, secondary and developing follicles in ovarian cortical tissue before and after 6 days of culture are shown in Figure 3. As shown, the percentages of primordial and developing follicles in non-cultured cortex were 80.23% and 19.20%, respectively. After 6 days of culture, a reduction in primordial follicles count was observed which was concomitant with a significant increase of developing follicles in the treatment vs. non-cultured group (P<0.05).

A strong reduction of primordial follicles was observed in tissues cultured in MEM plus 50 ng/mL of FSH. Furthermore, cortical tissues cultured with 50 ng/mL of FSH had a significant increase in the percentage of developing follicles after 6 days of culture when compared to control cultured group.

Significant increases in oocyte and follicle diameters were observed in primary follicles, when compared to non-cultured tissue (Table 1; P<0.05). In addition, the presence of FSH promoted a significant increase in follicular diameter of primary follicles after 6 days of culture when compared to control. Therefore, a significant increase in follicle diameter was observed with the increase of culture period from day 0 to 6 only in tissues cultured with 50 ng/mL of FSH. At day 6, tissue cultured in presence of 50 ng/mL of FSH had the highest follicular diameter (P<0.05).

The present study demonstrated the importance of FSH on *in vitro* activation and growth of ovine primordial follicles in a 6 days culture system.

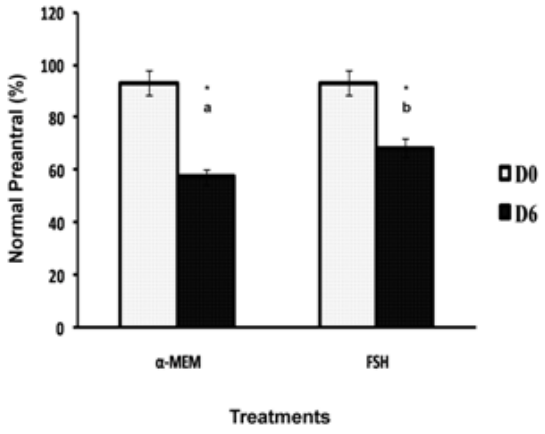

Figure 1 Percentages of histological normal preantral follicles in non-cultured tissue (control) and in tissue cultured for 6 days in α-MEM and α-MEM supplemented with 50 ng/mL FSH. (50 follicles evaluated in each one of three replicates per treatment). Asterisks (*) represent significant comparisons between non-cultured tissue and cultured groups for 6 days (P<0.05) Lowercase letters (a, b) were used for significant comparisons between groups on the same day (P<0.05)

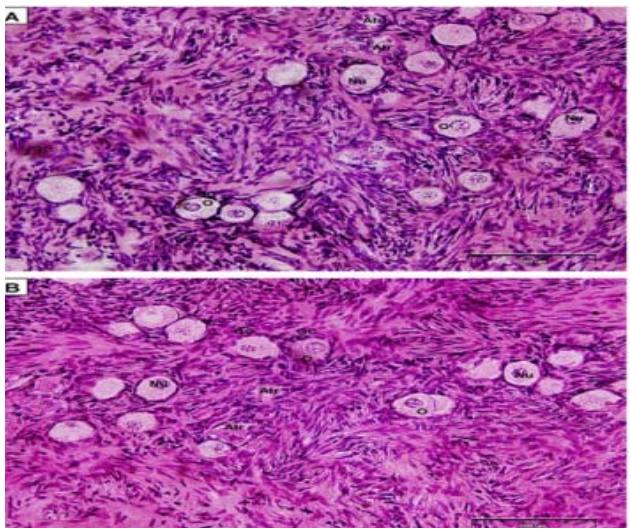

Figure 2 Histological section of A) non-cultured group and B) tested cultured tissue with FSH (50 ng/mL) for 6 days after staining with hematoxylin and eosin, showing normal primordial and primary follicles.
O: oocyte; NU: oocyte nucleus; GC: granulosa cells (400 x)

Although very little is known about the regulation of primordial follicle development, FSH seems to be a very effective factor in maintaining follicle viability (Saha *et al*. 2000). It was recently demonstrated that addition of FSH to the culture medium of ovine (Andrade *et al*. 2005) and cap-

rine (Matos *et al*. 2007a; Matos *et al*. 2007b; Magalhães *et al*. 2009) preantral follicles is important to the maintenance of viability and the activation of follicles, as well as for further follicular growth *in vitro* (Silva *et al*. 2004).

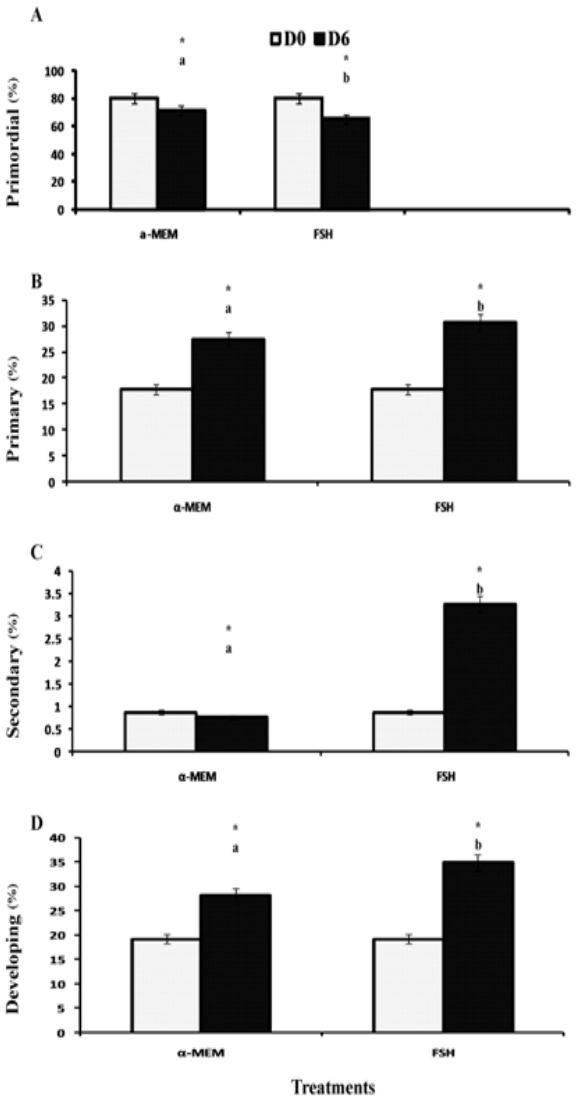

Figure 3 The A) primordial; B) primary; C) secondary and D) growing follicle percentages at 0, 6 days after organ culture. Asterisks (*) represent significant comparisons between different days of culture in the same group (P<0.05). Lowercase letters (a, b) were used for significant comparisons between groups on the same day (P<0.05)

However, Silva *et al*. (2004) did not observe a significant effect of FSH on follicle survival after 5 days culture, probably because a high concentration of FSH (100 ng/mL) was used.

In our study, however, addition of 50 ng/mL of FSH to culture medium was very important to maintain the percentage of normal follicles after 6 days of culture. Hsueh *et al*. (1994) and Chun *et al*. (1994) have suggested that the diffusion of several essential chemical and physical factors through the basal membrane could be compromised in the absence of FSH.

In addition, culture without FSH more frequently resulted in extrusion of the oocyte from its original follicular structure (Cortvrindt *et al*. 1997), which may be caused by either damage or reduction in the number of gap junctions (Hsueh *et al*. 1994). In addition, FSH inhibited apoptosis in preantral follicles cultured *in vitro* (McGee *et al*. 1997; Mao *et al*. 2002).

In addition to, Lima-Verde *et al*. (2010) showed that androstenedione at 50 or 100 ng/mL, either associated with FSH or at 50 ng/mL alone, plays an important role in the maintenance of caprine preantral follicle viability and activation after only a short *in vitro* culture period. Some studies demonstrated that.

The sequential addition of FSH to the culture medium maintained the survival of isolated canine preantral follicles and promoted an increased rate of follicular growth and antrum formation.

Andrade and *et al*. (2011) reported that the presence of IAA, EGF, and FSH helped to maintain ultrastructural integrity of sheep primordial follicles cultured *in vitro*. Addition of 50 ng/mL of FSH to the medium increased the activation rate of primordial follicles as early as day 0 of culture when compared to other treatments. However, after 6 days of culture, follicular activation was similar among all the treatments.

Ovarian follicular development is known to proceed to primordial and primary stages independently of the action of FSH. This has been observed in mice carrying invalidations of the FSHβ and FSHR genes (Dierich *et al*. 1998) and also in patients with mutations suppressing the function of the FSHR (Touraine *et al*. 1999).

Table 1 Mean oocyte and follicular diameters (Mean±S.E.M) of primordial, primary and secondary follicles in ovine cortical cultures at 0 and 6 days after organ culture. The first group was included α-MEM (G1). The secondary group was supplemented by 50 ng/mL FSH (G2). The small letters (a, b) were used for significant comparisons between groups on the same day (P<0.05). The star marks (*) were used for significant comparisons from the non-cultured group (P<0.05)

Day of culture and culture groups	Oocyte and follicular diameter					
	Primordial (n=60)		Primary (n=60)		Secondary (n=30)	
	Follicles	oocyte	Follicles	oocyte	Follicles	oocyte
0 day un-cultured group (G0)	40.73±1.31	39.10±1.90	50.04±2.11	40.72±1.82	80.14±3.70	57.89±2.94
6 days after culture						
Control group (G1)	41.82±1.12^{a}	38.32±1.53^{a}	53.22±1.95^{a*}	44.51±1.19^{a*}	79.27±2.82^{a}	58.90±3.14^{a}
FSH group (G2)	42.95±1.5^{a}	38.57±1.47^{a}	56.91±1.27^{b*}	46.72±1.71^{a*}	80.71±2.66^{a}	60.36±3.21^{a}

FSH binds to its receptor expressed on the granulosa cells (O'Shaughnessy *et al*. 1996) and oocytes (Meduri *et al*. 2002) from the primary follicles onward (Oktay *et al*. 1997). Matos *et al*. (2007a) demonstrated that FSH at 50 ng/mL concentration not only maintains the morphological integrity of 7 days cultured caprine preantral follicles, but also stimulates the activation of primordial follicles and the growth of activated follicles, which is in agreement with our results in sheep. In the current study, follicular diameter increased in presence of FSH. Itoh *et al*. (2002) also demonstrated that 50 ng/mL FSH increased both oocyte and follicular diameters in 13 days cultured bovine follicles. Silva *et al*. (2004) showed that both FSH and EGF stimulated an increase in follicle size by promoting oocyte growth in caprine preantral follicles. Matos *et al*. (2007a) demonstrated that FSH at concentration of 50 ng/mL increased oocyte and follicular diameters after 7 days culture in goat. FSH receptor expression has been reported to develop progressively during the transition from primordial to primary to secondary follicle (Oktay *et al*. 1997). The presence of FSH receptors in these early follicles presumably explains the effect of FSH on oocyte growth in preantral follicles. Since there are FSH receptors in oocytes, it is possible that FSH must act in both cell types to promote follicular growth and development (Méduri *et al*. 2002). Adriaens *et al*. (2004) reported that omission of FSH during the early preantral stage tends to compromise a maximal oocyte developmental competence.

CONCLUSION

This study showed that FSH promotes activation of primordial follicles and growth of activated preantral follicles. Furthermore, these data support the vital role of FSH on maintaining healthy oocyte growth and follicular normal structure after 6 days culture. This culture system should be useful for studying the regulation of early follicular growth and development, especially because these follicles represent a large source of oocytes that could be used *in vitro* for embryo production.

ACKNOWLEDGEMENT

This study was supported by a grant from Royan Institute of I.R.I. (fund item no. S/90/19/55501). The authors would like to thank Dr. H. Gurabi, A.H. Shahverid and Dr. A. Vosough for their full support.

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