EFFECT OF DIFFERENT DOSES OF FSH ON STEROIDOGENIC CAPABILITY, PROLIFERATION AND DIFFERENTIATION OF SHEEP GRANULOSA CELLS*

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Abstract

Sheep granulosa cells were cultured with three doses of FSH (0, 1ng and 10ng/ml) and for three different time periods of 48h, 96h and 144h to determine the effects of FSH on steroid production,cell proliferation, and gonadotrophins receptor expression. Cell number did not differ between any of the three time points except for cells without FSH in the media, for which there was a significant increase in cell number at 96h. Oestradiol production was found to decrease and progesterone production was found to increase with time, still not exhibiting any definite relation to the dose of FSH. There was a significant increase (P<0.001) in expression of FSHr with all doses of FSH with time. At 144hr there was a significant difference of FSHr expression in cells cultured with and without FSH. Without FSH in the media the receptor expression tends to decrease.No change in expression of LHr was noticed with time and different doses of FSH.

Keywords: *Granulosa cells, FSH, oestradiol, progesterone, FSHr, LHr*

Ovary is a complex endocrine gland responsible for production of sex steroids and is the source of fertilizable ova for reproduction. The ovary possesses two primary steroidogenic cell types. The theca cells are responsible for androgen synthesis, granulosa cells produce oestradiol by aromatisation of androgens secreted by the theca cells (Fortune, 1994). Granulosa cells constitute the vast majority of follicular cells in the mammalian ovary and they nurse the oocyte via gap junction communication and they are the main source of the female sex hormones oestradiol and progesterone, which control the oestrus cycle and reproduction (Amsterdam *et al*., 1989 and Robker *et al*., 2000). The development of granulosa cells is uniquely controlled by follicle-stimulating hormone (FSH) during folliculogenesis, causing them to proliferate and subsequently differentiate (Sasson *et al*., 2003). Many aspects of the process of granulosa cell proliferation and differentiation remain to be understood. This requires a dramatic plasticity in gene function, modulated predominantly by a single glycoprotein hormone, FSH, via activation of a G-coupled 7 transmembrane domain receptor located uniquely on the membrane of granulosa cells (Amsterdam *et al*., 2002). Therefore, it is important to analyze the effect of FSH on granulosa cell functions in a comprehensive manner. In this study we explore the effect of different doses of FSH on maintenance of granulosa cell function and differentiation, mainly in terms of proliferation, oestradiol production, progesterone secretion, *FSHr* expression, and *LHr* expression which is critical to the development of *in vitro* granulosa cell culture systems.

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Materials and Methods

Ovaries were collected from a local abattoir and, on arrival at the laboratory; they were washed with warm PBS (37^0C). Follicles were aspirated and the aspirants were decanted into a 60 mm petri dish and oocytes were removed. The remaining cells and follicular fluid were pipetted into an eppendorf tubes and centrifuged at 300g for 5 min. The Granulosa cells (GCs) were harvested and the GC pellets were re-suspended in medium and the viable GCs were counted by trypan-blue exclusion method. Viable cell counts were calculated for 1ml of the cell suspension and culture media and cell suspension mixed to provide a seeding density of $3.5x10^5$ cells/well in 500µl in 24-well plates. Cell culture medium used for granulosa cells consisted of 90% v/v TCM199 and 10% v/v of double distilled water supplemented with 0.01% w/v L-glutamine, 0.1% w/v BSA, 10ng/ml of insulin, 0.5µg/ml transferrin, 0.5ng/ml sodium selenite, 50IU/ml penicillin, 50µg/ml streptomycin. Cells were incubated at 38.8^0C in a humidified atmosphere of 5% $CO_2$for 144 h. Every 48 h, 80% of the medium was renewed with fresh medium. To establish the effects of FSH on granulosa cell proliferation, steroid production and gonadotrophin receptor (FSHr and LHr) expression, the cells were also cultured with different doses (0, 1 and 10ng/ml) of FSH for 48h, 96h and 144h to determine the effect of FSH. After 48, 96 and 144h of incubation, the spent media was collected from each well and cells were removed by incubating with Trypsin EDTA. Two wells from each treatment were used for the cell counting at each of the three time points. Cells were suspended in PBS and mixed with a fixed volume of trypan blue solution. Viable cells were counted in a haemocytometer and cell number/ml calculated. Culture media was collected every 48h, and oestradiol and progesterone concentrations determined by radioimmunoassay. Cells from 3 wells were pooled and RNA was extracted from samples using RNeasyÒ plus Mini kit (Qiagen Ltd, West Sussex, UK) for analysis of gene expression, by semi-quantitative PCR. RT reaction was carried out using Omniscript RT kit (Qiagen Ltd, West Sussex, UK).

The cDNA prepared from the RT reaction was then amplified with the gene specific primers which were designed by Primer3 software (Table1). BioMix Red (Bioline Ltd, London, UK) was used as the Mastermix for the PCR reaction. Multiplex PCR reaction was carried out to normalize the gene expression to the internal standard 18s rRNA (QuantumRNA Universal 18S Internal Standard; Applied Biosystems, Warrington, UK). Electrophoresis was conducted using a 1.5% agarose gel with ethidium bromide (Figure1). PCR products were confirmed by sequence analysis.

Data in all experiments were analysed by ANOVA using Genstat release 9.1 (Genstat, 2007). Experiments were analysed as a factorial arrangement of treatments with, for example, three doses of FSH and three time points (48, 96 and 144h). Oestradiol and progesterone concentration data were $\log_{10}$ transformed prior to analysis in order to normalize the data and standardise errors. Data are presented as simple means with their S.E.M.s.

Results and Discussion

Sheep granulosa cells were cultured with three different doses of FSH (0, 1ng and 10ng/ml) and for three different time periods of 48h, 96h and 144h to determine the effects of FSH on cell proliferation, steroid production and

Table 1. Primers for *FSHr* and *LHr* for semi quantitative PCR

Target gene	Primer sequence 5'-3'	Product size	NCBIAccession number
FSHr	CCTCTGGACCGGTCATTCTA TGTTGCAAATTGGATGAAGG	231bp	NM_001009289-Ovis aries
LHr	ACCTGCCAACAAAAGAGCAG CTTGGGTGAGCAGAAACCAT	167bp	L36329-Ovis aries
18s rRNA	QuantumRNAUniversal 18S Internal Standard (Ambion)	315bp	

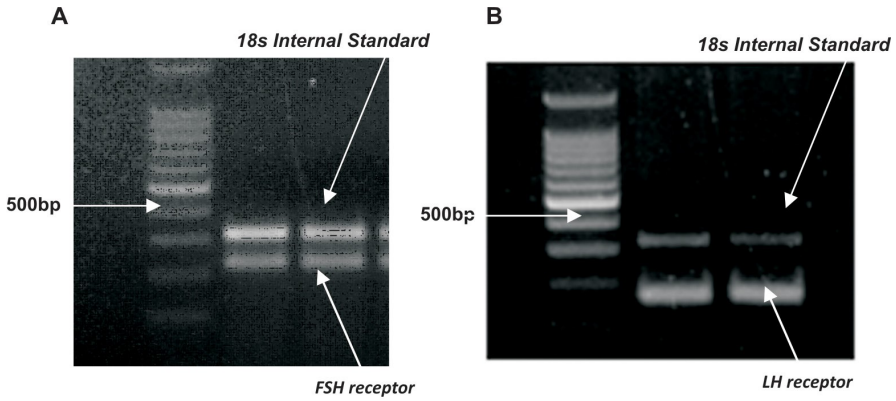

Fig.1. (A) Representative *FSHr* and *18s internal standard* expression in a multiplex PCR reaction (B) Representative *LHr* and *18s internal standard* expression in a multiplex PCR reaction

gonadotrophin receptor expression. A plating density of $3.5´10^5$ cells was selected initially. Granulosa cells were cultured for 48h, 96h and 144h to optimise the time point for study.

Cell number did not differ between any of the three time points except for cells without FSH in the media, for which there was a significant increase in cell number at 96h. There was no significant difference in cell number at any particular time point with different doses of FSH (Fig. 2.a). Monniaux and Pisselet (1992) noticed an increase in cell number at 96h with their ovine granulosa cell culture system. Studies by Rouillier *et al.* (1996) also observed a slight increase in the percentage of viable cells at 96h compared with 48h in their bovine granulosa cell culture system. In the current series of studies in order to give cells maximum exposure to the added FSH a culture period of 144h was eventually utilised. No dose effect on proliferation was noticed for FSH (Fig. 2.a), even though it has been shown that FSH can stimulate proliferative activity of granulosa cells *in vivo* (rats; Rao *et al*., 1978). However, *in vitro* studies failed to demonstrate such an effect in ewes (Monniaux and Pisselet, 1992). Monniaux (1987) found that FSH enhanced thymidine incorporation in sheep granulosa cells after 30 min of culture, but they also noticed that this effect was not sustained in longer incubations *in vitro*. Monniaux and Pisselet (1992) hypothesised that FSH *in vitro* makes some cells move from the G1 to the S-phase of the cell cycle, but that *in vitro* conditions are not capable of sustaining the subsequent events leading to cell multiplication.

Oestradiol concentrations in spent media were significantly reduced after 48hrs of culture. There was a significant decrease ($P<0.001$) in oestradiol production at 96h and 144h compared to 48h (Fig. 2.b). There was no effect of different doses of FSH on oestradiol production at either 48h or 144h. Campbell *et al*. (1996) also demonstrated a reduction in oestradiol production over time with ovine granulosa cells from >3.5mm size follicles, especially when they were cultured without FSH in the medium. Rouillier *et al.* (1996) observed a decline in oestradiol production from day 1 to day 4 during bovine granulosa cell culture in serum free condition, but they did not observe any dose effect of FSH on oestradiol production. They also found that the addition of 2ng/ml of FSH increased the production of oestradiol by the granulosa cells after four days of culture, whereas a negative effect was observed with the addition of 10ng/ml. As observed by Campbell *et al.* (1996), the dose of FSH required to obtain maximum stimulation in oestradiol production, in bovine cells, was 10 times lower than that required by sheep granulosa cells. In the present study, 10ng/ml of FSH showed a tendency to increase oestradiol production and this effect was significant at 96h. Although the cells exhibited the same trend in oestradiol production, the level of oestradiol observed in the current study was far below that reported by other workers (Campbell *et al*., 1996). This is probably due to the lack of testosterone in the culture medium which is the substrate for oestradiol production.

In the current study, there was no significant difference in progesterone production

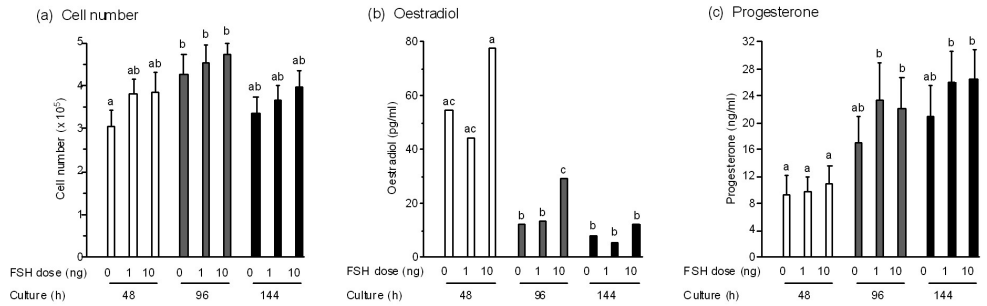

Fig. 2 .Mean (± S.E.M) effect of culture duration and dose of FSH on ovine granulosa cell proliferation and steroidogenesis. Data presented are cell number (a), oestradiol (b), and progesterone (c) concentrations in spent culture media. Oestradiol values are presented as geometric means. Oestradiol and progesterone levels expressed are for 3.5 x 10^5 cells.

due to different doses of FSH (0, 1 and 10ng). However, there was a significant increase (P<0.001) in progesterone production with time in culture (Fig. 2.c). Progesterone production was found to increase after 48h; however, the difference in production was not significant in cells without FSH in the medium. Khalid *et al*. (2000) also reported that there was no significant difference in progesterone production even after culturing ovine granulosa cells (first 48h of culture bovine calf serum was included in the medium) with 20ng/ml of FSH. Monniaux and Pisselet (1992) reported a dose-dependent effect of FSH on progesterone production by sheep granulosa cells which were cultured in a medium containing ovine foetal serum. Rouillier *et al.* (1996) reported that progesterone production in bovine granulosa cells cultured in a serum free medium increased in a dose dependent manner with 2ng/ml and 10ng/ml of FSH. In the current study, however, even though progesterone production was unaffected by the addition of FSH to medium, it was noticed that cells without FSH in the medium did not produce more progesterone over time, indicating an effect of FSH on temporal progesterone production. The decrease in oestradiol and increase in progesterone over time may indicate some level of luteinization of cells over time with this culture system.

FSHr expression was quantified by semi-quantitative RT-PCR and expression normalized to 18S *rRNA*. There was a significant increase (P<0.001) in expression of *FSHr* with time. The increase was significant for all doses of FSH (Fig. 3.a). There was no dose effect of FSH on *FSHr* expression at either 48 or 96 h. However, by 144h *FSHr* expression was lower in granulosa cells cultured without FSH in the media (Figure 3.a).Marsters *et al*. (2003) reported a decrease in expression of *FSHr* during the first 16h of a bovine granulosa cell culture. They then observed an increase in expression at 48h and 96h followed by a decline at 144h. They suggest that cells revert to an immature phenotype during the early stages of cell culture with marked down-regulation in the expression of *FSHr*; and that the expression of *FSHr* depends on the clumping of cells and their steroidogenic activity. There was no dose effect of FSH in the medium on the expression of *FSHr*. Gutierrez *et al.* (1997) suggested that ovine granulosa cells are less sensitive to FSH stimulation at all stages of follicle development compared to bovine cells. Dorrington *et al.* (1988) reported that FSH administered *in vivo* has a profound effect on follicular development in rats, but its effects during *in vitro* culture are modest. As observed in the studies of Campbell *et al.* (1996), the dose of FSH required to obtain maximum stimulation of oestradiol production in bovine cells was 10 times lower than that required by sheep granulosa cells. Therefore, in order to observe a difference in expression of *FSHr* in sheep granulosa cells, a higher dose of FSH might be required. However, there was a significant decrease in *FSHr* expression in cells cultured without FSH in medium at 144h of culture, indicating a positive effect of FSH on *FSHr* expression at least after 96h.

LHr expression was also determined by semi-quantitative RT-PCR and expression normalized to 18S rRNA. No change in expression of *LHr* was noticed with time.

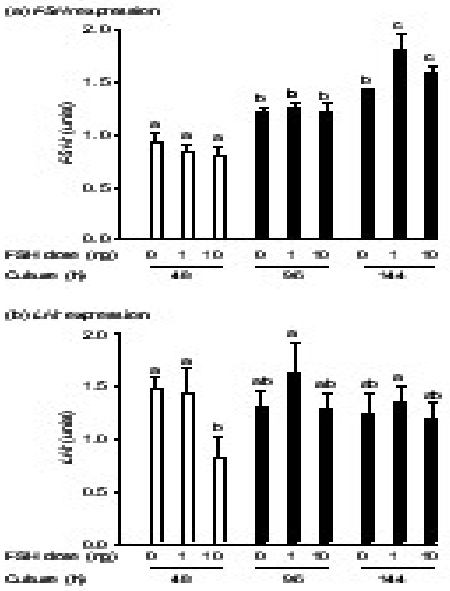

Fig. 3. FSH (a) and LH (b) receptor expression (Mean ± S.E.M) in ovine granulosa cells cultured for varying periods up to 144 h in the presence of differing doses of FSH.

Similarly, there was no effect of FSH dose on *LHr* expression (Figure 3.b).Piquette *et al.* (1991) observed that the treatment of rat granulosa cells with FSH can increase *LHr* expression in a dose- and time-dependent manner. Kanzaki *et al.* (1994) observed that cultured rat ovarian granulosa cells became differentiated and expressed *LHr* when they were incubated with high doses (200ng/ml), but not with low doses (20ng/ml) of FSH. Farookhi and Desjardins (1986) suggested that the disruption of the cell junctions, prior to granulosa cell culture, can affect FSH-stimulated induction of *LHr* expression. In the rat, *LHr* expression was induced in all FSH-containing cultures containing non-dispersed granulosa cells (Farookhi and Desjardins, 1986). In contrast, with dispersed cells, FSH alone failed to induce *LHr* expression. However, the inclusion of either oestradiol or testosterone with FSH restored *LHr* expression, indicating that cell-cell communication may be necessary for *LHr* induction in granulosa cells, and that oestradiol can promote intercellular interactions. Rani *et al.* (1981) also reported that the FSH-dependent induction of *LHr* expression in cultured rat granulosa cells can be blocked by an inhibitor of steroidogenesis. Kessel *et al.* (1985) observed that increasing concentrations of oestrogen maintained *LHr* expression in a dose-dependent fashion in rat granulosa cell cultures and that treatment with semi-purified oestrogen antibodies partially blocked the FSH stimulation of *LHr* expression. In the present study there was no effect of FSH on *LHr* expression. This may have been due to the initial dispersion of cells that was needed to plate equal numbers of cells in each well which may affect the steroidogenic response to FSH (Rouillier *et al*., 1996) and indirectly the expression of gonadotrophin receptors. Moreover, there was no testosterone in the culture medium in level of oestradiol was also low

In the present study there was no significant difference in number of granulosa cells due to culturing for 144hrs with FSH in the medium. However, there was increase in number when the cells were cultured for 96hrs without FSH in the medium. There was a decrease in oestrogen and increase in progesterone due to culturing of cells for 144hrs which indicated gradual luteinization of cells with the present culture conditions. There was increase in expression of *FSHr* with time and the inclusion of FSH in the medium at 1ng or 10ng was found to improve the expression of *FSHr* when the cells were cultured for 144hrs. There was no significant difference in expression level of *LHr* due to time or inclusion of FSH in the medium. The results of this study may form the basis of further studies inquiring the effect of FSH on the transcriptome profile of granulosa cells which maintain them in proliferative state or which take them to differentiated state which are necessary for proper development of follicle and fertilization potential of oocytes.

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