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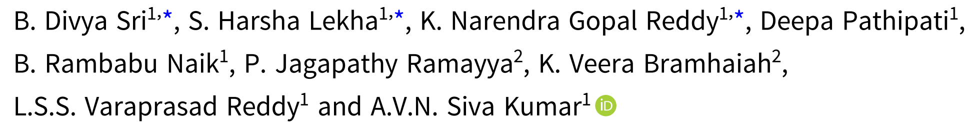
Kisspeptin stimulates sheep ovarian follicular development *in vitro* through homologous receptors



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Research Article

Cite this article: Divya Sri B *et al.* (2024) Kisspeptin stimulates sheep ovarian follicular development *in vitro* through homologous receptors. *Zygote*. **32**: 49–57. doi: 10.1017/S096719942300059X

Received: 3 August 2023
Revised: 3 November 2023
Accepted: 13 November 2023
First published online: 7 December 2023

Keywords:
Culture of ovarian follicles; Kisspeptin; Kisspeptin receptor; Sheep

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Summary

The present study was conducted to elucidate (1) the influence of kisspeptin (KP) on the *in vitro* development of preantral follicles (PFs) and (2) evolution of KP receptor gene (*KISS1R*) expression during ovarian follicular development in sheep. Kisspeptin was supplemented (0–100 μg/ml) in the culture medium of PFs for 6 days. The cumulus–oocyte complexes (COCs) from cultured PFs were subsequently matured to metaphase II (MII) for an additional 24 h. The proportions of PFs exhibiting growth, antrum formation, average increase in diameter, and maturation of oocytes to MII stage were the indicators of follicular development *in vitro*. The expression of the kisspeptin receptor gene at each development stages of *in vivo* developed (preantral, early antral, antral, large antral and COCs from Graafian follicles) and *in vitro* cultured PFs supplemented with KP was assessed using a real-time polymerase chain reaction. The best development in all the parameters under study was elicited with 10 μg/ml of KP. Supplementation of KP (10 μg/ml) in a medium containing other growth factors (insulin-like growth factor-I) and hormones (growth hormone, thyroxine, follicle-stimulating hormone) resulted in better PF development. The *KISS1R* gene was expressed in follicular cells and oocytes at all the development stages of both *in vivo* developed and *in vitro* cultured follicles. Higher *KISS1R* gene expression was supported by culture medium containing KP along with other hormones and growth factors. Accordingly, it is suggested that one of the mechanisms through which KP and other growth factors and hormones influence the ovarian follicular development in mammals is through the upregulation of expression of the KP receptor gene.

Introduction

The frequency of *in vitro* embryogenesis from cultured preantral follicles (PFs) is relatively poor (Arunakumari *et al.*, 2010). Efforts to improve culture conditions often involve supplementation of culture medium with different hormones and growth factors (Kona *et al.*, 2016). In this connection, kisspeptin a peptide hormone (145 amino acids), a product of the *KISS1* gene, synthesized and released from arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) of hypothalamus is an interesting but inadequately investigated growth factor. Kisspeptin was reported to be involved in upstream regulation of pulsatile and surge GnRH release, sex differentiation in the brain, gonadotropin secretion, onset of puberty, ovulation, metabolic regulation of fertility (Roa *et al.*, 2008a; Pinna *et al.*, 2012), seasonal reproduction, follicular development (Fernandois *et al.*, 2017), oocyte maturation (Lapatto *et al.*, 2007), implantation, and gonadal steroid feedback to the hypothalamus (Horikoshi *et al.*, 2003). However, the influence KP on the development of cultured PFs in sheep was never investigated.

Kisspeptin receptor (KISS1R) belongs to rhodopsin gamma family of G-protein coupled receptors and was identified for the first time as an orphan receptor in the rat brain (Lee *et al.*, 1999). The expression of *KISS1R* mRNA and its immunohistochemical localization in the ovaries of mice (Laoharatchatathanin *et al.*, 2015; Merhi *et al.*, 2016), hamster (Shahed and Young, 2009), rabbit (Maranesi *et al.*, 2019), cat (Cielesh *et al.*, 2017), dog (Tanyapanyachon *et al.*, 2018), swine (Saadeldin *et al.*, 2012), goat (Inoue *et al.*, 2009), bovine (Mishra *et al.*, 2019), and human (Gaytán *et al.*, 2009; García-Ortega *et al.*, 2014; Merhi *et al.*, 2016; Blasco *et al.*, 2019; Blasco *et al.*, 2020) suggest that kisspeptin (KP) may have direct gonadal effects (Clarke *et al.*, 2015). However, the evolution of KISS1R expression as the ovarian follicles and oocytes develop from preantral to Graafian follicle stage has not been reported previously. Furthermore it is unknown whether *in vitro* culture of ovarian follicles in mammals provides for the expression kisspeptin receptor.

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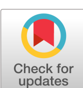

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In view of the above, the present study was conducted in sheep to (1) know the influence of kisspeptin on the development of cultured PFs, (2) evaluate the interaction of kisspeptin with other hormones and growth factors on *in vitro* growth of PFs, and (3) elucidate the expression of the *KISS1R* gene as the ovarian PFs developed to the Graafian follicle stage in *in vivo* developed and cultured PFs *in vitro*.

Materials and methods

All the methods described briefly here are routinely used in the culture of PFs and described in detail in several earlier publications from our laboratory (Arunakumari *et al.*, 2013; Kona *et al.*, 2016; Srividya *et al.*, 2017; Kumar *et al.*, 2019; Kona *et al.*, 2021). Unless otherwise stated, culture medium, hormones, growth factors, fetal calf serum and all the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and plastics from Nunclon (Roskilde, Denmark). All the hormones and growth factors used were cell culture tested and endotoxin free.

Collection, processing and isolation of different categories of follicles (PFs) from sheep ovaries

In total, 1148 ovaries recovered immediately after slaughter on different days were transported to the laboratory within 1 h in sterile, warm (37°C) phosphate-buffered saline. In total, 6592 intact PFs) isolated from the collected ovaries in the size range 250–400 µm (standardized in our laboratory and quoted in several earlier publications from our laboratory; Arunakumari *et al.*, 2013; Kona *et al.*, 2016; Kona *et al.*, 2021; Srividya *et al.*, 2017; Kumar *et al.*, 2019) were used in the study. PFs having visible centrally placed oocytes without any signs of atresia and with intact basement membrane and no antral cavity were considered to be good for the culture (Figure 1B, C). On average, the diameter of the PFs on day 0 was 321.3 ± 6.55 µm. In the size range of the follicles used here, a small proportion that may have initiated antrum formation were eliminated from *in vitro* culture. The PFs were cultured for 6 days in different groups (Tables 1–5) with subsequent *in vitro* maturation of the cumulus–oocytes complex (COC) for an additional 24 h, following methods developed in the laboratory (Arunakumari *et al.*, 2013; Kona *et al.*, 2016, 2021; Srividya *et al.*, 2017; Kumar *et al.*, 2019). These procedures are briefly mentioned in subsequent sections.

Culture of PFs, in vitro *maturation of COCs and evaluation*

Preantral follicles were cultured in bicarbonate-buffered tissue culture medium 199 (TCM199B) supplemented with 50 mg/ml gentamycin sulphate and different concentrations of kisspeptin-10 (KP; M 2816; Sigma-Aldrich, USA; Tables 1 and 2) and/or other growth factors and hormones (Table 3). Culture medium was pre-incubated for 1 h at 39°C under an humidified atmosphere of 5% CO_2 in air. The selected follicles were washed three times in culture medium and subsequently placed individually in 20-µl droplets of culture medium in 35-mm plastic culture dishes (cat. no. 153066, Nalge Nunc, Denmark). To avoid evaporation of the medium, the microdroplets were overlaid with autoclaved lightweight mineral oil (Sigma M8410) pre-equilibrated with the medium overnight at 39°C in 5% CO_2 in air. These culture dishes were incubated at 39°C under an humidified atmosphere in 5% CO_2 in air for up to 6 days. The day on which the PFs were placed in the culture was designated as day 0 and the subsequent days as day 1, day 2 and so on. Half the medium was replaced by an equal volume of fresh medium every 48 h.

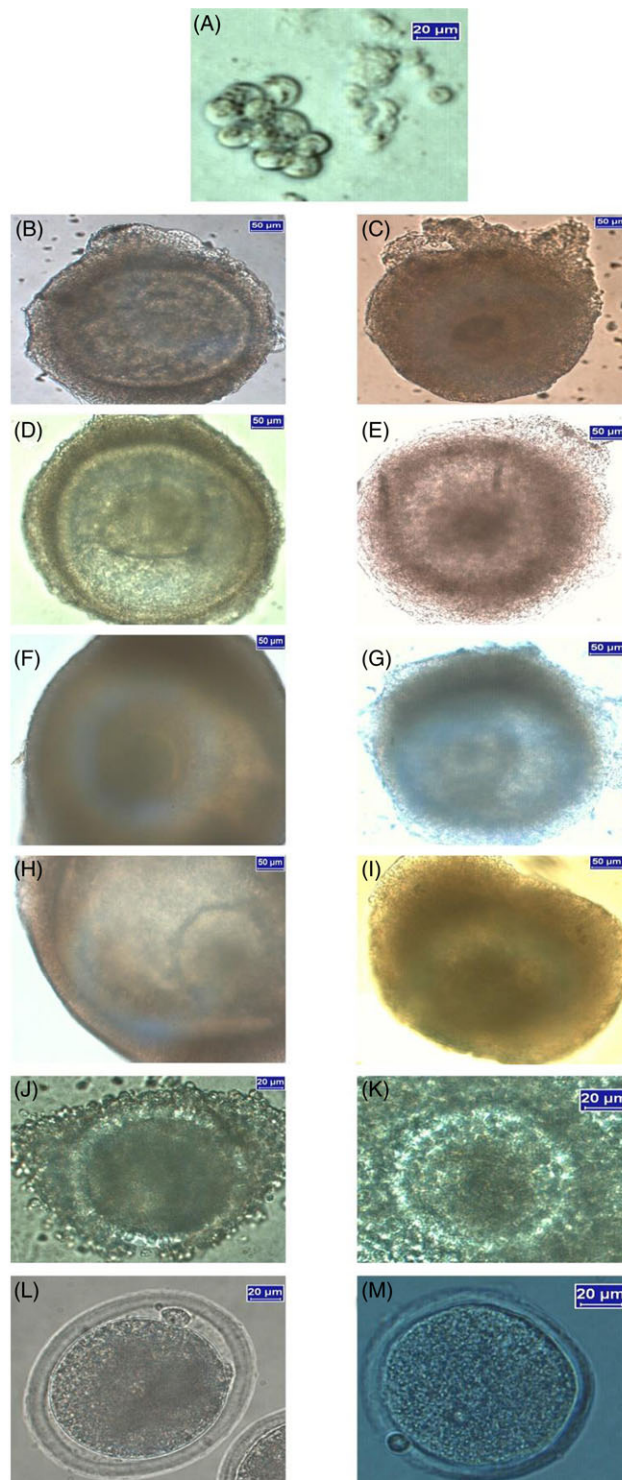

Figure 1. Different stages of *in vitro* and *in vivo* grown ovarian follicles used in the present studies. (A) Primordial follicles. (B, C) Preantral follicles at isolation 250–400 µm in diameter. (D) Early antral follicles. (E) Preantral follicles cultured for 2 days. (F) Antral follicles. (G) Preantral follicles cultured for 4 days. (H) Graafian follicle. (I) Preantral follicles cultured for 6 days. (J, K) Cumulus–oocyte complex from *in vivo* and *in vitro* cultured follicles. (L, M) Oocyte freed from follicular cells of (L) and (M) for the MII stage.

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Table 1. Effect of kisspeptin (0–100 μg/ml) on *in vitro* development of sheep preantral follicles (PFs). Each replicate: five preantral follicles; no. of replicates/group = 10

Concentration of kisspeptin (replicates/no. of follicles)	% PFs exhibiting growth (mean ± SE)	Average increase in diameter (μM) (mean ± SE)	% PFs exhibiting antrum formation (mean ± SE)	% oocytes from cultured PFs matured to MII stage (mean ± SE)
Control (TCM199B; 10/50)	49.62 ± 4.08[a]	23.20 ± 0.15[a]	26.67 ± 3.47[a]	6.34 ± 0.61[a]
1 μG/ml (10/50)	54.39 ± 5.79[c]	56.64 ± 4.21[d]	34.99 ± 3.69[b]	9.25 ± 0.16[b]
5 μG/ml (10/50)	66.67 ± 4.08[d]	59.98 ± 4.05[d]	46.67 ± 4.59[c]	13.20 ± 0.15[c]
10 μG/ml (10/50)	89.99 ± 3.49[g]	71.65 ± 2.41[f]	70.46 ± 2.41[e]	27.58 ± 0.30[f]
15 μG/ml (10/50)	76.67 ± 2.58[e]	69.98 ± 3.16[e]	55.37 ± 4.83[d]	21.05 ± 0.24[e]
20 μG/ml (10/50)	81.66 ± 2.83f	41.65 ± 4.85[c]	48.32 ± 3.68[cd]	22.41 ± 0.21[e]
25 μG/ml (10/50)	73.42 ± 2.58[e]	33.32 ± 4.08[b]	53.34 ± 3.94[d]	17.54 ± 0.21[d]
50 μG/ml (10/50)	43.35 ± 4.81[b]	26.67 ± 3.49[a]	31.66 ± 3.69[b]	6.77 ± 0.16[a]
100 μG/ml (10/50)	44.95 ± 4.11[b]	22.41 ± 0.21[a]	30.56 ± 4.08[b]	5.00 ± 0.15[a]

Control medium: TCM199B medium containing 50 μg/ml gentamycin sulphate.
Values with different superscripts within a column are significantly different ($P \leq 0.05$).

Table 2. Effect of kisspeptin (10–20 μg/ml) on *in vitro* development of sheep preantral follicles (PFs). Each replicate: five preantral follicles; no. of replicates/group = 10

Concentration of kisspeptin (replicates/no. of follicles)	% of PFs exhibiting growth (mean ± SE)	Average increase in diameter (μM) (mean ± SE)	% of PFs exhibiting antrum formation (mean ± SE)	% oocytes from cultured PFs matured to MII stage (mean ± SE)
Control (TCM 199B; 10/50)	47.86 ± 3.76[a]	15.15 ± 5.27[a]	34.07 ± 3.17[a]	7.09 ± 2.94[a]
10 μG/ml (10/50)	90.15 ± 2.54[e]	78.43 ± 1.89[f]	74.68 ± 5.33[e]	30.91 ± 5.05[d]
12.5 μG/ml (10/50)	83.67 ± 1.16[d]	69.98 ± 3.16[e]	65.29 ± 3.62[d]	24.78 ± 2.67[bc]
15 μG/ml (10/50)	81.65 ± 2.83[d]	64.56 ± 2.32[e]	58.37 ± 2.70[cd]	23.88 ± 2.99[bc]
17.5 μG/ml (10/50)	78.32 ± 3.37[c]	56.23 ± 4.83[d]	52.46 ± 7.12[c]	24.82 ± 5.30[bc]
20 μG/ml (10/50)	76.54 ± 2.58[c]	41.65 ± 4.85[c]	50.15 ± 7.85[c]	18.75 ± 7.41[b]

Control medium: TCM199B medium containing 50 μg/ml gentamycin sulphate.
Values with different superscripts with in a column are significantly different ($P \leq 0.05$).

Table 3. Effect of 10 μg/ml of kisspeptin in combination with standard medium (SM) on *in vitro* development of sheep preantral follicles (PFs). Each replicate: five preantral follicles; and no. of replicates/group = 10

Concentration of kisspeptin (replicates/no. of follicles)	% PFs exhibiting growth (mean ± SE)	Average increase in diameter (μM) (mean ± SE)	% PFs exhibiting antrum formation (mean ± SE)	% oocytes from cultured PFs matured to MII stage (mean ± SE)
Control (TCM; 10/50)	46.92 ± 6.61[a]	12.48 ± 2.70[a]	32.91 ± 9.98[a]	8.90 ± 0.07[a]
SM (10/50)[1]	65.98 ± 5.58[b]	34.07 ± 3.17[b]	51.33 ± 7.50[b]	22.62 ± 3.18[b]
SMKP (10/50)	94.98 ± 3.89[c]	66.14 ± 3.89[c]	71.29 ± 5.33[c]	38.36 ± 2.94[c]

Control medium: TCM199B medium containing 50 μg/ml gentamycin sulphate.
Standard medium composition (SM).
[1]TCM199B containing 1 μg/ml thyroxine, 2.5 μg/ml FSH, 10 ng/ml insulin-like growth factor-I and 1 mIU/ml of growth hormone.
Values with different superscripts within a column are significantly different ($P \leq 0.05$).

Each follicle was evaluated morphologically every 24 h during the culture period using an inverted microscope (Leica, DMIRB, Germany) for increase in the diameter and antrum formation. The average increase in diameter was calculated only for the growing follicles during the culture period in all the groups under study.

After 6 days of culture, the cumulus–oocyte complexes (COCs) were isolated by gently opening the cultured follicles (Figure 1J, K) mechanically with a 24G needle under a zoom stereomicroscope. The isolated COCs (Figure 1J, K) were subjected to further *in vitro* maturation for 24 h. The COCs were washed three times in *in vitro* maturation (IVM) medium (d'Anglemont de Tassigny *et al.*, 2008; TCM199B supplemented with 10 μg/ml follicle-stimulating hormone (FSH), 10 μg/ml luteinizing hormone, 1 μg/ml estradiol-17β, 50 μg/ml gentamycin sulphate, 10 μg/ml bovine serum albumin (BSA; A8412, Sigma, USA) and 10% (v/v) oestrous sheep serum. After washing the COCs were placed individually in 20-μl droplets of the same medium in 35-mm plastic culture dishes, covered with pre-equilibrated lightweight mineral oil, and incubated for 24 h as described above.

After IVM, the COCs were subjected to repeat pipetting through a fine bore glass pipette during which the oocytes were denuded of cumulus cells. Subsequently the oocytes were washed in Hoechst 33342 fluorescent stain solution (B2261; concentration of 5 μg /ml) and incubated in a 50-μl droplet of the same solution for 15 min at 39°C (d'Anglemont de Tassigny *et al.*, 2008) and

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Table 4. Design for *KISS1R* gene expression studies in *in vivo* developed and *in vitro* cultured PFs in sheep

In vivo	*In vitro*			
	Group I (TCM 199B)	Group II (TCM + SM)	Group III (TCM + KP at 10 µg/ml)	Group IV (SM + KP at 10 µg/ml)
Preantral follicles	PFs exposed to medium for 3 min	PFs exposed to medium for 3 min	PFs exposed to medium for 3 min	PFs exposed to medium for 3 min
Early antral follicles	PFs cultured for 2 days	PFs cultured for 2 days	PFs cultured for 2 days	PFs cultured for 2 days
Antral follicles	PFs cultured for 4 days	PFs cultured for 4 days	PFs cultured for 4 days	PFs cultured for 4 days
Large antral follicles	PFs cultured for 6 days	PFs cultured for 6 days	PFs cultured for 6 days	PFs cultured for 6 days
Cumulus–oocyte complexes (COCs) from Graafian follicles	COCs from 6-day cultured PFs after subjecting to IVM for 24 h	COCs from 6-day cultured PFs after subjecting to IVM for 24 h	COCs from 6-day cultured PFs after subjecting to IVM for 24 h	COCs from 6-day cultured PFs after subjecting to IVM for 24 h

For gene expression each sample of total RNA isolation from cumulus cells and oocytes, 50–60 cultured PFs were used for each respective culture duration of 20 stages. As the quantity of RNA acquired in the follicular cells is low, the RNA isolation was repeated four or five times for every stage, therefore almost 5082 follicles were used in the total RNA isolation.

examined under fluorescent light (510–560 nm wavelength) on an inverted microscope (Leica, Germany; excitation: 352–455 nm and emission 460–490 nm) for the MII stage (Figure 1L, M).

Quantitative expression of the KISS1R gene

Quantitative expression of the *KISS1R* gene was studied from the preantral follicle stage onwards in the *in vivo* developed and corresponding stages from four groups of cultured ovarian follicles (Table 4; see Supplementary Table and Figure 1 for equivalent stages between *in vivo* developed and *in vitro* cultured follicles). The entire experiment was repeated four times. Triplicate samples of complementary DNA (cDNA) from each replicate of the experiment ($4 \times 3 = 12$ cDNA) samples for each *in vivo* and *in vitro* stage were subjected to reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Isolation and quantification of total RNA from follicular cells and oocytes in each group

At each of the development stages of follicles (from PFs stage onwards) from *in vivo* developed and the cultured follicles (Table 4), follicular cells and oocytes were separately pooled from 30–50 follicles collected on the same day from the ovaries for the isolation of total RNA. RNA isolation was carried out using patented Medox-Easy spin column Total RNA Mini prep Kits (Srividya *et al.*, 2017; Kumar *et al.*, 2019) (26–27) according to the manufacturer's instructions (Medox Biotech India Pvt. Ltd, Chennai, India). The RNA concentration and purity were determined using a NanoDrop lite spectrophotometer (Thermo Scientific S. No.1354). RNA samples with purity (absorbance at 260/280) only in the 1.8–2.1 range were used in the expression studies. The RNA sample was stored at −70°C until analyzed.

Reverse transcription and real-time PCR

High-capacity reverse transcription kit (Applied Biosystems part no. 4368814) was used for the reverse transcription. RT reaction was carried out for 10 min at 25°C, for 120 min at 37°C and for 5 min at 85°C in a thermocycler (Eppendorf Mastercycler Gradient) according to the manufacturer's instructions. In a comparison of 12 commonly used reference genes, *RPLPO*, *HPRT1* and 18S rRNA were the three most stably expressed genes in sheep ovarian follicles under the current experimental conditions (unpublished observations in our laboratory). Therefore, the geometric mean of these three genes (Khamsi and Roberge, 2001) was used as the normalizer in the analysis of the expression of kisspeptin receptor gene. Primer and probe details for kisspeptin receptor and the reference genes are given in Table 6. Real-time RT-qPCR was performed on an Applied Biosystems 7500 machine. Each 25-µl reaction mix contained 12.5 µl of TaqMan Universal PCR Master Mix (2×), 1.25 µl of 20× gene expression assay mixture, and 25 ng of cDNA sample in nuclease-free water. Thermal cycling conditions were Erase UNG (uracil *N*-glycosylase) activation 2 min at 50°C, AmpliTaq Gold DNA polymerase activation10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Extreme Ct (threshold cycle number) values and 'no detection' found for some of the samples meant that these were discarded prior to calculation of relative quantification (RQ) values, resulting in unequal numbers of observations in different groups. To calculate the expression levels (RQ values) of different target genes, first the Ct values of the target and reference genes were converted to quantity inputs using the formula $2^{\text{minimumCt} - \text{sampleCt}}$. Expression of the target genes was the ratio of target quantity input to that of geometric mean of quantity inputs of the reference genes.

Experiments

In total, four experiments were conducted to study the influence of KP on *in vitro* development of PFs and *KISS1R* gene expression in sheep.

Experiment 1

Experiment 1 was initially conducted with KP (concentration ranges 1, 5, 10, 15, 20, 25, 50 and 100 µg/ml) to ascertain whether KP could influence the *in vitro* development of PFs (Table 1)

Experiment 2

Based on the results of Experiment 1, and having confirmed in Experiment 1 that KP could influence the development of PFs, Experiment 2 was conducted to determine the best concentration for KP for PFs development in *in vitro* culture (Table 2).

Experiment 3

It was undertaken to discover whether KP at 10 µg/ml (as determined from Experiment 2) interacted with other hormones and growth factors [standard medium (SM)] on the *in vitro* development of sheep PFs (Table 3).

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Table 5. Expression of kisspeptin receptor (*KISS1R*) gene in follicular cells and oocytes in *in vivo developed and in vitro* cultured PFs follicles

Source		Kisspeptin receptor gene expression (log_{10} relative quantification)				
		In vitro				
Follicular cells from	*In vivo*		Group -1 (TCM199 B)	Group- II standard medium	Group III (TCM + KP).	Group IV (SM + KP)
Preantral follicles	3.49 ± 0.06^{a1}	PFs exposed to medium for 3 min	1.83 ± 0.11^{a1}	3.11 ± 0.21^{a1}	7.94 ± 0.05^{a2}	1.45 ± 0.15^{a1}
Early antral follicles	12.38 ± 1.09^{b3}	PFs cultured *in vitro* for 2 days	2.07 ± 0.04^{a1}	9.05 ± 0.86^{b2}	19.76 ± 0.74^{b3}	1.88 ± 0.12^{a1}
Antral follicles	59.07 ± 4.52^{c3}	PFs cultured *in vitro* for 4 days	19.56 ± 2.41^{b1}	28.10 ± 2.76^{c2}	35.27 ± 0.24^{c2}	20.99 ± 0.48^{b1}
Large antral follicles	75.31 ± 0.18^{d3}	PFs cultured *in vitro* for 6 days	16.89 ± 0.89^{b1}	23.32 ± 0.53^{c1}	37.29 ± 0.21^{c2}	72.61 ± 0.29^{c3}
Cumulus cells from COCs after IVM	594.2 ± 1.45^{e5}	cumulus cells from of 6-day cultured PFs matured *in vitro* for 24 h	1.52 ± 0.09^{a1}	10.26 ± 1.45^{b2}	136.9 ± 1.51^{d3}	163.56 ± 2.98^{d4}
Oocytes from						
Preantral follicles	1.98 ± 0.07^{a1}	PFs exposed to medium for 3 min	2.69 ± 0.15^{a1}	4.56 ± 0.23^{a2}	8.50 ± 1.11^{a3}	97.75 ± 9.14^{a4}
Early antral follicles	2.14 ± 0.67^{a1}	PFs cultured *in vitro* for 2 days	2.21 ± 0.14^{a1}	8.49 ± 0.49^{ab2}	13.15 ± 1.09^{a3}	30.65 ± 1.20^{b4}
Antral follicles	6.93 ± 0.92^{b1}	PFs cultured *in vitro* for 4 days	16.00 ± 2.0^{b2}	13.32 ± 0.53^{b2}	42.09 ± 2.56^{c3}	203.04 ± 9.07^{c4}
Large antral follicles	44.53 ± 2.0^{c4}	PFs cultured *in vitro* for 6 days	2.08 ± 0.11^{a1}	5.98 ± 0.83^{a2}	29.99 ± 2.73^{b3}	282.00 ± 12.8^{d5}
COCs collected from Graafian follicles after IVM	94.03 ± 0.66^{d3}	COCs from 6-day cultured PFs matured *in vitro* for 24 h	26.9 ± 2.34^{c2}	25.27 ± 0.24^{c2}	21.42 ± 0.31^{b1}	449.18 ± 3.03^{e4}

Values with different superscripts within a column are significantly different (depicted with a, b, c, d, e).
Values with different superscripts within a column are significantly different (depicted with 1, 2, 3, 4, 5).

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Table 6. Primers and probe sequences of target gene (kisspeptin receptor, *KISS1R*) and reference genes

Gene name and accession number	Symbol	Primers and probe sequences	Amplicon size (base pairs)	Assay on demand lot no. (Applied Biosystems)
Kisspeptin-10 receptor	*KISS-1R*	F primer: CTGGTGCAGCGGGAGAAG	57	7542879–2E3, 3A5
		R primer: GCGAGGCCGAAGGA		
		Probe: 6FAM-ACGTGTCCGCCTACA-TAMRA		
Reference (housekeeping) genes				
Hypoxanthine-guanine phosphoribosyl transferase	*HPRT1*	F primer: GCTCGAGATGTGATGAAGGAGAT	92	P210421–000 E09
		R primer: TCCAACAGGTCGGCAAAGAA		
		Probe: FAM-AGCCCCCCTTGAGCACACAGA—NFQ		
18S ribosomal RNA	18S rRNA	F primer: AACAATACAGGACTCTTTCGAGGC	86	P210421–000 E10
		R primer: CAGACTTGCCCTCCAATGGA		
		Probe: FAM-CCACTTTAAATCCTTCCGCGAGGAT-NFQ		
Large ribosomal protein	*RPLPO*	F primer: GCTCTGGAGAAACTGTTGCC	91	P210421–000 E11
		R primer: CCAGCAGCATGTCCCTGAT		
		Probe: FAM-AGGTCCTCCTTGGTGAACACGAAGC-NFQ		

Experiment 4

This experiment was conducted to elucidate whether observed improvement of KP on *in vitro* development of PFs was due to the expression of its receptor through gene expression using RT-PCR studies (Tables 4 and 5). Receptor expression was studied in *in vitro* cultured follicles and compared with the *in vivo* developed PFs

Statistical analysis

Dependent variables are the development parameters of the follicles, and independent variables are the different treatments in the experiments. A comparison of the proportion of PFs exhibiting growth, average increase in diameter, and antrum formation in the *in vitro* cultured PFs among different treatment groups was undertaken separately using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS 20 software. The meiotic maturation of the oocytes to MII stage was calculated using simple percentage analysis at the end of the culture period. For gene expression studies, the stage of development and source (*in vivo* or *in vitro*) were the independent variables, and expression of the genes was the dependent variable. Log RQ values were analyzed using two-way ANOVA [General Linear Model (GLM)] with unequal numbers of observations followed by the Tukey honestly significant difference (HSD) multiple comparison test (SPSS version 20, IBM Corp., USA). A P-value ≤ 0.05 was considered statistically significant.

Results

Influence of kisspeptin on in vitro growth of PFs in sheep

The influence of KP on preantral follicular development is shown in Tables 1 and 2. It was observed that KP at 10 μg/ml supported a significantly better development of PFs (Tables 1 and 2) compared with all other concentrations for all the parameters studied in both Experiments 1 and 2. Furthermore the SM (growth factors and hormones) supplemented with KP 10 μg/ml supported the best development of PFs *in vitro* (Table 3). Therefore 10 μg/ml of KP was subsequently used in the *KISS1R* gene expression studies.

Quantitative expression of KISS1R gene in in vitro developed ovarian follicles and in vitro cultured PFs in sheep

Expression of kisspeptin receptor gene in follicular cells and oocytes from in vivo developed ovarian follicles and in vitro cultured PFs

While *KISS1R* expression in follicular cells was quantitatively different among different development stages of *in vivo* developed and *in vitro* cultured follicles, it increased as the development progressed in both groups (Table 5). While the follicular (cumulus cells) cells in *in vitro* matured COCs from the follicles cultured in Groups 3 and 4 exhibited a significant increase in the expression (Table 5), the follicular cells (cumulus cells) in *in vitro* matured COCs from the follicles cultured in Groups 1 and 2 exhibited a significant drop in expression (Table 5). Furthermore the development from the early antral to the antral stage marked the transition to better expression of KISS1R in the follicular cells among all groups of follicles either *in vivo* developed or *in vitro* cultured (Table 5).

The expression patterns of KISS1R in the oocytes from different development stages of *in vivo* developed and *in vitro* cultured follicles was parallel to that observed in the follicular cells (Table 5), increasing as development progressed from the preantral stage. Similar to the follicular cells, the transition to better expression occurred as the follicles developed to the antral stage (Table 5). However, unlike the follicular cells, a significant drop in the expression of KISS1R was observed in the oocytes cultured in Groups 1–3 media as they developed from the antral to the large antral stage (Table 5). The pattern of gene expression of *KISS1R* in oocytes as observed in *in vivo*

was mimicked at all development stages in the oocytes from the follicles cultured in Group 4 (Table 5)

Discussion

This is the first study to examine the direct effects of kisspeptin (KP) on the *in vitro* development of PFs in sheep. It was observed in this study that KP at lower concentrations promoted the growth of PFs and yielded better results compared with that of higher concentrations (Table 1). As we investigated a reasonably large number of different concentrations, it may be suggested that 10 µg/ml appears to be the best, if not optimum, for the *in vitro* development of sheep PFs (Tables 1 and 2). It must be pointed out that the KP concentration that supported follicular growth *in vivo* might be higher, as suggested by the high concentrations of KP in the follicular fluid compared with serum in the pig (Saadeldin *et al.*, 2012) and women (Singh *et al.*, 2021). However, intrafollicular concentrations of KP in sheep are unknown.

The detrimental effects of higher doses of kisspeptin on follicular growth *in vitro* have also been reported earlier (Mamo *et al.*, 2007; Byri *et al.*, 2017; Fernandois *et al.*, 2017). In a study in goats, supplementation of kisspeptin at 1 and 10 µM concentrations facilitated the development of primordial follicles, while higher doses of 100 µM resulted in the degeneration of follicles when ovarian cortical strips were cultured *in vitro* for 7 days (Magamage *et al.*, 2021). Previously it was hypothesized that the inhibitory effects of KP at higher doses on cellular proliferation and viability might be due to the stimulation of apoptotic markers (Fernandois *et al.*, 2017).

In another study conducted in our laboratory, 10 µg/ml KP favoured the expression of antiapoptotic genes (*Bcl-xl*, *Bcl-2*) but inhibited proapoptotic genes (*BAX*, *BAD*, *p53* and *BAK*) in the cultured PFs (unpublished data). Therefore, it is possible that improvement in PF growth at 10 µg/ml in the present study might be due to the protective effect of KP against apoptosis. From among the thousands to hundreds of thousands of PFs present in the ovary at birth in mammals, almost 99% undergo apoptosis before reaching the Graafian follicle stage (Hsueh *et al.*, 1994). Therefore KP might rescue the PFs from undergoing apoptosis.

Although there have been no published reports on the use of KP in combination with other hormones and growth factors in the culture of PFs, the present results conform with earlier reports on the *in vitro* development of cumulus/granulosa cells in medium supplemented with KP and other hormones (Saadeldin *et al.*, 2012; Mamo *et al.*, 2007; Byri *et al.*, 2017; Fabová and Sirotkin, 2021). The positive effect of FSH in culture medium along with KP has been reported previously in sheep (Fabová and Sirotkin, 2021), in pig (Mamo *et al.*, 2007; Saadeldin *et al.*, 2012) and in human (Byri *et al.*, 2017) in controlling oocyte maturation and KP activation. Recent studies have also demonstrated the role of KP in regulating the action and secretion of GH and T_4 in cultured pituitary cells (Jayasena *et al.*, 2014). As the culture medium (SM) in the present study contained FSH, GH, IGF-1 and T_4, therefore it is assumed that KP could have a synergistic/additive effect with above growth factors and hormones for the better observed development of PFs and oocyte maturation (Table 3).

KISS1R mRNA expression in different stages of in vitro *developed and* in vitro *cultured ovarian follicles*

This study was used to elucidate the quantitative expression of kisspeptin receptor (KISS1R) in different development stages of *in vivo* developed follicles and their corresponding *in vitro* stages (see Table S1) and as evidence for the observed results in Experiments 1–3. *KISS1R* mRNA expression at different stages of development of *in vivo* developed ovarian follicles (from preantral stage onwards) in the present study alludes to (1) that KP acts through its own receptors, and (2) therefore supports the earlier view (Shahed and Young, 2009) that KP might play a direct role in follicular development. Only few reports are available to support this observation of expressing KISSR at different stages of follicular development (Liu *et al.*, 2017; Tanyapanyachon *et al.*, 2018). However, previous findings have demonstrated that KP receptor deficiency in mice resulted in the depletion of preantral and antral follicles, as well as the arrest of follicular development (Castellano *et al.*, 2006). It may be argued that *KISS1R* gene expression need not necessarily result in the synthesis of the receptor protein, as gene transcription does not always lead to translation. However, in another study in the laboratory (unpublished data), the immunoreactivity of the KISS1R protein was observed to increase, along with the development of PFs, both in *in vivo* developed and cultured ovarian follicles.

A previous report observed that follicular cells (cumulus cells) were the main site for kisspeptin synthesis (Xiao *et al.*, 2011), which explains the significantly abundant expression of *KISS1R* mRNA in the follicular cells observed in the present study. This finding is further supported by the earlier study that explained the basis of kisspeptin secreted in granulosa cells with regard to progesterone secretion and associated actions during steroidogenesis by regulating the steroidogenic enzymes Erk1/2 in the mitogen-activated protein kinase pathway (Ricu *et al.*, 2012). It was also reported that KP exhibited a dose-dependent regulation of estradiol-17β secretion, in which the low doses significantly increased estradiol-17β compared with a high dose of the KP thereby suggesting the role of KP in gonadal steroid production at lower doses, which further increased the LH response to kisspeptin (Peng *et al.*, 2013). Also, it was apparent that KISS1R expression was progressive with the formation of the antrum, which indicates the accumulation of kisspeptin in the follicular fluid, as confirmed in previous studies (Saadeldin *et al.*, 2012; Singh *et al.*, 2021).

Of the four groups, Group 4 showed marked increase for *KISS1R* mRNA expression compared with the other *in vitro* cultured groups. Although meagre reports are available to date to support this observation, one study showed a positive correlation between FSH and KP, which reflects the permissive effect of FSH for KP action, thereby leading to an increase in KISS1R expression (Saadeldin *et al.*, 2012). It was also reported previously that a blockade in FSH secretion suppressed KISS1R expression (Roa *et al.*, 2008a). Reports are available to indicate the role of LH in increasing the kisspeptin expression responsiveness to FSH (Saadeldin *et al.*, 2012; Babitha *et al.*, 2014; Fabová and Sirotkin, 2021). As FSH is a component of SM (Groups 2 and 4) used in this study, it is possible that FSH shows synergism with kisspeptin and also suggests a carryover effect between FSH and LH, which might be responsible for the highest expression of KISS1R in the PFs cultured in Group 4 compared with all other groups. mRNA expression of KISS1R was found to be highest in the COCs aspirated from *in vivo* developed large antral follicles, cumulus cells and oocytes from 6-day cultured PFs subjected to an additional 24 h of IVM subsequently during different culture conditions in this study. This observation highlights the possibility of bidirectional communication of kisspeptin in regulating the proliferation and differentiation of granulosa cells and therefore its receptor expression, as reported in previous studies (Roa *et al.*, 2008b; Levine, 2015; Cielesh *et al.*, 2017). Therefore, KISS1R expression in

https://doi.org/10.1017/S096719942300059X Published online by Cambridge University Press

the oocytes and granulosa cells appears to regulate the preovulatory surge of gonadotrophins, which could promote oocyte survival in the later stages of follicular growth (Dorfman *et al.*, 2014). As LH is one of the hormones supplemented to IVM medium, the high expression of KISS1R in the oocytes subjected to *in vitro* maturation could be attributed to the LH-dependent matrix metalloproteinase (MMP) activity, which partly supports the KISS1R expression (Shahed and Young, 2009; Liu *et al.*, 2017) and the role of LH in the KISS1R expression ovaries as was reported previously (Roa *et al.*, 2008a). Currently detailed quantitative investigations into the levels of gene expression of various factors related to kisspeptin in connection with ovulation and associated events of female reproduction in different stages of *in vivo* grown and *in vitro* cultured PFs in various culture media is under progress in our laboratory, and could provide a better understanding of the local role of kisspeptin in ovine reproduction.

In the present study, although the pattern of expression of KISS1R was similar among different development stages of *in vivo* developed and *in vitro* cultured ovarian follicles, quantitatively, the expression was better in the *in vivo* grown follicles. This suggests (1) the present culture conditions may not support normal expression, or (2) *in vivo* secretion of KP might be higher as observed in pig and human (Saadeldin *et al.*, 2012; Singh *et al.*, 2021) requiring higher concentration of homologous receptors. These facets would form interesting subjects for future studies.

In conclusion, KP has a direct influence on the growth of ovarian follicles through the stimulation of the expression of homologous receptors. It could also have a synergistic/additive effect with other growth factors and hormones in stimulating the growth of PFs under *in vitro* culture.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S096719942300059X

Acknowledgements. This work was supported by a research grant from the Science and Engineering Research Board (DST No. EMR/2017/000851) to A.V.N. Siva Kumar.

Competing interests. We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

References

Arunakumari, G., Shanmugasundaram, N. and Rao, V. H. (2010). Development of morulae from the oocytes of cultured sheep preantral follicles. *Theriogenology*, **74**(5), 884–894. doi: 10.1016/j.theriogenology.2010.04.013

Arunakumari, G., Amin, R. U., Sadasiva Rao, K., Teja, A. and Ramesh, T. (2013). Effects of isolation methods and different concentrations of hormones and growth factors on in vitro development of preantral follicles from goat fetuses. *International Journal of Recent Scientific Research*, **4**, 259–265.

Babitha, V., Yadav, V. P., Chouhan, V. S., Hyder, I., Dangi, S. S., Gupta, M., Khan, F. A., Taru Sharma, G. T. and Sarkar, M. (2014). Luteinizing hormone, insulin like growth factor-1, and epidermal growth factor stimulate vascular endothelial growth factor production in cultured bubaline granulosa cells. *General and Comparative Endocrinology*, **198**, 1–12. doi: 10.1016/j.ygcen.2013.12.004

Blasco, V., Pinto, F. M., Fernández-Atucha, A., Prados, N., Tena-Sempere, M., Fernández-Sánchez, M. and Candenas, L. (2019). Altered expression of the kisspeptin/KISS1R and neurokinin B/NK3R systems in mural granulosa and cumulus cells of patients with polycystic ovarian syndrome. *Journal of Assisted Reproduction and Genetics*, **36**(1), 113–120. doi: 10.1007/s10815-018-1338-7

Blasco, V., Pinto, F. M., Fernández-Atucha, A., González-Ravina, C., Fernández-Sánchez, M. and Candenas, L. (2020). Female infertility is associated with an altered expression of the neurokinin B/neurokinin B receptor and kisspeptin/kisspeptin receptor systems in ovarian granulosa and cumulus cells. *Fertility and Sterility*, **114**(4), 869–878. doi: 10.1016/j.fertnstert.2020.05.006

Byri, P., Gangineni, A., Reddy, K. R. and Raghavender, K. B. P. (2017). Effect of kisspeptin on in vitro maturation of sheep oocytes. *Veterinary World*, **10**(3), 276–280. doi: 10.14202/vetworld.2017.276-280

Castellano, J. M., Gaytan, M., Roa, J., Vigo, E., Navarro, V. M., Bellido, C., Dieguez, C., Aguilar, E., Sánchez-Criado, J. E., Pellicer, A., Pinilla, L., Gaytan, F. and Tena-Sempere, M. (2006). Expression of KiSS-1 in rat ovary: Putative local regulator of ovulation? *Endocrinology*, **147**(10), 4852–4862. doi: 10.1210/en.2006-0117

Cielesh, M. E., McGrath, B. M., Scott, C. J., Norman, S. T. and Stephen, C. P. (2017). The localization of kisspeptin and kisspeptin receptor in the canine ovary during different stages of the reproductive cycle. *Reproduction in Domestic Animals*, **52** Suppl. 2, 24–28. doi: 10.1111/rda.12841

Clarke, H., Dhillo, W. S. and Jayasena, C. N. (2015). Comprehensive review on kisspeptin and its role in reproductive disorders. *Endocrinology and Metabolism*, **30**(2), 124–141. doi: 10.3803/EnM.2015.30.2.124

d'Anglemont de Tassigny, X., Fagg, L. A., Carlton, M. B. and Colledge, W. H. (2008). Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. *Endocrinology*, **149**(8), 3926–3932. doi: 10.1210/en.2007-1487

Dorfman, M. D., Garcia-Rudaz, C., Alderman, Z., Kerr, B., Lomniczi, A., Dissen, G. A., Castellano, J. M., Garcia-Galiano, D., Gaytan, F., Xu, B., Tena-Sempere, M. and Ojeda, S. R. (2014). Loss of Ntrk2/Kiss1r signaling in oocytes causes premature ovarian failure. *Endocrinology*, **155**(8), 3098–3111. doi: 10.1210/en.2014-1111

Fabová, Z. and Sirotkin, A. V. (2021). Interrelationships between kisspeptin and FSH in control of porcine ovarian cell functions. *Domestic Animal Endocrinology*, **74**, 106520. doi: 10.1016/j.domaniend.2020.106520

Fernandois, D., Cruz, G., Na, E. K., Lara, H. E. and Paredes, A. H. (2017). Kisspeptin level in the aging ovary is regulated by the sympathetic nervous system. *Journal of Endocrinology*, **232**(1), 97–105. doi: 10.1530/JOE-16-0181

García-Ortega, J., Pinto, F. M., Fernández-Sánchez, M., Prados, N., Cejudo-Román, A., Almeida, T. A., Hernández, M., Romero, M., Tena-Sempere, M. and Candenas, L. (2014). Expression of neurokinin B/NK3 receptor and kisspeptin/KISS1 receptor in human granulosa cells. *Human Reproduction*, **29**(12), 2736–2746. doi: 10.1093/humrep/deu247

Gaytán, F., Gaytán, M., Castellano, J. M., Romero, M., Roa, J., Aparicio, B., Garrido, N., Sánchez-Criado, J. E., Millar, R. P., Pellicer, A., Fraser, H. M. and Tena-Sempere, M. (2009). KiSS-1 in the mammalian ovary: Distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. *American Journal of Physiology. Endocrinology and Metabolism*, **296**(3), E520–E531. doi: 10.1152/ajpendo.90895.2008

Horikoshi, Y., Matsumoto, H., Takatsu, Y., Ohtaki, T., Kitada, C., Usuki, S. and Fujino, M. (2003). Dramatic elevation of plasma metastin concentrations in human pregnancy: Metastin as a novel placenta-derived hormone in humans. *Journal of Clinical Endocrinology and Metabolism*, **88**(2), 914–919. doi: 10.1210/jc.2002-021235

Hsueh, A. J., Billig, H. and Tsafriri, A. (1994). Ovarian follicle atresia: A hormonally controlled apoptotic process. *Endocrine Reviews*, **15**(6), 707–724. doi: 10.1210/edrv-15-6-707

Inoue, N., Hirano, T., Uenoyama, Y., Tsukamura, H., Okamura, H. and Maeda, K. I. (2009). Localization of kisspeptin and gonadotropin-releasing hormone (GnRH) in developing ovarian follicles of pigs and goats. *Biology of Reproduction*, 81(**1**) Suppl. 1, 551. doi: 10.1093/biolreprod/81.s1.551

Jayasena, C. N., Abbara, A., Comninos, A. N., Nijher, G. M., Christopoulos, G., Narayanaswamy, S., Izzi-Engbeaya, C., Sridharan, M., Mason, A. J., Warwick, J., Ashby, D., Ghatei, M. A., Bloom, S. R., Carby, A., Trew, G. H. and Dhillo, W. S. (2014). Kisspeptin-54 triggers egg maturation in women undergoing in vitro fertilization. *Journal of Clinical Investigation*, **124**(8), 3667–3677. doi: 10.1172/JCI75730

Khamsi, F. and Roberge, S. (2001). Granulosa cells of the cumulus oophorus are different from mural granulosa cells in their response to gonadotrophins

and IGF-I. *Journal of Endocrinology*, **170**(3), 565–573. doi: 10.1677/joe.0.1700565

Kona, S. S., Praveen Chakravarthi, V., Siva Kumar, A. V., Srividya, D., Padmaja, K. and Rao, V. H. (2016). Quantitative expression patterns of GDF9 and BMP15 genes in sheep ovarian follicles grown in vivo or cultured in vitro. *Theriogenology*, **85**(2), 315–322. doi: 10.1016/j.theriogenology.2015.09.022

Kona, S. S. R., Kumar, A. S., Punyakumari, B., Kumar, R. S. and Rao, V. H. (2021) Influence of TCM 199B, α-MEM, Waymouth MB 752/1 culture medium, VEGF, Estradiol-17β, GDF-9 and FGF on in vitro development of preantral follicles in sheep. *Veterinary and Animal Science*, **13**, 100189.

Kumar, P. A., Sivakumar, A. V. N., Pathipati, D., Chakravarthi, V. P., Brahmaiah, K. V. and Rao, V. H. (2019). Leptin induced in vitro development of ovarian follicles in sheep is related to the expression of P450 aromatase and steroidogenesis. *Theriogenology*, **136**, 1–6. doi: 10.1016/j.theriogenology.2019.06.017

Laoharatchatathanin, T., Terashima, R., Yonezawa, T., Kurusu, S. and Kawaminami, M. (2015). Augmentation of metastin/kisspeptin mRNA expression by the proestrous luteinizing hormone surge in granulosa cells of rats: Implications for luteinization. *Biology of Reproduction*, **93**(1), 15. doi: 10.1095/biolreprod.115.127902

Lapatto, R., Pallais, J. C., Zhang, D., Chan, Y. M., Mahan, A., Cerrato, F., Le, W. W., Hoffman, G. E. and Seminara, S. B. (2007). Kiss1−/− mice exhibit more variable hypogonadism than Gpr54−/− mice. *Endocrinology*, **148**(10), 4927–4936. doi: 10.1210/en.2007-0078

Lee, D. K., Nguyen, T., O'Neill, G. P., Cheng, R., Liu, Y., Howard, A. D., Coulombe, N., Tan, C. P., Tang-Nguyen, A. T., George, S. R. and O'Dowd, B. F. (1999). Discovery of a receptor related to the galanin receptors. *FEBS Letters*, **446**(1), 103–107. doi: 10.1016/s0014-5793(99)00009-5

Levine, J. E. (2015). Chapter 26. *Neuroendocrine control of the ovarian cycle of the Rat Knobil and Neill's physiology of reproduction* (4th edn) (pp. 1199–1257). Academic Press.

Liu, H., Xu, G., Yuan, Z., Dong, Y., Wang, J. and Lu, W. (2017). Effect of kisspeptin on the proliferation and apoptosis of bovine granulosa cells. *Animal Reproduction Science*, **185**, 1–7. doi: 10.1016/j.anireprosci.2017.07.008

Magamage, M. P. S., Sathagopam, S., Avula, K., Madushanka, D. N. N. and Velmurugan, S. (2021). Kisspeptin regulates the development of caprine primordial follicles in vitro. *Journal of Animal Reproduction and Biotechnology*, **36**(1), 51–58. doi: 10.12750/JARB.36.1.51

Mamo, S., Gal, A. B., Bodo, S. and Dinnyes, A. (2007). Quantitative evaluation and selection of reference genes in mouse oocytes and embryos cultured in vivo and in vitro. *BMC Developmental Biology*, **7**, 14. doi: 10.1186/1471-213X-7-14

Maranesi, M., Petrucci, L., Leonardi, L., Bufalari, A., Parillo, F., Boiti, C. and Zerani, M. (2019). Kisspeptin/kisspeptin receptor system in pseudopregnant rabbit corpora lutea: Presence and function. *Scientific Reports*, **9**(1), 5044. doi: 10.1038/s41598-019-41623-1

Merhi, Z., Thornton, K., Bonney, E., Cipolla, M. J., Charron, M. J. and Buyuk, E. (2016). Ovarian kisspeptin expression is related to age and to monocyte chemoattractant protein-1. *Journal of Assisted Reproduction and Genetics*, **33**(4), 535–543. doi: 10.1007/s10815-016-0672-x

Mishra, G. K., Patra, M. K., Singh, L. K., Upmanyu, V., Chakravarti, S., M, K., Singh, S. K., Das, G. K., Kumar, H. and Krisnaswami, N. (2019). Kiss1 and its receptor: Molecular characterization and immunolocalization in the hypothalamus and corpus luteum of the buffalo. *Animal Biotechnology*, **30**(4), 342–351. doi: 10.1080/10495398.2018.1520715

Peng, J., Tang, M., Zhang, B. P., Zhang, P., Zhong, T., Zong, T., Yang, B. and Kuang, H. B. (2013). Kisspeptin stimulates progesterone secretion via the Erk1/2 mitogen-activated protein kinase signaling pathway in rat luteal cells. *Fertility and Sterility*, **99**(5), 1436–1443.e1. doi: 10.1016/j.fertnstert.2012.12.008

Rao, B. S., Naidu, K. S., Amarnath, D., Vagdevi, R., Rao, A. S., Brahmaiah, K. V. and Rao, V. H. (2002). In vitro maturation of sheep oocytes in different media during breeding and non-breeding seasons. *Small Ruminant Research*, **43**(1), 31–36. doi: 10.1016/S0921-4488(01)00254-1

Ricu, M. A., Ramirez, V. D., Paredes, A. H. and Lara, H. E. (2012). Evidence for a celiac ganglion-ovarian kisspeptin neural network in the rat: Intraovarian anti-kisspeptin delays vaginal opening and alters estrous cyclicity. *Endocrinology*, **153**(10), 4966–4977. doi: 10.1210/en.2012-1279

Roa, J., Vigo, E., Castellano, J. M., Gaytan, F., García-Galiano, D., Navarro, V. M., Aguilar, E., Dijcks, F. A., Ederveen, A. G., Pinilla, L., van Noort, P. I. and Tena-Sempere, M. (2008a). Follicle-stimulating hormone responses to kisspeptin in the female rat at the preovulatory period: Modulation by estrogen and progesterone receptors. *Endocrinology*, **149**(11), 5783–5790. doi: 10.1210/en.2008-0604

Roa, J., Aguilar, E., Dieguez, C., Pinilla, L. and Tena-Sempere, M. (2008b). New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. *Frontiers in Neuroendocrinology*, **29**(1), 48–69. doi: 10.1016/j.yfrne.2007.07.002

Saadeldin, I. M., Koo, O. J., Kang, J. T., Kwon, D. K., Park, S. J., Kim, S. J., Moon, J. H., Oh, H. J., Jang, G. and Lee, B. C. (2012). Paradoxical effects of kisspeptin: It enhances oocyte in vitro maturation but has an adverse impact on hatched blastocysts during in vitro culture. *Reproduction, Fertility, and Development*, **24**(5), 656–668. doi: 10.1071/RD11118

Shahed, A. and Young, K. A. (2009). Differential ovarian expression of KiSS-1 and GPR-54 during the estrous cycle and photoperiod induced recrudescence in Siberian hamsters (Phodopus sungorus). *Molecular Reproduction and Development*, **76**(5), 444–452. doi: 10.1002/mrd.20972

Singh, A., Lal, B., Parkash, J. and Millar, R. P. (2021). Gametogenic and steroidogenic action of kisspeptin-10 in the Asian catfish, Clarias batrachus: Putative underlying mechanistic cascade. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology*, **256**, 110642. doi: 10.1016/j.cbpb.2021.110642

Srividya, D., Praveen Chakravarthi, V., Kona, S., Siva Kumar, A., Brahmaiah, K. V. and Rao, V. H. (2017) Expression of kit ligand and insulin-like growth factor binding protein 3 during in vivo or in vitro development of ovarian follicles in sheep. *Reproduction in Domestic Animals*, **52**(4), 661–671. doi: 10.1111/rda.12965

Tanyapanyachon, P., Amelkina, O. and Chatdarong, K. (2018). The expression of kisspeptin and its receptor in the domestic cat ovary and uterus in different stages of the ovarian cycle. *Theriogenology*, **117**, 40–48. doi: 10.1016/j.theriogenology.2018.05.019

Xiao, Y., Ni, Y., Huang, Y., Wu, J., Grossmann, R. and Zhao, R. (2011). Effects of kisspeptin-10 on progesterone secretion in cultured chicken ovarian granulosa cells from preovulatory (F1-F3) follicles. *Peptides*, **32**(10), 2091–2097. doi: 10.1016/j.peptides.2011.09.001

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