Effect of different concentrations of kisspeptin on in vitro maturation rate of buffalo oocytes

Kolipaka Rajesh, B Swathi, G Aruna Kumari and M Shanmugam

Abstract
Culture grade oocytes collected from slaughtered healthy buffaloes were randomly allotted to 4 treatment (T) groups containing oocyte maturation media and were allowed to mature at 38.5 °C and 5% CO₂ in humidified atmosphere for 22 hours. Tissue Culture Medium 199 (TCM 199B), supplemented with Gentamicin was used as control (T1); while for groups T2, T3 and T4 kisspeptin (Kp) was supplemented to control media at 5 µg/ml, 10 µg/ml and 15 µg/ml respectively. The oocytes were evaluated based on the percentages of Cumulus Cell Expansion (CCE) and 1st Polar Body extrusion (PB). Addition of Kp to the maturation media observed that the proportion of oocytes showing CCE and PB was significantly (P<0.5) higher when compared to the control. Among all the three concentrations of Kp, the proportion of oocytes showing the percentage of CCE and PB was highest in T1 (73.21% and 22.11%, respectively) compared to the T2 and T3. Inferring that 10 µg/ml concentration of Kp would yield optimum in vitro maturation of buffalo oocytes.

Keywords: Kisspeptin, in vitro maturation (IVM), Buffalo oocytes, Cumulus Cell Expansion (CCE), 1st Polar Body extrusion (PB)

Introduction
Kisspeptin (Kp) plays an important role in controlling GnRH release. It is thought to be involved in all phases of reproductive life and hence has attracted the interest of many reproductive neuro-endocrinologists (Caraty and Franceschini, 2008) [4]. Kisspeptin appears to be involved in onset of puberty, initiation of breeding season and dynamic changes of gonadotropin secretion throughout the oestrous cycle, pregnancy and implantation (Clarke et al., 2015 and Decourt et al., 2016) [12, 13]. Exogenous administration of Kp evidently stimulated gonadotropin release in many species, including monkey (Ramaswamy et al., 2007) [23], mare (Wilborn, 2008) [29], pig (Lents et al., 2008) [16], bovine (Whitlock et al., 2011; Naniwa et al., 2013) [28, 19], ewe, canine (Albers-Wolthers et al., 2014) [3], woman (Jayasena et al., 2014) [14] which is ultimately responsible for the maturation and ovulation of oocyte.

Genes encoding Kp and its receptors (KISS1 and KISS1R) have been documented in the ovaries of rat (Terao et al., 2004 and Roa et al., 2007) [26, 21], mouse (Castellano et al., 2006) [5], fish (Filby et al., 2008 and Elakkanai et al., 2015) [10, 9], hamster (Shahed and Young, 2009) [24], pig and goat (Inoue et al., 2009) [13], primate and human ovaries (Gaytan et al., 2009; Hameed et al., 2011 and Cejudo Roman et al., 2012) [11, 12, 6]. This indicates that Kp may also have direct gonadal effects and may interact with metabolic pathways (Clarke et al., 2015) [12]. The presence of Kp at higher concentrations in porcine follicular fluid than in serum reveals the involvement of Kp during follicular development and suggests an intra-follicular or systemic origin of action (Saadeldin et al., 2012) [23]. Very few in vitro studies have been conducted to see the effect of Kp on in vitro maturation (IVM) and fertilization (IVF) of oocytes in farm animals. The expression of genes encoding Kp and its receptors was detected during the IVM period in both oocytes and cumulus cells of pig (Saadeldin et al., 2012) [23] bovine (Ming et al., 2015) [18] suggesting that the supplementation of Kp in IVM media may improve the oocyte maturation rates in pigs and bovines. Saadeldin et al., 2012 [23] further opined that Kp may have continuous and direct action on oocytes and cumulus cells in an autocrine-paracrine fashion. However literature in buffalo is limited. Hence the present study was taken up to know the effect of kisspeptin on in vitro maturation rate of buffalo oocytes.

Material and methods
Chemicals and media
All media, hormones and chemicals were sourced from Sigma Chemical Co., USA, and plastic...
ware was from Nunc, Denmark, Kp-10 (TyrAsn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH2) was purchased from Auropeptides, Hyderabad, India. HEPES buffered tissue culture medium 199 supplemented with 10% PBS (Handling medium) was used for washing and handling of oocytes. Heparin (25 IU/ml) was additionally added to the handling medium for collection of oocytes. Bicarbonate buffered tissue culture medium 199 (TCM199B) supplemented with gentamicin (50 µg/ml) was used as control medium for maturation of oocytes. The media used for transport and washing of ovaries, collection, handling, and maturation of oocytes were supplemented with gentamicin (50 µg/ml) and filter sterilized (0.22 µm) before use. Handling, collection, and maturation media were equilibrated with 5% carbon dioxide in air, in a humidified atmosphere at 38.5 °C for at least 2 h before use.

Oocyte collection and in vitro maturation
Collection and processing of ovaries for aspiration of cumulus oocyte complexes (COCs) were carried out as described by Shahid et al., 2014 [25]. The ovaries were washed with phosphate buffered saline (PBS P 4417, Sigma USA) and kept in sterile polythene sacs containing warm (37 °C) PBS. These sacs were transported to the laboratory within 1-2 h after collection in a thermos flask containing warm water (37 °C). External surface of the each ovary was sterilized by rinsing once in 70% alcohol and thrice in D-PBS. The COCs were aspirated aseptically from the follicles of >6 mm diameter present on the surface of the ovary by using 18 G needle attached to 5 ml disposable syringe containing 2 ml of collection medium. The COCs having homogenous cytoplasm and surrounded by more than three layers of compact cumulus cells were considered as good quality oocytes (Fig-A). The isolated COCs were then washed three times in 100 µl droplets of handling medium. Subsequently, the 8-10 COCs were transferred into 50 µl droplets of different IVM media in 35 mm culture dishes. The droplets were overlaid with autoclaved pre-equilibrated mineral oil. The dishes with COCs were cultured in an incubator with 5% CO2 under humidified air at 38.5 °C for 22 hours.

Experimental design
To identify the optimal concentration of kisspeptin for in vitro maturation of buffalo oocytes, COCs were matured in control medium supplemented with three different concentrations of Kp 5, 10 and 15µg/ml in T2, T3 and T4 respectively. TCM 199 supplemented with Gentamicin was served as control (Table-1).

Evaluation of in vitro matured oocytes
At the end of in vitro maturation, the COCs were examined for cumulus cell expansion (CCE) (Fig-B). After evaluation of the CCE the COCs were placed in 200 µl of hyaluronidase (100 IU/ml) solution and incubated for 15 minutes at 37°C and then washed twice in handling medium. The cumulus cells were denuded off the oocytes (Fig-C) by repeated pipetting through a fine bore glass capillary which were examined through phase contrast microscope (TH4- 200, Olympus, Japan) for presence of first polar body (Fig-D) in the perivitelline space.

Statistical analysis
The percentage of oocytes exhibiting Cumulus cell expansion and extrusion of first polar body were analyzed by ANOVA (SPSS, v. 17.0). Duncan’s Multiple Range test was used to test the significant difference between control and treatment groups respectively.

Results and discussion
Buffalo oocytes were cultured in media containing TCM 199 as control (T1), while for T2, T3 and T4 Kp was added at 5µg/ml, 10µg/ml and 15µg/ml respectively. Based on the percentages of CCE and PB, Kp concentration for IVM of buffalo oocytes has been standardized. All the three Kp supplemented media showed significantly (P<0.05) higher proportion of CCE and PB compared to the control. Among the three Kp treated media viz., T2, T3 and T4, COCs were significantly highest (73.21%) in T3 medium supplemented with 10µg/ml of Kp followed by T1 (68.77%) and T2 (65.32%). Similar trend was observed with PB extrusion with the significantly highest value (22.11%) in T3 compared to other treatment groups T4 (16.32%) and T2 (11.70%) supplemented with Kp at 15 and 5 µg/ml respectively (Table No-2). TCM 199 supplemented with 10 µg/ml of Kp was found to be the optimum concentration for IVM of buffalo oocytes based on CCE and PB extrusion percentage. The present findings were in accordance with that obtained by Ming et al., 2015 [18] in buffalo and Byrt et al., 2017 [3] in ovine oocytes, who reported improved maturation rates of oocytes in TCM-199 media supplemented with 10 nM (13 µg) of Kp. On contrary, supplementation of pig oocyte maturation medium with Kp did not result in extrusion of PB (0%) as reported by Saadeldin et al., 2012 [23]. The local action of Kp on cumulus cells and oocytes might be species specific. For example, in bovines (Kadokawa et al., 2008) [15] and mares (McGrath et al., 2016) [14], Kp increased LH secretions. In ovariectomized sheep, the increased LH response to Kp administration was decreased by administration of Kp antagonist (p234) (Roseweir et al., 2009) [22], whereas in dogs it was not altered by Kp antagonist (p271) (Albers-Wolthers et al., 2016) [1]. In the present study, the percentage of oocyte maturation has increased significantly by increasing Kp concentration from 5 to10 µg/ml, but decreased at 15µg/ml suggesting that 10 µg/ml concentration of Kp would yield optimum in vitro maturation of buffalo oocytes.

Conclusion
Inferring that 10 µg/ml concentration of Kp would yield optimum in vitro maturation of buffalo oocytes.

Table 1: Details of different Kp concentrations (Treatments: T1-T4) used in the experiment.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>IVM Media Treatments</th>
<th>composition of different IVM media combinations</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>T1</td>
<td>Control medium (TCM 199B + Gentamicin sulfate (50 µg/ml))</td>
</tr>
<tr>
<td>2</td>
<td>T2</td>
<td>Control medium + Kp (5 µg/ml)</td>
</tr>
<tr>
<td>3</td>
<td>T3</td>
<td>Control medium + Kp (10 µg/ml)</td>
</tr>
<tr>
<td>4</td>
<td>T4</td>
<td>Control medium + Kp (15 µg/ml)</td>
</tr>
</tbody>
</table>
Table 2: Efficacy of different concentrations of Kp on In Vitro maturation of buffalo oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of Kp (µg/ml)</th>
<th>No. of Oocytes/Replicates</th>
<th>Percentage of Cumulus Cell Expansions (CCE)</th>
<th>Percentage of Extrusion of 1st Polar body (PB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Control (TCM199)</td>
<td>150/6</td>
<td>61.26±0.56*</td>
<td>5.23±0.65*</td>
</tr>
<tr>
<td>T2</td>
<td>Kp-5</td>
<td>150/6</td>
<td>65.32±0.49b</td>
<td>11.70±0.45b</td>
</tr>
<tr>
<td>T3</td>
<td>Kp-10</td>
<td>150/6</td>
<td>73.21±1.74d</td>
<td>22.11±0.60d</td>
</tr>
<tr>
<td>T4</td>
<td>Kp-15</td>
<td>150/6</td>
<td>68.77±0.58c</td>
<td>16.32±0.81c</td>
</tr>
</tbody>
</table>

Values with different superscripts within the column are significantly different. One way ANOVA followed by Duncan’s multiple range tests (P≤0.05) KP=Kisspeptin, CCE=Cumulus cell expansion, PB=Polar body.

References

13. Inoue N, Hirano T, Uenoyama Y, Tsukamura X,


