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

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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 ºC and 5% CO_2 in humidified atmosphere for 22 hours. Tissue Culture Medium 199 (TCM 199B), supplemented with Gentamicin was used as control (T_1), while for groups T_2, T_3 and T_4 kisspepetin (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 ($P \leq 0.5$) 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 T_3 (73.21% and 22.11%, respectively) compared to the T_2 and T_4. 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) [7, 8]. Exogenous administration of Kp evidently stimulated gonadotropin release in many species, including monkey (Ramaswamy *et al*., 2007) [20], 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) [2], 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) [7].
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 sachets containing warm (37 °C) PBS. These sachets were transported to the laboratory within 1-2 hr 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 T_2, T_3 and T_4 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 (T_1), while for T_2, T_3 and T_4 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 ($P \leq 0.05$) higher proportion of CCE and PB compared to the control. Among the three Kp treated media *viz.*, T_2, T_3 and T_4, COCs were significantly highest (73.21%) in T_3 medium supplemented with 10µg/ml of Kp followed by T_4 (68.77%) and T_2 (65.32%). Similar trend was observed with PB extrusion with the significantly highest value (22.11%) in T_3 compared to other treatment groups T_4 (16.32%) and T_2 (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 Byri *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 contraries, 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) [17], 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: T_1-T_4) used in the experiment.

S. No.	IVM Media Treatments	composition of different IVM media combinations
1	T_1	Control medium (TCM 199B + Gentamicin sulfate (50 µg/ml))
2	T_2	Control medium + Kp (5 µg/ml)
3	T_3	Control medium + Kp (10 µg/ml)
4	T_4	Control medium + Kp (15 µg/ml)

Table 2: Efficacy of different concentrations of Kp on *In Vitro* maturation of buffalo oocytes

Treatment	Concentration of Kp (µg/ml)	No. of Oocytes/ Replicates	Percentage of Cumulus Cell Expansions(CCE)	Percentage of Extrusion of 1st Polar body (PB)
T1	Control(TCM199)	150/6	61.26±0.56[a]	5.23±0.65[a]
T2	Kp-5	150/6	65.32±0.49[b]	11.70±0.45[b]
T3	Kp-10	150/6	73.21±0.76[d]	22.11±0.60[d]
T4	Kp-15	150/6	68.77±0.58[c]	16.32±0.81[c]

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

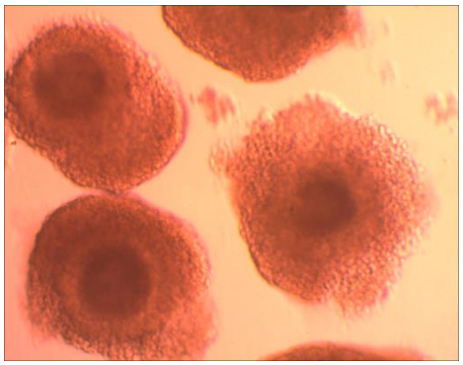

Fig A: Grade -I Oocyte

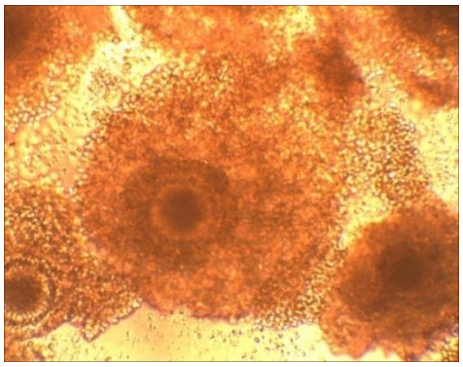

Fig B: Cumulus Cell Full Expansion

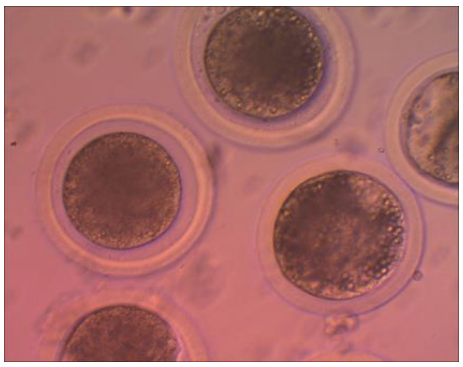

Fig C: Pool of Oocytes Post- Denaturation

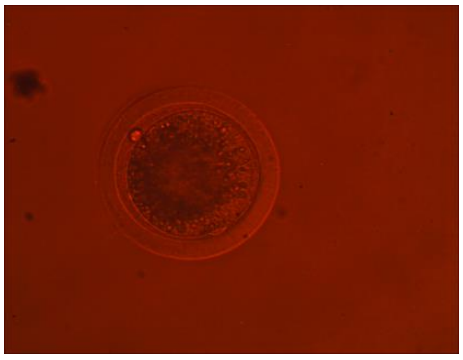

Fig D: Oocyte with Polar Body

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