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Nuclear and Cytoplasmic Maturation of *In Vitro* Matured Denuded Oocytes in the Presence of Gonadotrophins, Oestradiol and Insulin in Nili Ravi Buffalo

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ABSTRACT

The lower recovery of competent oocytes in buffalo species limits the commercialization of *in vitro* embryo production technology in field conditions. The present study aimed to improve the cytoplasmic and nuclear maturation of *in vitro* matured oocytes of Nili Ravi buffalo in the presence of hormones. The denuded oocytes (DOs) obtained by repeated pipetting were collected from 2-8mm follicles. A series of experiments were conducted to evaluate the effects of oestradiol (2 µg/ml), recombinant human follicle stimulating hormone (0.05 IU/ml), human chorionic gonadotrophin (2 IU/ml) and insulin (0.12 IU/ml) alone or together in different combinations at intervals of 8, 16 and 24 h of incubation period during *in vitro* maturation on buffalo oocyte. The supplementation of TCM-199 with E_2+rhFSH showed a highly significant increase in the diameter and maturation of oocytes ($P<0.0001$), as a greater number of the oocytes progressed to the metaphase II stage, and a lower proportion of the oocytes became degenerated ($P<0.0001$) after 24 h of incubation. The supplementation of medium with E_2+rhFSH+hCG or E_2+rhFSH+hCG+insulin also showed a significant increase in the meiotic maturation rate after 24 h ($P<0.01$ and $P=0.04$ respectively) and a significant decrease in the degeneration of the oocytes ($P=0.001$). The addition of insulin was not found to be effective for *in vitro* maturation. It is concluded that the addition of E_2+rhFSH in culture media was found to be the best combination of hormones for *in vitro* maturation of denuded buffalo oocytes.

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Key words
Nuclear maturation, Oocytes, Nili Ravi buffalo, Gonadotrophins, Oestradiol

INTRODUCTION

The reproductive efficiency in livestock species can be improved by applying assisted reproductive technologies (ARTs) and has significant roles in the preservation of endangered species (Herrick, 2019). Recently, *in vitro* embryo production has increased rapidly with an average rate of 12% (Viana, 2019; Ferré *et al*., 2020). Despite the success of *in vitro* fertilization, embryo culture, and transgenesis, the *in vitro* systems still have a very low efficiency, with a small percentage of embryos produced from *in vitro* matured oocytes (El-Magd *et al*., 2019). Because of this limited success in bubaline species (Drost, 2007; Baruselli *et al*., 2013) the *in vitro* embryo production is the available alternative to support breeding program for the rapid propagation and upgradation of buffalo (Gasparrini, 2013; Saliba *et al*., 2013; Galli *et al*., 2014; Ferraz *et al*., 2015; Ohash *et al*., 2017). The maturation of oocytes that are cultured *in vitro,* is a technique of assisted reproduction. It involves the use of controlled ovarian hyperstimulation for *in vitro* fertilization through the collection of immature oocytes at the prophase I stage, which are then matured *in vitro* until they reach metaphase II (MII) stage (Paulson *et al*., 2016).

A period of 24 h is necessary for a bovine oocyte to complete nuclear maturation (Pioltine *et al*., 2021). The cytoplasmic maturation includes the progression of the oocyte from germinal vesicle (GV) to the next stage (MII) accompanied by a remarkable reorganization, concomitant with chromosome condensation and migration and the acquisition of developmental competence (Conti and Franciosi, 2018). Cytoplasmic maturation involves the

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synthesis, activation, and degradation of maternal mRNA (Sun *et al*., 2020). The addition of a maturation medium with bovine fetal calf serum and follicular fluid (Lopes *et al*., 2019) can affect the *in vitro* maturation of oocytes. Moreover, the subsequent fertilization and development can be enhanced by the selection of protein supplements and hormones (Wrenzycki and Stinshoff, 2013) such as gonadotropin (Widayati and Pangestu, 2020), steroids and insulin growth factor-I (Anisworth *et al*., 1980; Pereira *et al*., 2019). The nuclear maturation and blastocyst formation of sow oocytes during in-vitro culture was improved by the addition of follicle-stimulating hormone (FSH) in the culture medium (Lima *et al*., 2018). The presence of equine chorionic gonadotropin (eCG) improved the cleavage and developmental rates of in-vitro fertilized buffalo embryos (Abdoon *et al*., 2001).

The steroids hormone such as oestradiol enhanced the maturation of oocytes in buffalo (Anisworth *et al*., 1980; Abdoon *et al*., 2001). The proliferation of granulosa cells and the production of progesterone in bovine is stimulated by insulin and insulin-like growth factor-I (Gong *et al*., 1993; Spicer *et al*., 1993). The aim of this study was to optimize the culture medium for *in-vitro* maturation of Nili Ravi buffalo oocytes by supplementation with oestradiol (E_2), recombinant FSH, hCG and insulin (I). The objective was to determine the effect of hormones in different combinations on the nuclear and cytoplasmic maturation of denuded oocytes.

MATERIALS AND METHODS

Collection of ovaries

The ovaries used in this study were obtained from the slaughtered buffaloes, therefore there was no need of approval from the ethical committee of the university. Approximately 560 buffalo (*Bubalus bubalis*) ovaries were obtained from a local slaughterhouse, Sihala Rawalpindi immediately after slaughtering of 280 culled dairy buffaloes during the period of three months. All the culled buffaloes that seize producing milk having age 6-12 years were brought to slaughterhouse from the adjoining areas of Islamabad, Rawalpindi, and Punjab. Rectal palpation that is a part of routine procedure at slaughterhouse was done to remove any pregnant buffalo from the slat of slaughtering. The ovaries with cysts or any other abnormalities were discarded and only 400 ovaries with normal shape and visible follicles were selected. The ovaries were transported to the laboratory in an insulated flask containing sterile normal 0.9% saline and antibiotics (100 IU/ml penicillin G; 100 µg/ml streptomycin sulphate, Zafa Pharmaceuticals) at 38.5°C.

Recovery of oocytes

The oocytes were recovered from 2200 ovarian follicle (2-8 mm in diameter) by aspiration method using a 5 ml syringe with 16- gauge needle into a beaker containing supplemented oocyte culture medium (Shahid *et al*., 2014). The denuded oocytes with 48 % recovery rate were obtained by pipetting into the intergrid plate through pasteur pipette by removing most of cumulus layers. The oocytes with single layer of cumulus were selected. Oocytes were transferred to a drop of oocyte collection medium.

Preparation of culture media

Ten liter double distilled water (ddH_2O) was used to dissolve TCM -199 and 3.50 g $NaHCO_3$ by adjusting the pH at 7.2-7.4. The medium (400 ml) was sterile filtered and kept at 4°C. The medium was supplemented one day before use with 1 aliquot of bovine steer serum (BSS) + N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 aliquot of glutamine (4 ml), 0.4 ml of penicillin G (100 IU/ml) and 0.04 g of streptomycin sulphate (100 µg/ml) to the next leaving all debris behind.

In vitro *maturation of oocytes*

After oocytes had been cleaned of debris, a group of 10 oocytes was transferred a 50 µl micro drop of oocyte maturation medium (OMM; 4.45 ml aliquots of TCM-199 were prepared and stored at 4°C until use. On night before commencing *in-vitro* maturation, 500 µl of bovine serum albumen (BSA), 50 µl of gentamicin) supplemented with sodium pyruvate (0.23 mmol/l), BSA (4.0 mg/ml; Sigma), oestradiol (1 mg/100ml; Merck) and rhFSH (0.05 IU/ml; Organon) were added to an aliquot of TCM-199. The droplet of the maturation medium containing oocytes was covered with mineral oil and incubated at 38.5˚C with 5% of CO_2 for 24 h.

Experimental design

The nuclear and cytoplasmic maturation of DOs were assessed by examining the impact of E_2 (2 µg/ml; Maksura *et al*., 2021), rhFSH (0.05 IU/ml; Ullah *et al*., 2006), hCG (2 IU/ml) and insulin (0.12 IU/ml; Shahid *et al*., 2015) alone or in different combinations.

Group A (control) The DOs cultured in TCM-199 containing BSA, sodium pyruvate, gentamicin and without any addition of E_2, rhFSH, hCG and insulin considered as control.

Group B: The DOs cultured *in vitro* in TCM-199 + 2 µg/ml E_2.

Group C: The nuclear and cytoplasmic maturation of DOs was studied inTCM-199 containing 0.05 IU/ml rhFSH.

Group D: The DOs were cultured in TCM-199 containing 2 IU/ml hCG.

Group E: The effect of 0.12 IU/ml of beef insulin on nuclear and cytoplasmic maturation of DOs were studied in supplemented TCM-199.

Group F: The denuded oocytes were grown in TCM-199 containing E_2 and rhFSH.

Group G: The oocytes were matured in a medium-199 supplemented with E_2 and hCG.

Group H: The DOs were matured *in vitro* in TCM-199 containing E_2 and beef insulin.

Group I: The DOs were matured in a medium-199 containing rhFSH and hCG.

Group J: The DOs were cultured in medium-199 containing rhFSH and beef insulin.

Group K: The DOs were grown in a medium-199 containing hCG and beef insulin.

Group L: The DOs were cultured in medium-199 containing E_2, rhFSH and hCG.

Group M: The DOs were matured *in vitro* in TCM-199 supplemented with oestradiol, rhFSH, hCG and beef insulin.

Meiotic competence and oocyte expansion

The meiotic competence of the oocyte was examined using inverted phase contrast microscope (Nikon) at incubation times of 8, 16, and 24 h. Stages of nuclear maturation were classified into germinal vesicle (GV), germinal vesicle breakdown (GVBD), and metaphase II (MII) stages. In GV stage, the oocyte had a single prominent nucleus. In the GVBD stage, the oocyte underwent GVBD, but no polar body has been extruded. In MII stage, the oocyte had a polar body extrusion. The abnormal or degenerated oocytes displayed both multipolar meiotic spindle or abnormal chromatin clumps or no chromatin with fragmented or shrunken cytoplasm (Figure 1a-d; Dell'Aquila *et al*., 2003).

The oocyte growth (including zona pellucida) was assessed by measuring the diameter of the oocyte using an ocular micrometer calibrated stage micrometer by taking readings in two perpendicular directions, at the incubation time of 8, 16 and 24 h.

Statistical analysis

The mean diameter of DOs from zero to 24 h of culture period with different treatments was analyzed by two ways ANOVA. The overall expansion between control and various treatment groups was analyzed by ANOVA. The percentage of GVDB, MII and degenerated oocytes were compared among control and different treatment groups by analysis of variance and for comparing means, Tukey test was applied. The data were presented as mean±SEM and value of $P \leq 0.05$ was considered significant. All the statistical analysis was implemented using Graph Pad Prism version 6.

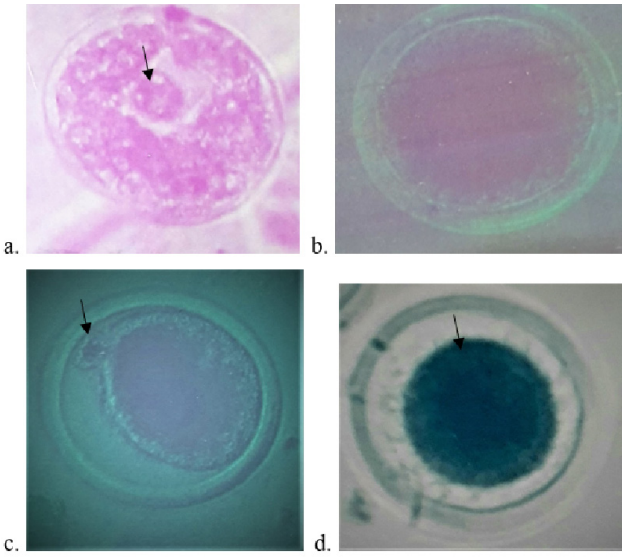

Fig. 1. Photomicrographs of fully grown Nili Ravi buffalo oocytes during *in vitro* culture: (a) An intact germinal vesicle (indicated by arrow) with a single prominent nucleus arrested in dictyate stage of the first meiotic prophase. (b) Oocyte undergoing GVBD showing complete dissolution of the GV with only a small remnant of the nucleolus. (c) MII oocyte arrested at metaphase II stage and omitted a polar body (indicated by arrow). (d) Degenerated oocyte showing shrinkage of ooplasm. Photographs were taken from an inverted phase contrast (Nikon) microscope (objectives: a-d, X20).

RESULTS

Effect of hormones on oocyte expansion

Effect of different hormones alone and in different combinations on the expansion (mean ± SEM) of cultured DOs for a period of 8, 16 and 24 h is presented in Table I. Two-way analysis of variance indicated that the effect of different treatment of hormones did not significantly affect the increase in diameter of denuded oocytes at different time periods. However, analysis of variance showed that all the hormones highly significantly ($P<0.0001$) increase the growth of denuded oocytes indicated by increase in diameter compared with control group.

Effect of hormones on nuclear maturation

The effect of hormones alone or in various combinations on the nuclear maturation of Nili Ravi buffalo denuded oocytes (DOs) cultured *in vitro* is given in Table II. The result showed that after 24 h of incubation

Table I. Effect of different hormones alone or in various combinations on the cytoplasmic maturation (mean ± SEM) of cultured denuded oocytes in Nili Ravi buffalo.

Treatment group	Diameter of denuded oocytes (µm)				
	0 h	8 h	16 h	24 h	Growth of the oocytes
Control (15)	155±2.95	154.5±2.62	152.66±2.39	152.0±2.25	0±0.7
Oestradiol (10)	153.25±1.75	156.25±1.63	156.50±2.01	157.5±1.86	4.25±0.11
rhFSH (10)	156.25±1.71	157.75±1.72	158.25±1.53	158.5±1.24	2.25±0.47
hCG (25)	159.5±1.56	159.1±1.87	158.4±1.38	157.1±1.43	0±0.13
Insulin (10)	156.25±1.63	155.75±2.44	155.0±2.47	153.75±2.77	0±1.14
E_2 and rhFSH (16)	151.87±1.0	152.65±1.05	153.75±1.14	156.25±1.44	4.38±0.44
E_2 and hCG (38)	156.97±1.12	157.03±1.33	157.63±1.07	159.14±1.32	2.17±0.2
E_2 and insulin (26)	157.01±1.67	157.21±1.22	157.5±1.10	158.07±1.28	1.06±0.39
rhFSH and hCG (43)	157.15±1.04	157.61±0.99	158.0±0.90	158.13±0.84	0.98±0.2
rhFSH and insulin (31)	158.30±1.69	157.41±1.36	157.0±1.57	156.29±1.32	0±0.37
hCG and insulin (37)	160.13±1.36	159.66±1.39	159.52±1.26	159.05±1.41	0±0.05
E_2, rhFSH and hCG (36)	156.52±1.06	156.94±1.09	158.61±1.16	158.81±1.17	2.29±0.11
E_2, rhFSH, hCG and insulin (34)	154.85±1.28	155.88±1.07	157.79±0.91	158.82±1.15	3.97±0.13

The mean growth of the denuded oocytes is the final mean diameter after maturation minus initial mean diameter of oocytes before maturation. Values in parenthesis ( )= Number of Dos.

Table II. Effect of different hormones alone or in various combinations on the nuclear maturation of Nili Ravi buffalo denuded oocytes (DOs) cultured in-vitro after 24 h of incubation.

Treatment	No. of DOs	Rate of nuclear maturation (%) (Mean ±SEM)		
		GVBD	MII	Degenerated
Control	22	$27.65±1.97^{a}$ (6)	$30.87±4.92^{a}$ (7)	41.48±2.95 (9)
E_2	41	26.84±1.57 (11)	$48.86±1.76^{a***}$ (20)	$24.30±3.25^{a***}$ (10)
rhFSH	44	22.71±1.43 (10)	$47.77±0.79^{a***}$ (21)	$29.53±1.4^{a***}$ (13)
hCG	25	24.06±0.66 (6)	$51.89±1.31^{a***}$ (13)	$24.06±0.66^{a***}$ (6)
Insulin	37	21.75±2.27 (8)	$43.18±1.06^{a*}$ (16)	$35.14±1.2^{b**d*}$ (13)
E_2+rhFSH	40	$9.92±1.56^{a,b***}$ (4)	$82.58±1.43^{a,b,c,d,e***}$ (33)	$7.51±0.13^{a,c,e***b,d**}$ (3)
E_2+hCG	38	23.71±0.45 (9)	$50.00±1.57^{a,f***}$ (19)	$26.29±1.63^{a,f***e*}$ (10)
E_2+Insulin	26	23.13±0.66 (6)	$50.35±4.26^{a,f***}$ (13)	$26.87±2.36^{a,f***}$ (7)
rhFSH+hCG	43	$16.33±1.81^{a*b**}$ (7)	$51.12±0.78^{a,f***e*}$ (22)	$32.54±1.48^{a*f***}$ (14)
rhFSH+Insulin	31	$35.46±2.07^{b*c,e***}$ (11)	$35.46±2.07^{b,c,d,f,g,h,i***}$ (11)	$29.08±0.64^{a**f***}$ (9)
hCG+Insulin	37	$35.07±1.21^{c,e***}$ (13)	$35.23±2.33^{b,c,d,f,g,h,i***}$ (13)	$29.71±1.74^{a**f***}$ (11)
E_2+rhFSH+hCG	36	$38.89±1.97^{a**b,c,d,e***}$ (14)	$50.0±0.00^{a,f,j,k***}$ (18)	$11.11±1.97^{a,c,e,i,j,k***b,d,g,h**}$ (4)
E_2+rhFSH+hCG+Insulin	34	32.33±1.89 (11)	$53.01±1.07^{a,f,j,k***e**}$ (18)	$14.66±1.99^{a,c,e,i,k***g,h,j*}$ (5)

Means with different superscripts within the same column differ significantly (*, P<0.05; **, P<0.001; ***, P<0.0001) and the figure in the parenthesis indicates the total number of oocytes.

the percentage of oocytes progressed to MII stage was significantly higher in all treatments groups except treatment of the culture media with rhFSH+insulin and hCG+insulin compared with control group. The degeneration was significantly lower (P<0.001) in all treatment groups except insulin group compared with control group.

The maximum maturation (82.58±1.43%) and minimum degeneration (7.51±0.13%) was observed by the supplementation of E_2+rhFSH hormones in culture media

compared with all treatment groups (P<0.0001). Whare as, the addition of single hormone in culture media showed the lowest maturation rate (35.23±2.33%) compared with all treatment groups. The highest degeneration (35.14±1.20%) of oocytes was observed in culture media treated with insulin alone (P<0.001).

DISCUSSION

In current study numerous hormones were utilized with different combinations to discover which one could improve the increase in the diameter and nuclear maturation of Nili Ravi buffalo DOs cultured *in vitro*. The study revealed that the addition of oestradiol alone in a medium caused significant difference in the diameter of DOs and caused non-significant increase in the percentage of GVBD stage oocytes after 24 h of incubation. Whereas the percentage of MII stage oocytes increased significantly. However, the survival of DOs was enhanced, indicated by a significant decreased in percentage of degenerated oocytes compared to control. The present results in agreement with other findings in which it was reported that more prominent numbers of buffalo oocytes reached the MII stage with treatment of 1.5 µg/ml of estradiol compared with other groups (Maksura *et al*., 2021). Similarly, the addition of 1 mg/100 ml oestradiol in TCM-199, the percentage of oocytes undergoing germinal vesicle breakdown (GVBD), MII and degenerated oocytes at 24 h of incubation was 29%, 47.3% and 23.65% respectively in Nili Ravi buffalo oocytes (Khan *et al*., 2009). The maturation rate was enhanced by the supplementation of 1 µg/ml of oestradiol to the maturation medium. Whereas, in another study it was demonstrated that the supplementation of oestradiol in culture media had a negative effect on *in vitro* nuclear maturation of bovine denuded oocyte (Younis *et al*., 1989; Woudenberg *et al*., 2004). This discrepancy may reflect species-specific differences in the nuclear maturation mechanism.

The addition of rhFSH alone in the medium was observed to have a positive effect on the nuclear and cytoplasmic maturation of DOs, while percentage of degenerated oocytes was non-significantly decreased. These results agree with the study in which it was observed that FSH stimulated the resumption of meiosis in oocytes cultured as COC attached to membrana granulosa (COCGs) in the absence of thecal tissue (Van Tol *et al*., 1996). The FSH supplementation in culture medium stimulated the nuclear and cytoplasmic maturation during *in-vitro* grown of equine cumulus oocytes complexes (Tremoleda *et al*., 2003).

The present study revealed that hCG treatment was not found to improve the nuclear and cytoplasmic maturation of buffalo oocytes. Although the supplementation of hCG promotes oocytes maturation by reducing the synthesis of α-subunit of inhibin, thereby favouring secretion of β-subunit dimmers (Newton *et al*., 2002).

Our study indicated that supplementation of a medium with hormones in all combination showed a significant increase in the diameter of DOs after 8, 16 and 24 h of incubation time. However, all the combinations effect similarly, and no difference was observed within the treatment groups. When oocytes matured in the presence of bovine insulin and human insulin, no significant differences were observed between the two insulin supplemented oocyte groups (Ocaña-Quero *et al*., 1998).

In present study maximum nuclear maturation was achieved by the addition of E_2 and rhFSH in a medium after 24 h of incubation time. In this combination maximum number of oocytes progressed to MII stage. The present results are similar to another study in which it was observed that the addition of 1 mg/100ml oestradiol and 0.05 IU/ml rhFSH in TCM-199 enhanced the meiotic maturation of Nili Ravi buffalo oocytes (82.02% oocytes were progressed to MII stage). Although, 1 µg/ml of oestradiol in serum free medium TCM-199 clearly reduces the maturation rate of bovine oocytes, it also appeared that supplementation of the medium with FSH suppresses inhibition by oestradiol, restoring the percentage of MII oocytes to control levels (Beker *et al*., 2002). In the present study minimum percentage (7.5%) of degenerated oocytes was obtained with E_2 and FSH treatment. Our study agrees with who reported that after 24 h the percentage of degenerated oocytes of Nili Ravi buffalo was 7.8% in E_2+FSH combination (Khan *et al*., 2009).

Supplementation of E_2+hCG in medium showed decrease in percentage of GVBD oocytes and increase in percentage of MII oocytes but non-significantly. The number of MII stage oocytes were not increased during *in-vitro* culture of bovine oocytes by supplementation of oestrogen and human chorionic gonadotrophin did therefore in combination of E_2+hCG when hCG was replaced with insulin it had a significant effect on increase in the diameter and percentage of MII oocytes (Bahrami *et al*., 2019). The supplementation of recombinant LH to recombinant FSH could increase the meiotic development rate of oocytes significantly during *in vitro* (Cortvrindt *et al*., 1998).

In the present study addition of rhFSH and hCG together in a medium caused no significant difference in the cytoplasmic maturation of DOs till 8 h of incubation however, after 24 h a significant increase on meiotic maturation rate was observed. The present findings showed that the supplementation of a medium with rhFSH and insulin caused no change in the cytoplasmic maturation.

The presence of insulin, the addition of LH or FSH alone had no significant effect oocyte diameter expansion (Itoh *et al*., 2002). A non-significant decrease in the percentage of GVBD, MII and degenerated oocytes in the medium with rhFSH+insulin and hCG+insulin in the present study was observed.

The present study indicated that addition of E_2, rhFSH and hCG in a medium showed significant difference in the diameter of DOs. In case of nuclear maturation, there was non-significant decrease in the GVBD stage oocytes after 16 and 24 h compared to control. The percentage of MII stage oocytes was significantly (P=0.018) increased after 16 h and non-significantly increased after 24 h of incubation. When human oocytes matured in TC 199-S medium containing 1 µg/ml 17β-oestradiol, 0.075 IU/ml FSH, 0.5 IU/ml hCG and 10 ng/ml EGF, results found that overall mean diameter of oocytes was 111.0±6.6 µm (Cortvrindt *et al*., 1998). This shows that mean diameter of human DOs is smaller than buffalo oocytes. The present study showed 38.88% oocytes at GVBD stage and 50% oocytes at MII stage after 24 h of incubation. After 28 h the number of MII oocytes increased to 53.8%. In human oocytes cultured *in vitro* the resumption of meiosis occurred after 48 h in a similar percentage from both the FSH-treated (84.2%) and control (71.7%) groups. This shows that in human degeneration rate is higher than buffaloes while the percentage of MII oocytes is nearly same after 24 h of incubation.

This study shows that by the supplementation of TCM-199 with single hormones, the optimum maturation was obtained by oestradiol as degeneration was less by this hormone. By the addition of double hormones combination most of the oocytes reached to metaphase II stage, and lower proportion of the oocytes became degenerated. The highest increase in diameter of DOs and maturation rate was obtained with combination of E_2+rhFSH. However, maturation rate of DOs was not so much affected by addition of the E_2+rhFSH+hCG, but lower percentage of degenerated oocytes were observed. Similar results were found in combination of E_2+rhFSH+hCG+insulin.

In conclusion, the combination of E_2+rhFSH is most suitable to improve culture medium for nuclear maturation of the Nili Ravi buffalo oocytes cultured *in vitro*.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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