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Effect of *in vitro* culture on the expression of genes enhanced meiotic progression in Egyptian buffalo oocytes

Wagdy K. B. Khalil[1]*, Sally S. Alam[1], Ibrahim A. H. Barakat[2], Aziza M. Hassan[1], Karima F. Mahrous[1]

[1]Cell Biology Department, National Research Center, 12622 Dokki, Giza, Egypt
[2]Zoology Department, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Kingdom of Saudi Arabia
Corresponding author E-mail: wagdykh@yahoo.com Tel: +202-33252126; Mobile: +2012-7410600, Fax: +202-33370931

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ABSTRACT
Objective: Factors such as hormonal alterations and culture conditions can have a profound effect on oocyte quality and subsequently embryo development. Numerous efforts to identify candidate genes for the developmental competence of bovine oocytes have been made by employing different strategies. Gene expression techniques have become a powerful tool to analyze the relative abundance of transcripts related to quality of oocytes. In the present study, the relationship between mRNA expression profiles of Cyclin B, cell division control (CDC2), extracellular signal-regulated kinases (ERK2) and c-mos genes and time of *in vitro* culture of buffalo oocytes was evaluated. *Methodology and results:* To carry out this study 1899 Cumulus–oocyte complexes (COCs) aspirated from 709 buffalo ovaries were used. The results indicated that the relative abundance of Cyclin B, CDC2, ERK2 and *c-mos* was affected by the maturation time and meiotic progression. The expression level of all genes was significantly higher in buffalo oocytes after collection and gradually decreased after *in vitro* culture. *Conclusion and application of findings:* The time of culture was shown to regulate the mRNA translation to synthesize the maturation proteins which stimulate the buffalo oocytes to achieve meiotic progression up to the Metaphase II (MII) stage.
Key words: Buffalo; IVM; Oocytes; Cyclin B, CDC2, ERK2 and *c-mos* genes; RT-PCR

INTRODUCTION
In vitro embryo production (IVEP) technology represents the best tool to improve maternal contribution to genetic progress in buffalo, due to limitations with multiple ovulation programs in this species (Zicarelli, 1997). One of the important factors that might affect the IVEP efficiency is inadequate oocyte maturation (Gasparrini, 2002). It is known that the time necessary for accomplishment of maturation *in vitro* differs among species, varying from 18–24h in cattle (Ward et al., 2002; Park et al., 2005), to 36–48h in pig (Prather and Day, 1998). In buffalo, there are discrepancies regarding the *in vitro* oocyte maturation rate reported by different authors, with the highest proportion of MII oocytes observed between 15h (Neglia et al., 2001) and 24h (Gasparrini et al., 2008). Despite these conflicting data, the majority of the authors inseminate buffalo oocytes *in vitro* 24h after the start of *in vitro* maturation (IVM).

The developmental competence of oocytes may be a function of the presence or abundance of specific transcripts in their mRNA pools, since the earliest stages of embryogenesis in mammals and other

animals are regulated by maternally-inherited RNAs and proteins stored within the oocyte (Bachvarova, 1985). These pools of stored RNA are essential to ensure proper protein synthesis during transcriptional silencing of the bovine embryonic genome that occurs during meiotic maturation, fertilization, and the first embryonic cell divisions. The main transcripts produced during maturation of the cumulus oocyte complexes (COCs) encode regulators of the cell cycle: maturation promoting factor (MPF), *c-mos* proto-oncogene (MOS) protein, and mitogen-activated protein kinase (MAPK) (Calder et al., 2003). MPF is a protein complex composed of two subunits, (a) a catalytic subunit [$p34^{cdc2}$ kinase, a 34 kD protein]. Its gene is a homologue of the yeast gene CDC2 (cdc= cell division control); (b) a regulatory subunit [Cyclin B, a 45 kD protein]. Its gene is a homologue of the yeast cdc_{13} product (Gautier et al., 1990; Taieb et al., 1997). The MPF activity in oocytes has been well characterized by fusion experiments between meiotically incompetent and competent oocytes, demonstrating that active MPF from a competent oocyte can induce condensation of chromatin in the immature oocyte nucleus and reorganization of the cytoplasm (Motlik and Kubelka, 1990). Therefore, the ability of the oocyte to resume meiosis is associated with MPF activity (Yamashita et al., 2000).

The family of mitogen-activated protein (MAP) kinases (42 and 44 kD), also termed extracellular signal-regulated kinases (ERKs), has been shown to have an important role during the M-phase (Kubelka et al., 2002). The activation of MAP kinase occurs on the onset of oocyte maturation in a number of species including mouse (Gavin et al., 1994), cattle (Fissore et al., 1996), goat (Dedieu et al., 1996), and pig (Tatemoto and Muto, 2001). The activation of MAP kinase is important for the transition of oocytes from Metaphase I (MI) to MII (Verlhac et al., 1994). In mouse oocytes, it is MAP kinase, not MPF that is responsible for the transition from MI to MII, because MPF activity declines between Anaphase I (AI) and Telophase I (TI) while MAP activity continues to be high during maturation.

Activation of MAP kinase may be regulated by a signaling pathway. In maturing mouse oocytes active MAP kinase occurs after synthesis of Mos kinase (proto-oncogene Mos kinase) (Verlhac et al., 2000; Peter et al., 2002). The mos proto-oncogene encodes the protein Mos (Maxwell and Arlinghaus, 1985), which is expressed at high levels in oocytes undergoing maturation (Gebauer and Richter, 1997). In species with a longer interval between the stimulus of MPF and germinal vesicle breakdown (GVBD), such as pig, cow and sheep, the Mos protein showed to be required as a regulator of MPF activity throughout meiosis. Mos protein was also shown to affect the stability of the meiotic spindle (Gandolfi and Gandolfi, 2001).

The Mediterranean buffalo is important for the local economy and, because of its high performances in terms of milk production; its genotype is highly requested around the world (Gasparrini et al., 2004). The success rate of *in vitro* fertilization (IVF) in buffalo is reported to be low and is still under experimentation (Gasparrini et al., 2006). Information on gene expression profiling in the course of IVM and IVF procedures in buffalo is still an unexplored area which promises to provide valuable leads for not only optimizing the IVF procedure in buffalo but will also help in answering some physiological questions and to explain the reproduction potential in this species. The present work was undertaken to evaluate the expression of important genes (Cyclin B, CDC2, ERK2 and *c-mos*) which play a critical role in IVM of follicular oocytes in buffalo which will help in characterization of gene expression quantification strategy in this species.

MATERIALS AND METHODS

Chemicals: All media and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Dynabead. mRNA DIRECT Kit was purchased from Dynal company (Dynal, Oslo, Norway).

Oocyte recovery and *in vitro* maturation: Buffalo ovaries (n= 709) were collected from a local abattoir immediately after slaughter and were transported within 2–3 h from the slaughterhouse to the laboratory in

physiological saline at approximately 35-38°C. Cumulus–oocyte complexes (COCs, n= 1899, Table 1) were recovered by aspiration of 2 to 8 mm follicles using an 18-gauge needle attached to a 10-ml syringe.

Table 1: Experimental design clarifies replications and number of ovaries and oocytes used.

Groups (Culture time h)	Meiotic progression analysis			mRNA analysis		
	No of replications*	No of ovaries	No of oocytes	No of replications*	No of ovaries	No of oocytes
0	11	56	146	10	52	132
10	12	57	152	11	53	138
20	11	59	144	10	54	129
22	14	62	178	12	58	152
24	14	67	187	13	61	165
26	15	68	199	14	62	177

*For each replicate about 10-15 oocytes were used

Intact COCs were collected in tissue culture medium (TCM 199) supplemented with 0.4% Bovine serum albumin (BSA), 25mm HEPES, 100ugml−1 streptomycin sulphate, 100Uml−1 penicillin and 10 µgml^{-1} heparin. The COCs were evaluated on the basis of their morphology: only those with a compact, non-atretic cumulus and a homogeneous cytoplasm, previously classified as grades A and B (Neglia et al. 2003), were selected. The COCs were then washed three times in the same medium. Groups of 25–35 COCs were matured in 0.5 ml TCM 199 buffered with 25 mM sodium bicarbonate supplemented with 10% fetal calf serum (FCS), 0.2 mM sodium pyruvate, 0.5 µgml^{-1} FSH, 10 IUml^{-1} LH, 1 µgml^{-1} 17 β –Estradiol and 50 µgml^{-1} Gentamicin in four-well dishes (Nunc, Roskilde, Denmark). IVM was carried out at 38.5 °C for several time intervals (0, 10, 20, 22, 24 and 26 h) under a controlled gas atmosphere of 5% Carbon Dioxide (CO_2) in humidified air. At the end of the maturation period, the oocytes morphology was assessed by observing cumulus expansion, size of the perivitelline space, and presence of an intact oolemma.

After *in vitro* maturation, cumulus cells were removed by incubating COCs in mDPBS supplemented with 1 mgml^{-1} polyvinyl alcohol (Av. Mol. Wt., 30.000 to 70.000), 0,1% (w/v) porcine trypsin and 0,2% EDTA (w/v) (100 g of disodium salt, pH 4.6 at 25°C) for 20 min at 37°C. Oocytes were denuded mechanically by repeatedly pipetting with a fine Pasteur pipette under a stereomicroscope, followed by 3 washes in mDPBS supplemented with 1 mgml^{-1} polyvinyl alcohol. Approximately, half number of the denuded oocytes of each category was frozen at -80°C in approximately 4 µl mDPBS supplemented with 1 mgml^{-1} polyvinyl alcohol in a 0.5 ml siliconized polypropyl tube to be used for mRNA analysis. The other number of oocytes was subject to cytogenetical analysis to determine the nuclear maturation state.

Evaluation of nuclear maturation: To assess the rate of meiosis at the end of IVM, a total of 1006 buffalo oocytes, separated in groups according to time of maturation, were analyzed. Immediately after removal of cumulus cells denuded oocytes were transferred onto a glass slide. Silicone was used to maintain a coverslip in contact with the oocytes without exerting excessive pressure. The slides were immersed in a 3:1 fixative solution of ethanol/acetic acid for a minimum of 24 h. Fixed oocytes were stained with 1% (w/v) orcein in 45% (v/v) acetic acid for 30 min. Oocytes were analyzed cytogenetically for different stages of meiosis under a phase-contrast microscope (Zeiss) at magnifications x 200 and x 400. For classification of different meiotic stages, the system described by Hunter and Polge (1966) and Polański and Kubiak (1999) was adopted.

RNA extraction and cDNA synthesis: To determine the gene expression of selected genes, a total of 893 buffalo oocytes were used. Poly (A)-RNA from pooled COCs were extracted using the Dynabeads mRNA DIRECT kit according to the manufacturer's instructions with minor modifications (Wrenzycki et al. 1999). Briefly, pools of 10 – 15 frozen oocytes (Table 1) from each group were lysed by adding 30 µl of lysis/binding buffer (cell lysis/mRNA binding buffer, 100 mM Tris-

HCl, pH 8.0; 500 mM LiCL; 10 mM EDTA; 1% LiDS (SDS); 5 mM DTT) in 0.6 ml siliconized polypropyl tubes. Then, 0.1 pg rabbit Globin mRNA (BRL, Gaithersburg, MD, USA) per one oocyte was added to each tube as internal standard. After vortexing for 10 sec, centrifugation for 30 sec at 1000 g and incubation at room temperature for 10 min, 5 µl of Dynabeads Oligo (dT) 25, prewashed twice with 60 µl of lysis/binding buffer, were pipetted into the fluid. After 5 min of incubation at 20°C under constant shaking to induce binding of poly(A)- RNAs to oligo (dT)25 Dynabeads, the beads were separated on ice by a Dynal MPC-E-1 magnetic separator (Dynal, Hamburg, Germany). After washing once with 40 µl washing buffer 1 (10 mM Tris /HCl, pH 8.0; 0.15 M LiCl; 1 mM EDTA; 0.1% LiDS) and three times with 40 µl washing buffer 2 (10 mM Tris /HCl, pH 8.0; 0.15 M LiCl; 1 mM EDTA), Poly(A)- RNAs were then eluted from the beads by incubation with 11 µl sterile water at 65°C for 2 min. Aliquots were immediately used for reverse transcription (RT) using the PCR RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany), to obtain the widest array of cDNAs. To create a cDNA copy using 1 µl oligo dT primer, the RT reaction was carried out in a final volume of 20 ul at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min and immediate cooling on ice. The cDNA then was used immediately in the following PCR reaction or stored at -20°C until use.

Semi-quantitative RT-PCR: The first strand cDNA (2 µg) from different categories of buffalo oocytes was used as templates for PCR with a pair of specific primers. The sequences of specific primers and product sizes are listed in Table 2.

Table 2: Primer sequences used to amplify the studied genes.

Genes	**Primer sequences and positions**	**Fragment Size (bp)**	**Sequence references [EMBL accession no.]**
CDC2	5′ primer (392-416) = upper primer 5′ -ATTCTATCCCTCCTGGTCAGTTCAT- 3′ 3′ primer (786-810) = lower primer 3′ -GTTATTACTTCACACCGGTCTTCAC- 5′	419	Yang and Farin (1994) [L26547]
Cyclin B	5′ primer (85-109) = upper primer 5′ -GAGGGGATCCAAACCTTTGTAGTGA- 3′ 3′ primer (378-402) = lower primer 3′ -CTTCTTTACATGGGAGGTCTTTAAC- 5′	318	Anderson et al. (1995) [L48205]
c-*mos*	5′ primer (998-1022) = upper primer 5′ -CTTGGACCTGAAGCCAGCGAACATT- 3′ 3′ primer (1274-1298) = lower primer 3′ -GTTAGAGGCAGGCAGGGAGAGCCGC- 5′	301	Newman and Dai (1994) [X78318]
ERK2	5′ primer (430-454) = upper primer 5′ -CACCGACCATCGAGCAGATGAAAGA- 3′ 3′ primer (873-897) = lower primer 3′ -TAAGACCCATAAGAACCTAGGGGCA- 5′	468	Ely et al. (1992) [Z14089]
Globin	5′ primer (241-260) = upper primer 5′ -GCAGCCACGGTGGCGAGTAT- 3′ 3′ primer (555-657) = lower primer 3′ -GTGGGACAGGAGCTTGAAAT- 5′	257	Cheng et al. (1986) [X04751]

The reaction mixture for RT-PCR was consisted of 10 mM dNTP's, 50 mM MgCl2, 10x PCR buffer, and autoclaved water. The PCR cycling parameters were one cycle of 94 °C for 3 min, 33 (ERK2), 29 (CDC2, Cyclin B and c-mos) or 27 (Globin) cycles of 94 °C for 30 s, 59 °C to 60 °C for 30 s, 72 °C for 90 s, and a final cycle of 72 °C for 7 min. A volume of 20ul/reaction was subjected to electrophoresis on a 2% agarose gel in TBE buffer (90 mM Tris; 90 mM borate; 2 mM EDTA, pH 8.3), containing 0.2 µg/ml ethidium bromide. The

intensity of each band was quantified by densitometry using an image analysis program (IP Lab Gel). The relative abundances for mRNA were estimated as the ratio between the intensity of each mRNA-gene (CDC2, Cyclin B, ERK2 and c-mos) and Globin fragment (Wrenzycki et al., 1999). For each mRNA-gene, analysis was repeated five times where for each replication a minimum of ten oocytes were used.

Statistical analysis: Data of mRNAs abundance in the oocytes were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS) (SAS, 1982), followed by Scheffé-test to assess significant differences between groups. The values were expressed as Mean±SEM. All statements of significant were based on probability of $P < 0.05$. Data of time of the culture were expressed as percentage.

RESULTS

Evaluation of meiotic progression of buffalo oocytes during IVM

Table 3: Meiotic progression of the Egyptian buffalo oocytes at different times of the *in vitro* culture.

Groups (Culture time h)	No. of ovaries	No. of oocytes	State of nucleus									
			GV		GVBD		MI		AI/TI		MII	
			No	%	No	%	No	%	No	%	No	%
0	56	146	113	77.4	33	22.6	0	0	0	0	0	0
10	57	152	0	0	76	50.0	62	40.8	10	6.6	4	2.6
20	59	144	0	0	12	8.3	62	43.1	42	29.2	28	19.4
22	62	178	0	0	8	4.5	56	31.5	32	18.0	82	46.1
24	67	187	1	0.5	21	11.2	24	12.8	25	13.4	116	62.0
26	68	199	0	0	0	0	14	7.0	23	11.6	162	81.4

GV= Germinal vesicle, GV= Germinal vesicle breakdown, MI= Metaphasel, AI/TI= Anaphasel/ Telophasel, MII= Metaphase II

Table 3 shows the state of meiotic progression of the buffalo oocytes *in vitro* cultured at different time intervals (0, 10, 20, 22, 24 or 26 h). The results indicated that, most oocytes were arrested in the GV stage immediately after collection; however, 22.6% of oocytes had resumed meiosis before IVM culture. After 10 h of IVM culture, 50% of the buffalo oocytes arrived at the (GVBD) stage. After 20 h of the culture 43.1% of the total oocytes were arrested in (MI) stage. However, most oocytes cultured for 22, 24 or 26 h were completely matured, where the nuclei of these oocytes were arrested in (M II) (46.1%, 62% and 81.4%, respectively, Fig. 1).

Expression of CDC2, Cyclin B, ERK2 and *c-mos* genes at different maturation times: A semi-quantitative RT-PCR assay was used to assess the relative abundance (RA) of mRNA of CDC2, Cyclin B, ERK2 and *c-mos* genes which are known to be involved in mammalian oocyte maturation. Total RNA was isolated from buffalo oocytes after collection and after IVM at different time intervals from 10-26 h. Afterwards, the isolated mRNA transformed to cDNA using a reverse transcription reaction. Amplification of cDNA with CDC2, Cyclin B, ERK2 and *c-mos* specific primers resulted in one PCR product with the expected size of 419, 318, 468 and 301bp, respectively (Fig. 2 and 4). These products of the studied genes were detected in all oocytes whether they were analyzed after collection or after IVM from 10-26 h.

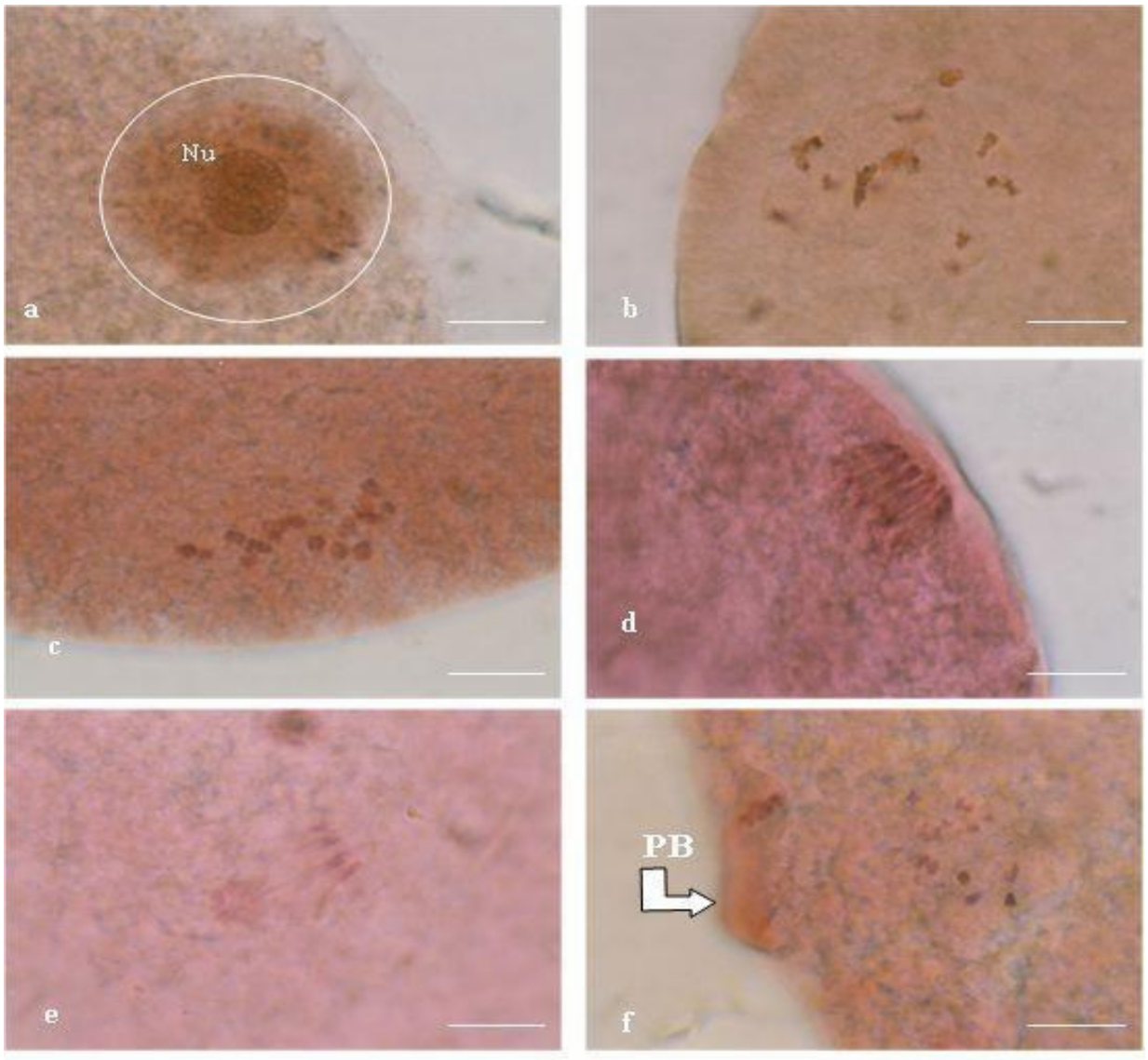

Figure 1: Photomicrographs of stained whole mounted buffalo oocytes representing various nuclear stages during maturation. (a) Germinal vesicle stage , the nucleus containing a nucleolus (Nu) (bar = 10 µm). (b) Germinal vesicle break down (bar = 15 µm). (c) Metaphase I (bar = 10 µm). (d) Anaphase I (bar = 15 µm).(e) Telophase I (bar = 15 µm). (f) Oocyte at Metaphase II and abstriction of first polar body (PB) (bar = 20 µm).

The relative abundance of CDC2, Cyclin B, ERK2 and *c-mos* mRNA was affected by maturation time (P< 0.05) (Fig. 3 and 5). The expression of CDC2, Cyclin B, ERK2 and *c-mos* mRNA was higher in buffalo oocytes after collection and gradually decreased with increment the time of oocyte maturation. There were no differences in the relative abundance of CDC2 or Cyclin B mRNA after collection, 10, 20 and 22h of maturation times. However, the relative abundance was significantly higher (P< 0.05) after collection compared with 24 and 26 h maturation times in these genes. Results of the ERK2 mRNA expression showed a high level in buffalo oocytes after oocyte collection and after IVM from (10 to 20h). While *c-mos* mRNA expression showed a high level after oocyte collection up to 24h of maturation times. However, a significant (P< 0.05) decline of the relative abundance of ERK2 and *c-mos* mRNA was found at 22 to 26h and at 26h of the maturation times, respectively.

DISCUSSION

The duration of IVM may play a critical role for subsequent development, since an inappropriate timing of maturation results in abnormal chromatin (Dominko and First, 1997), oocyte aging (Hunter and Greve, 1997) and reduced development (Marston and Chang, 1964). Although the sperm can penetrate oocytes prior to completion of oocyte maturation, subsequent development is generally reduced and, hence, it appears that the optimum time for IVF is at completion of meiosis (Chian et al., 1992). In the present study the attainment of the MII stage commenced after 20h maturation but the majority of oocytes completed nuclear maturation between 24 and 26h. These results were in agreement with those reported by Gasparrini et al. (2008), who found that the majority of buffalo oocytes accomplish nuclear maturation between 21 and 24h after the start of IVM. In another study, Nandi et al. (2002) found that cumulus expansion and extrusion of first polar body in buffalo oocytes commence at 6–17h post-maturation to reach the maximum levels at 22–24 h. However, the results of the present study were in disagreement with the study of Neglia et al. (2001), in which the majority of buffalo oocytes reached the MII stage between 15 and 19 h after the start of IVM and an increased incidence of degenerated oocytes was observed at later times. Large variations in the timing of the oocyte maturation process *in vitro* have also been reported in cattle (Ward et al. 2002; Park et al. 2005). The different oocyte maturation time-scale recorded among buffalo studies may be accounted for by different conditions of IVM and particularly by oocyte quality, which in this species, is also likely to be affected by seasonal factors.

The cytoplasmic maturation of the oocytes is a key parameter, which determines the success rate of *in vitro* production of embryos (Blondin and Sirard, 1995). Gaining knowledge about the variation/chronology of gene expression during oocyte maturation is crucial for optimization of IVF and other artificial reproduction technologies. Studies involving expression pattern of developmentally important genes in oocytes and embryos have not been widely investigated (Gaudette et al. 1993; Lechniak, 2002). Also, study the expression of CDC2, Cyclin B, ERK2 and *c-mos* genes during IVM of the buffalo oocytes has not been investigated yet.

Based on the mRNA transcripts in buffalo oocytes in our study, mRNAs for genes that encode components of MPF were detected immediately after oocytes collection, at 0 h of IVM. Wu et al. (1997) reported that activity of MPF is low in GV-stage bovine oocytes and increases around GVBD to peak at the MI stage. In the current study, Cyclin B and CDC2 mRNA were high at 10 h of IVM and decreased gradually until 26 h, where most of oocytes reached the MII. This result correlates the resumption of meiosis with the abundance of MPF component mRNAs. The depletion of these mRNAs could be associated with mRNA degradation or translation into MPF-component proteins.

In agreement with the results of this study, Robert et al. (2000) reported that bovine GV-stage oocytes, recovered from ovaries immediately after slaughter, possess a stockpile of maternal Cyclin B mRNA, but no measurable protein (Levesque and Sirard, 1996). Interestingly, it seems that Cyclin B protein can accumulate before oocyte maturation, when the ovary transportation time is long (Levesque and Sirard, 1996). Cyclin B mRNA probably is in a translationally inactive state (masked) in immature GV-stage bovine oocytes. Therefore, initiation of Cyclin B translation could be one of the early events leading to oocyte meiotic resumption. Tremblay et al. (2005) mentioned that oocytes can synthesize and store maternal mRNA in an inactive translational state until the start of IVM.

Similarly, with results of Dedieu et al. (1998) and Kanatsu-Shinoh ara et al. (2000) the current study indicted a statistical ($P< 0.01$) decrease in the level of CDC2 mRNA abundance with progression of maturation time which could be used in p34cdc2 protein synthesis. In goat, Anguita et al. (2007) reported that the amount of p34cdc2 protein was detected after *in vitro* maturation of goat oocytes which clearly shows that the transcript has been translated during the culture period and was associated with the ability to complete meiosis and to develop into embryos.

Extracellular signal-regulated kinase is required to convert the pre-MPF to the active form. In the current study it was found that the relative abundance of ERK2 mRNA was significantly ($P< 0.001$) high from 0 to 20 h, and then declined ($P< 0.01$) from 22 to 26 h of IVM. In consistent with our results, ERK2-mRNA in the bovine oocytes was decreased so that the synthesis of the MAP protein required for the activation of the MPF and for the assembly spindle of MI stage (Krischek and Meinecke, 2002). Salamone et al. (2001) observed the kinetics of MPF and MAPK activity during oocyte maturation in calf and cow oocytes, concluding that kinase activity was low at the GV stage (0 h) and increased several fold at 24 h after maturation in both oocytes groups.

On the other hand, MAP protein could be stimulated by

c-MOS protein (Inoue et al., 1998; Motlik et al., 1998; Lee et al., 2000). The MOS protein is also coded by maternal RNA stored during oocyte growth. In the present study high-level accumulation of *c-mos* transcripts was up to 24 h, and then declines at 26 h of IVM is consistent with previous studies in various species including bovine (Nganvongpanit et al., 2006) and human oocytes (Pal et al. 1994). Hirao and Eppig (1997) reported that, the c-Mos protein plays a main role as a cytostatic factor (CSF) to prevent the MII oocyte from developing parthenogenetically until the time of fertilization. This role of c-Mos protein may explain why the level of c-mos-mRNA in the present study remained high until the end of oocyte maturation compared to CDC2- or ERK2-mRNA

CONCLUSION,

There is an effect of the culture time on the mobilization of masked mRNA during the IVM. It could be suggested that time of culture was shown to regulate the mRNA translation to synthesize the maturation proteins which may stimulate the competence of the buffalo oocytes to achieve meiotic progression to the MII stage at 24 to 26 h of culture.

REFERENCES

Anderson JE, Matteri RL, Prather RS, 1995. Partial cDNA encoding a porcine cyclin B homolog. Unpublished.

Anguita B, Jimenez-Macedo AR, Izquierdo D, Mogas T, Paramio MT, 2007. Effect of oocyte diameter on meiotic competence, embryo development, p34 (cdc2) expression and MPF activity in prepubertal goat oocytes. Theriogenology 67, 526–536.

Bachvarova R, 1985. Gene expression during oogenesis and oocyte development in mammals, in: Browder, L.W, (Ed.), Developmental Biology: A Comprehensive Synthesis. Vol. 1: Oogenesis. New York: Plenum Press, pp.453–524.

Blondin P and Sirard MA, 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Molecular Reproduction & Development 41, 54–62.

Calder MD, Caveney AN, Smith LC, Watson AJ, 2003. Responsiveness of bovine cumulus-oocyte-complexes (COC) to porcine and recombinant human FSH, and the effect of COC quality on gonadotropin receptor and Cx43 marker gene mRNAs during maturation in vitro. Reproductive Biology and Endocrinology, 1, 1–14.

Cheng JF, Raid L, Hardison RC, 1986. Isolation and nucleotide sequence of rabbit globin gene cluster Ψζ-α1Ψα: absence of a pair of α-globin genes evolving in concert. Journal of Biological Chemistry, 261, 839-848.

Chian RC, Niwa K, Nakahara H, 1992. Effect of sperm penetration *in vitro* on completion of first meiosis by bovine oocytes arrested at various stages in culture. Journal of Reproduction and Fertility, 96, 73–78.

Dedieu T, Gall L, Crozet N, Sevellec C, Ruffini S, 1996. Mitogen-activated protein kinase activity during goat oocyte maturation and the acquisition of meiotic competence. Molecular Reproduction & Development, 45, 351-358.

Dedieu T, Gall L, Hue I, Ledan E, Crozet N, Ruffini S, Sevellec C, 1998. p34cdc2 expression and meiotic competence in growing goat oocytes. Molecular Reproduction & Development, 50, 251–262.

Dominko T. and First NL, 1997. Kinetics of bovine oocyte maturation allows selection for developmental competence and is affected by gonadotrophins. Theriogenology, 37, 203.

Ely CM, Cox ME, Her J, Parsons SJ, 1992. Cloning and sequencing of ERK2 from a bovine adrenal medulla cDNA library. Unpublished.

Fissore RA, He CL, Vande Woude GF, 1996. Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. Biology of Reproduction, 55, 1261-1270.

Gandolfi TA and Gandolfi F, 2001. The maternal legacy to the embryo: cytoplasmic components and their effects on early development. Theriogenology, 55, 1255-1276.

Gasparrini B, 2002. *In vitro* embryo production in buffalo species: state of the art. Theriogenology, 57, 237–256.

Gasparrini B, Boccia L, De Rosa A, Di Palo R, Campanile G, Zicarelli L, 2004. Chemical activation of buffalo (Bubalus bubalis) oocytes by different methods: effects of aging on post-

parthenogenetic development. Theriogenology, 62, 1627–1637.

Gasparrini B, Boccia L, Marchandise J, Di Palo R, George F, Donnay I, Zicarelli L, 2006. Enrichment of *in vitro* maturation medium for buffalo (Bubalus bubalis) oocytes with thiol compounds: effects of cystine on glutathione synthesis and embryo development. Theriogenology, 65, 275–287.

Gasparrini B, De Rosa A, Attanasio L, Boccia L, Di Palo R, Campanile G, Zicarelli L, 2008. Influence of the duration of *in vitro* maturation and gamete co-incubation on the efficiency of *in vitro* embryo development in Italian Mediterranean buffalo (Bubalus bubalis). Animal Reproduction Science, 105, 354–364.

Gaudette MF, Cao QP, Crain WR, 1993. Quantitative analysis of specific messenger RNAs in small numbers of preimplantation embryos. Methods in Enzymology, 225, 328–344.

Gautier J, Minshull J, Lohka M, Glotzer M, Hunt T, Maller JL, 1990. Cyclin is a component of maturation-promoting factor from Xenopus. Cell, 60, 487–494.

Gavin AC, Cavadore JC, Schorderet-Slatkine S, 1994. Histone H1 kinase activity, germinal vesicle breakdown and M phase entry in mouse oocytes. Journal of Cell Science, 107, 275-283.

Gebauer F, Richter JD, 1997. Synthesis and function of Mos: the control switch of vertebrate oocyte meiosis. Bioessays, 19, 23-28.

Hirao Y and Eppig JJ, 1997. Parthenogenetic development of Mos-deficient mouse oocytes. Molecular Reproduction & Development, 48, 391-396.

Hunter RH and Polge C, 1966. Maturation of follicular oocytes in the pig after injection of human chorionic gonadotrophin. Journal of Reproduction and Fertility, 12, 525-531.

Hunter RHF and Greve T, 1997. Could artificial insemination of cattle be more fruitful? Penalties associated with aging eggs. Reproduction in Domestic Animals, 32, 137–142.

Inoue M, Naito K, Nakayama T, Sato E, 1998. Mitogen-activated protein kinase translocates into the germinal vesicle and induces germinal vesicle breakdown in porcine oocytes. Biology of Reproduction, 58, 130-136.

Kanatsu-Shinohara M, Schultz RM, Kopf GS, 2000. Acquisition of meiotic competence in mouse oocytes: absolute amounts of p34(cdc2), cyclin B1, cdc25C, and wee1 in meiotically incompetent and competent oocytes. Biology of Reproduction, 63, 1610–1616.

Krischek C and Meinecke B, 2002. *In vitro* maturation of bovine oocytes requires polyadenylation of mRNAs coding proteins for chromatin condensation, spindle assembly, MPF and MAP kinase activation. Animal Reproduction Science, 73, 129-140.

Kubelka M, Anger M, Kalous J, Schultz RM, Motlik J, 2002. Chromosome condensation in pig oocytes: lack of a requirement for either cdc2 kinase or MAP kinase activity. Molecular Reproduction & Development, 63, 110-118.

Lechniak D, 2002. Quantitative aspect of gene expression analysis in mammalian oocytes and embryos. Reproductive Biology, 2, 229–241.

Lee J, Miyano T, Moor RM, 2000. Localisation of phosphorylated MAP kinase during the transition from meiosis I to meiosis II in pig oocytes. Zygote, 8, 119-125.

Levesque JT and Sirard MA, 1996. Resumption of meiosis is initiated by the accumulation of cyclin B in bovine oocytes. Biology of Reproduction, 55, 1427–1436.

Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, Ahn NG, 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science, 265, 966-970.

Marston JH and Chang MC, 1964. The fertilizable life of ova and their morphology following delayed insemination in mature and immature mice. Journal of Experimental Zoology, 15, 237–251.

Maxwell SA and Arlinghaus RB, 1985. Serine kinase activity associated with Maloney murine sarcoma virus-124-encoded p37mos. Virology, 143: 321-333.

Motlik J and Kubelka M, 1990. Cell-cycle aspects of growth and maturation of mammalian oocytes. Molecular Reproduction & Development, 27, 366–375.

Motlik J, Pavlok A, Kubelka M, Kalous J, Kalab P, 1998. Interplay between CDC2 kinase and MAP kinase pathway during maturation of mammalian oocytes. Theriogenology, 49, 461-469.

Nandi S, Ravindranatha BM, Gupta PSP, Sarma PV, 2002. Timing of sequential changes in cumulus cells and first polar body extrusion during *in vitro* maturation of buffalo oocytes. Theriogenology, 57, 1151–1159.

Neglia G, Gasparrini B, Caracciolo di Brienza V, Di Palo R, Campanile G, Presicce GA, Zicarelli L, 2003. Bovine and buffalo *in vitro* embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration. Theriogenology, 59, 1123–1130.

Neglia G, Marino M, Di Palo R, Wilding M, Caracciolo di Brienza V, Dale B, Gasparrini B, Zicarelli L, 2001. A comparison of *in vitro* maturation in buffalo (Bubalus Bubalis) and bovine oocytes using confocal microscopy. Theriogenology, 55, 488.

Newman BJ and Dai Y, 1994. Expression of *c-mos* in pig ovary and oocytes. Unpublished.

Nganvongpanit K, Müller H, Rings F, Gilles M, Jennen D, Hölker M, Tholen E, Schellander K, Tesfaye D, 2006. Targeted suppression of E-cadherin gene expression in bovine preimplantation embryo by RNA interference technology using double-stranded RNA. Molecular Reproduction & Development, 73,153-163.

Pal SK, Torry D, Serta R, Crowell RD, Siebel MM, Cooper, GM, Kiessling AA, 1994. Expression and potential function of the *c-mos* proto-oncogene in human eggs. Fertility and Sterility, 61, 496–503.

Park YS, Kim SS, Kim JM, Park HD, Byun MD, 2005. The effects of duration of *in vitro* maturation of bovine oocytes on subsequent development, quality and transfer of embryos. Theriogenology, 64, 123–134.

Peter M, Labbé JC, Dorée M, Mandart E, 2002. A new role for Mos in Xenopus oocyte maturation: targeting Myt1 independently of MAPK. Development; 129, 2129-2139.

Polański Z and Kubiak JZ, 1999. Meiosis, in: Knobil, E. (Ed.), Encyclopedia of Reproduction, San Diego, California, Academic Press, Vol, III, pp. 160-167.

Prather RS and Day BN, 1998. Practical considerations for the *in vitro* production of pig embryos. Theriogenology, 49, 23-32.

Robert C, Barnes FL, Hue I, Sirard MA, 2000. Subtractive hybridization used to identify mRNA associated with the maturation of bovine oocytes. Molecular Reproduction & Development, 57,167–175.

Salamone DF, Damiani P, Fissore RA, Robl JM, Duby RT, 2001. Biochemical and Developmental evidence that ooplasmic maturation of prepubertal bovine oocytes is compromised. Biology of Reproduction, 64, 761–768.

SAS, 1982. SAS user's guide: statistics, 1982 edn. SAS Institute Inc., Cary, NC.

Taieb R, Thibier C, Jessus C, 1997. On cyclins, oocytes, and eggs. Molecular Reproduction & Development, 48, 397–411.

Tatemoto H and Muto N, 2001. Mitogen-activated protein kinase regulates normal transition from metaphase to interphase following parthenogenetic activation in porcine oocytes. Zygote, 9, 15-23.

Tremblay K, Vigneault C, McGraw S, Sirard M, 2005. Expression of Cyclin B1 Messenger RNA Isoforms and Initiation of Cytoplasmic Polyadenylation in the Bovine Oocyte. Biology of Reproduction, 72, 1037–1044 .

Verlhac MH, Kubiak JZ, Clarke HJ, Maro B, 1994. Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Development, 120, 1017-1025.

Verlhac MH, Lefebvre C, Kubiak JZ, Umbhauer M, Rassinier P, Colledge W, Maro B, 2000. Mos activates MAP kinase in mouse oocytes through two opposite pathways. European Molecular Biology Organization Journal, 19, 6065-6074.

Ward F, Enright B, Rizos D, Boland M, Lonergan P, 2002. Optimization of *in vitro* bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. Theriogenology, 57, 2105–2117.

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H, 1999. Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. Molecular Reproduction & Development, 53, 8-18.

Wu B, Ignotz G, Currie WB, Yang X, 1997. Dynamics of maturation-promoting factor and its constituent proteins during *in vitro* maturation of bovine oocytes. Biology of Reproduction, 56, 253–259

Yamashita M, Mita K, Yoshida N, Kondo T, 2000.

Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects. Progress in Cell Cycle Research, 4,115–129.

Yang L and Farin CE, 1994. Identification of cDNAs encoding bovine cyclin B and Cdk1/Cdc2. Gene, 141, 283-286.

Zicarelli L, 1997. Superovulatory response in buffaloes bred in Italy. In: Third Course on Biotechnology of Reproduction in Buffaloes. Caserta, Italy, 167–188.

APPENDIX:

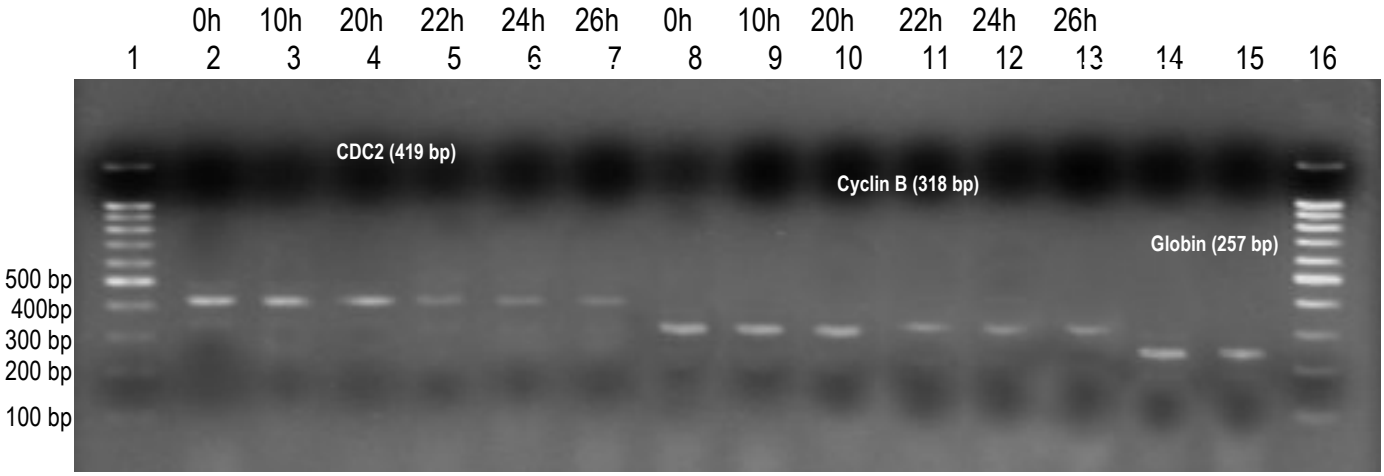

Figure 2: Semi-quantitative RT-PCR analysis of CDC2-, Cyclin B- and Globin-mRNAs in buffalo oocytes cultured for different time intervals. Gel electrophoresis represents mRNA of CDC2 (lanes 2-7), Cyclin B (Lanes 8-13) and Globin (lanes 14-15) genes. Each lane represents the RT-PCR products derived from Poly(A)-RNA from the equivalent of 8-10 oocytes. Lanes 14 and 15 are 80 ng Globin as positive controls. Lanes 1 and 16 represent DNA ladder. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from the specific genes used and Globin.

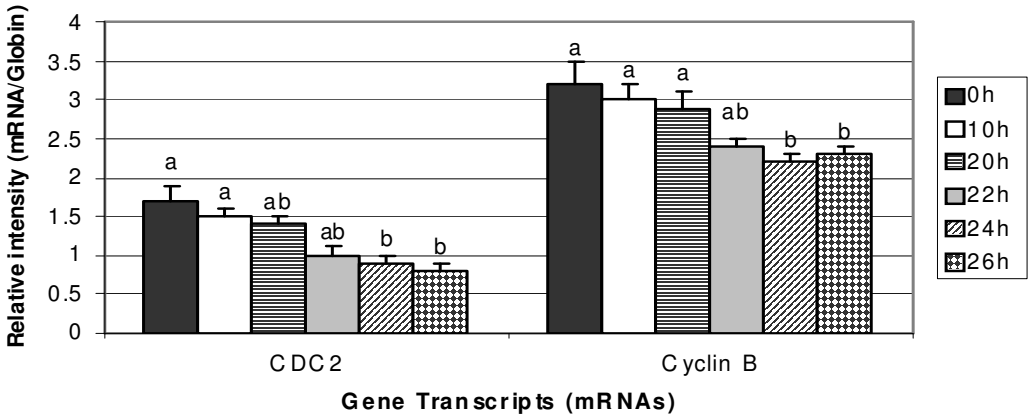

Figure 3: Relative abundance of CDC2 and Cyclin B mRNAs in buffalo oocytes cultured *in vitro* at different time intervals. The CDC2 or Cyclin B/ Globin densitometric ratio is shown (Mean ± SEM). The Superscript "a, b" shows bars with no superscripts in common are different at $P<0.05$.

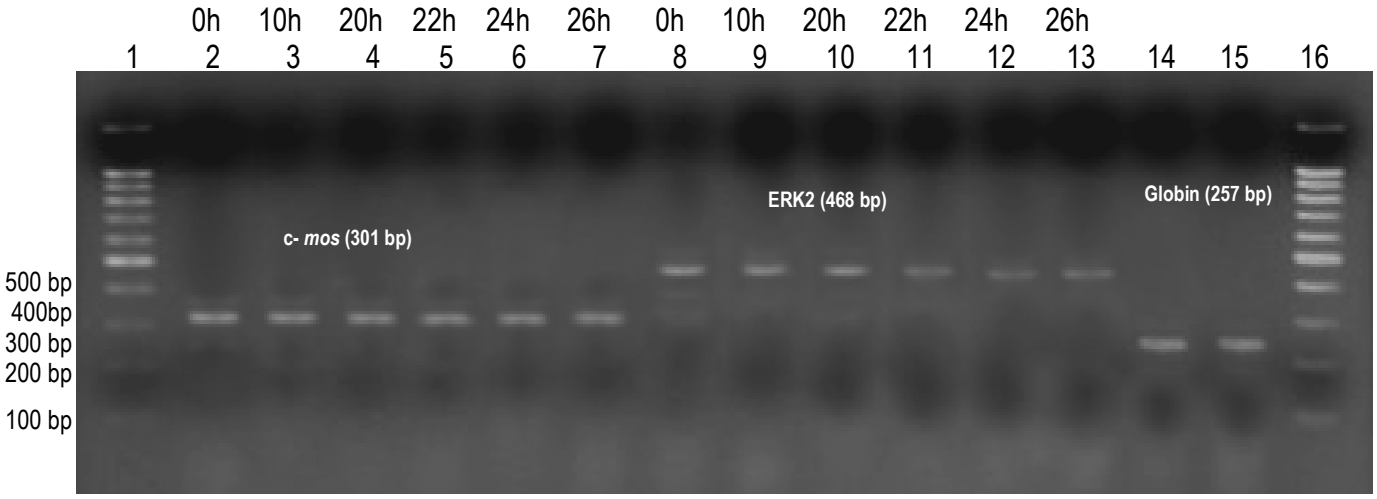

Fig. 4: Semi-quantitative RT-PCR analysis of *c- mos*-, ERK2- and Globin-mRNAs in buffalo oocytes cultured for different time intervals. Gel electrophoresis represents mRNA of *c- mos* (lanes 2-7), ERK2 (Lanes 8-13) and Globin (lanes 14-15) genes. Each lane represents the RT-PCR products derived from Poly(A)-RNA from the equivalent of 8-10 oocytes. Lanes 14 and 15 are 80 ng Globin as positive controls. Lanes 1 and 16 represent DNA ladder. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from the specific genes used and Globin.

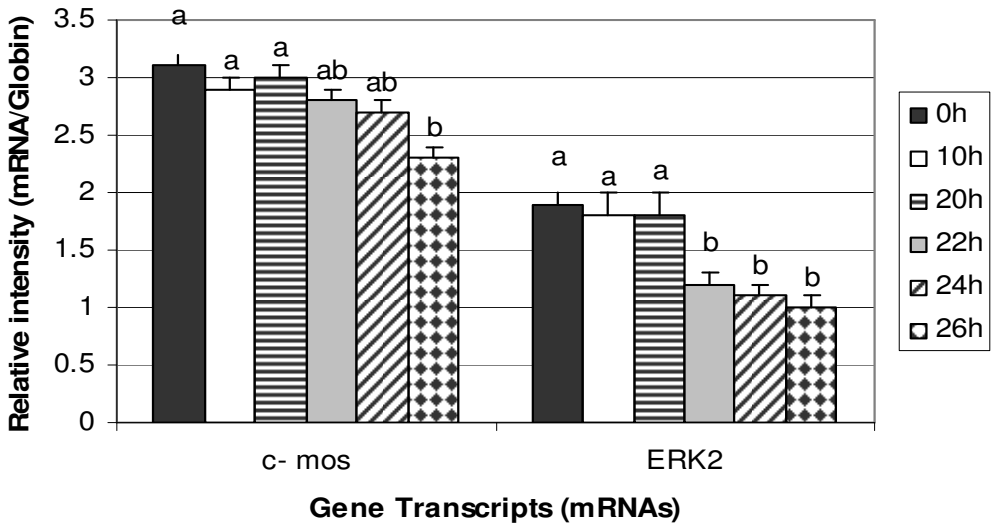

Figure 5: Relative abundance of *c-mos* and ERK2 mRNAs in buffalo oocytes cultured *in vitro* at different time intervals. The *c-mos* or ERK2/ Globin densitometric ratio is shown (mean ± SEM). The Superscript "a, b" shows bars with no superscripts in common are different at $P < 0.05$.