Journal of Applied Biosciences 24: 1520 - 1534
ISSN 1997–5902

Effect of hormones, culture media and oocyte quality on in vitro maturation of Egyptian Sheep oocytes

I.M. Farag[1] , S.M. Girgis[*1] , W.K.B.Khalil[1], N.H.A. Hassan[2], A.A.M.Sakr [1], S.M. Abd Allah [3], N. I. Ali[1]
[1]Cell Biology Department, National Research Centre, 12622- Dokki, Cairo, Egypt.
[2]Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt.
[3]Theriogenology Department., Faculty of Veterinary Medicine, Benisuef University Benisuef, Egypt.

***Corresponding author**: Dr. S. M. Girgis, E-mail: shenoudagirgis10@yahoo.com
Published online at www.biosciences.elewa.org on December 7, 2009

ABSTRACT

Objective: The present study was carried out to investigate the role of adding hormone combinations (PMSG+hCG+E2) alone with or without fetal bovine serum (FBS) to the culture media (TCM-199 or RPMI-1640) on the in vitro maturation (IVM) of sheep oocytes. The effects of type of culture media and oocyte quality were also investigated.

Methodology and results: Sheep ovaries were collected form local slaughterhouse. Cumulus oocyte complexes COCs and cumulus denuded oocytes [CDOs] were recovered from visible antral follicles (2-6mm) by aspiration method. The collected oocytes were matured in culture media for 26-29h at 39℃ under 5% CO_2 in air and 95% humidity. The results indicated that the addition of hormones combined with FBS to the basic medium (TCM-199) significantly ($P<0.05$) improved the IVM of COCs as compared to the control (medium alone) (41.25 vs. 3.50, respectively). Supplementing TCM – 199 with hormones alone (PMSG+hCG+E_2) insignificantly increased the IVM of COCs compared to the control (14.75 vs. 3.50, respectively). However, supplementing TCM – 199 medium with hormones alone or hormones combined with FBS did not improve the IVM of denuded oocytes. On the other hand, the addition of hormones combined with FBS to RPMI medium significantly ($P<0.05$) improved the IVM of COCs as compared to the control (medium alone) (7.22 vs. 4.69, respectively). However, the addition of hormones alone to RPMI medium did not improve the IVM of COCs. Supplementing RPMI medium with hormones alone significantly ($P<0.05$) improved the IVM of denuded oocytes as compared to the control group (3.30 vs. 0.00, respectively). In contrast, the addition of hormones plus FBS did not improve the IVM of denuded oocytes. Concerning, the effect of type of culture media on the IVM of sheep oocytes, the results showed that the proportion of oocytes (COCs or denuded oocytes) reaching MII significantly increased ($P<0.05$) in the group that were matured in TCM-199 medium compared to RPMI medium (19.84 vs. 3.98; or 2.50 vs. 1.07, respectively). The effect of oocye quality on IVM showed that COCs had higher maturation rates compared to denuded oocytes in either TCM-199 ($P<0.01$) supplemented groups (23.98 vs. 2.57%, respectively) or in RPMI supplemented groups (2.59 vs. 1.36%, respectively).

Conclusion and application of findings: The present study demonstrated that the addition of hormone combinations (PMSG + hCG + E_2) with FBS to culture media (TCM- 199 or RPMI- 1640) could significantly improve the IVM of sheep oocytes especially COCs). TCM – 199 medium is more effective for in vitro maturation of sheep oocytes than RPMI- 1640 medium. For optimum oocyte nuclear maturation, the use of COCs is recommended.

Key words: Hormones, culture media, oocyte quality, sheep,cytogenetics, nuclear maturation.

INTRODUCTION

In the Egyptian economy, sheep play a role as suppliers of meat, milk and wool. Since sheep have their place in agriculture, ways have to be found to improve their level and efficiency of production and quality of their products. Efficient production depends among others, genetic improvement, and in vitro embryo production technology is a useful tool in this respect (Gilchrist & Thompson, 2007).

Ovine in vitro embryo production is one of the future sheep breeding strategies for the development of biotechnologies in which gene transfer by zygote microinjection is of high importance. Following the first successful in vitro fertilization of bovine oocytes (Brackett *et al*., 1982), in which in vivo matured bovine oocytes were used, researchers have focused on in vitro fertilization (IVF) of in vitro matured mammalian oocytes (Sirard, 1989). Since the maturation of oocytes is one of the prerequisites of successful IVF, many workers have studied the different aspects of in vitro maturation (IVM) of mammalian oocyles (Yadave *et al*., 1997).

In some studies, the supplementation of the IVM media with different combinations of hormones such as gonadotropins plus estradiol have been found to be essential for acquisition of developmental capacity of oocytes in cattle (Henderson *et al*., 1982; Fukushima & Fukui, 1985; Brackett *et al*., 1989) and buffaloes (Totey *et al*., 1992, 1993; Chauhan *et al*., 1997). The addition of hormone combinations (gonadotropins plus estradiol) with a source of sera such as estrous goat serum (EGS) to TCM-199 medium has also been found to be necessary for achieving high maturation rates (72.4%) for goat oocytes (Mogas *et al*., 1997 a-b). Moreover, the cleavage rate of fertilized goat oocytes was significantly higher (69%) when hormone combinations (LH + FSH + E_2) plus fetal bovine serum (FBS) were added to maturation medium than those supplemented with hormone combinations (LH + FSH + E_2) plus goat serum (GS) (54%; $P<0.05$) (Seydou *et al*., 1999).

Gonadotropins are the primary regulators of nuclear maturation in mammalian oocytes in vitro, and one of the functions of its preovulatory surge is to suppress the granulose cell factors that inhibit meiosis (Moor & Trounson, 1977; Pawshe *et al*., 1996; Gilchrist & Thompson, 2007). Also, in many mammalian species, gonadotropins have been found to stimulate cumulus cells to synthesize molecules able to drive germinal vesicle breakdown GVBD as meiosis- activating sterols (Tsafriri *et al*., 2005). Estradiol has been found to improve the completion of maturational changes and also to support the synthesis of presumed male pronuclear growth factor (Moor & Warnes, 1978; Fukui & Ono, 1989). The importance of sera may be due to its contents of hormones, trace nutrients and proteins such as globulin, albumin and futuin (Hsu *et al*., 1987; Tajik & Shams-Esfandabadi, 2003).

Although the importance of hormone combinations (gonadotropins plus estradiol) alone or with a source of sera in the oocyte maturation and development of some species of farm animals has been reported (Henderson *et al*., 1982; Fukushima & Fukui, 1985; Totey *et al*., 1993), it has never been used in the forms (PMSG+ hCG +E_2) with or without fetal bovine serum (FBS) in the culture media for IVM of Egyptian sheep oocytes.

On the other hand, the type of medium is also an important factor that can affect the IVM of mammalian oocytes. The culture media employed in IVM not only affects the proportion of mammalian oocytes undergoing fertilization but it also influences the subsequent cleavage and developmental competency (Madan *et al*., 1994; Tajik & Shams-Esfandabadi, 2003). Different culture media such as TCM-199 (Yamauchi & Nagi,1999; Kharche *et al*., 2006), minimum essential medium (MEM) (Bavister *et al*., 1992) and Ham's F-10 (Tamilmani *et al*., 2005 and Arunakumari *et al*. (2007) have been used for in vitro maturation of mammalian oocytes. TCM-199 is the most widely used culture medium for such purposes (Tamilmani *et al*., 2005; Arunakumari *et al*., 2007).

Some studies have showed that the TCM-199 medium is superior to Ham's F.10 in promoting

IVM of buffalo (Totey *et al*., 1993) and goat (Pawshe *et al*., 1996) oocytes. Also, TCM- 199 medium has been found to support more proportions of MII (metaphase II) of bovine oocytes than did minimum essential medium (8.57 vs. 60.3%, respectively) (Sahoo *et al*., 1998) or M16 (95 vs. 13.6%, respectively) (Bilodeau- Goeseels, 2006).

The beneficial effect of TCM-199 medium on IVM of animal oocytes may be related to some factors in its composition such as essential amino acids and glutamine that stimulate DNA and RNA synthesis and enhance cell division (Pawshe *et al*.,1996; Gordon, 2003). However, the differences between TCM-199 medium and RPMI-1640 on improving IVM of Egyptian sheep oocytes is not apparent.

The oocyte quality certainly also plays a key role in the acquisition of oocyte developmental competence in vitro (Totey *et al*., 1993). Although the techniques of in vitro maturation (IVM), fertilization (IVF) and Culture (IVC) have been used for production of embryos in cattle and buffalo, success in terms of birth of calves through this technology is limited in these animals. The important factor, among several, that is known to affect success of IVM and consequently IVF is the cumulus cells that surround the recovered oocytes (Bilodeau-Goeseels & Panich, 2002, Lonergan *et al*., 2003; Ge *et al*., 2008). The ability of the oocyte to undergo maturation and reach metaphase II (MII) depends on the presence of an intact complement of the cumulus cells during maturation (Mori *et al*., 2000; Webb *et al*., 2002; Shirazi *et al*., 2007). Various roles reported for cumulus cells include prevention of the hardening of zona pellucida (De Felici & Siracusa, 1982), the provision of energy for oocyte maturation and production of cytoplasmic maturation factors (Vanderhyden & Armstrong, 1989), and the uptake of nutrients for oocytes during maturation in culture medium (Mori *et al*., 2000). On the other hand, there are some reports showing that cumulus-denuded oocytes (CDOs) can complete their meiotic maturation as in mice (Binor & Wolf, 1979), rat (Magnusson, 1980) and cattle (Chian *et al*., 1994) in vitro. However, other reports indicate a lower developmental competence of CDOs after in vitro fertilization (IVF) compared to (COCs) (Vanderhyden & Armstrong, 1989; Chian *et al*., 1994, Yamauchi & Nagai, 1999). The lower developmental competence of CDOs might reflect a difference in cytoplamic maturation of oocytes (Geshi *et al*., 2000). Such effect of oocyte quality on IVM of Egyptian sheep oocytes has not been obvious in previous studies.

Therefore, the present study was designed to evaluate the effect of addition of hormone combinations (PMSG + hCG + E_2) alone or with fetal bovine serum (FBS) to culture media (TCM-199 or RPMI – 1640), on IVM of Egyptian sheep oocytes (COCs or CDOs). The effect of type of culture media and oocyte quality on IVM of sheep oocytes was also investigated.

MATERIALS AND METHODS

Chemicals and plastics: TCM -199 medium (M-4530), RPMI – 1640 medium (R-8758), fetal bovine serum (F-7524), 178-estradiol (E-2758), and mineral oil (M-8410) were purchased from Sigma Chemicals Co. (St. Louis, Mo., USA). hCG Pregnyl(R), was provided from Nile Co. for Pharmaceutical & Chemical Industries A.R.E, PMSG Folligon(R), Intervet International B.V., Boxmeer, Holland D-PBS (Cat. No: 21300-017) was obtained from GIBCO/BRL (Grand Island, N.Y, USA). Polystyrene Plastic culture dishes (35x 10mm, 60x 10 mm) and 0.22 µm millipore membrane filters were purchased from Nunclon, Nalge Nunc International, Roskide, Denmark.

Oocyte collection and maturation: Sheep ovaries were collected at a local abattoir about 15 min after slaughter. The ovaries were transported to the laboratory in 0.9% saline supplemented with 50µg/ml gentamycin sulfate at 30 to 35°C within 2-3h. Oocytes from all visible antral follicles (2 to 6 mm in a diameter) were aspirated with a 20-gauge hypodermic needle attached to a 5 ml disposable syringe containing 1ml of aspiration medium. The aspiration medium consisted of Dulbecco's phosphate buffer saline (D-PBS) supplemented with 0.03g/ml bovine serum albumin and 50µg/ml gentamycin sulfate (Chauhan *et al*., 1997a).

Cumulus oocyte complexes (COCs,) (with an unexpanded mass of cumulus cells and homogenous

cytoplasm) and cumulus –denuded oocytes (CDOs, (with homogenous cytoplasm) were recovered under a stereomicroscope. Both the COCs and CDOs were washed once with aspiration medium and twice in basic culture medium TCM-199 or in tested culture medum RPMI-1640. These media (TCM-199 or RPMI – 1640) were enriched with 50µg/ml gentamycin sulfate and were without any hormone or serum supplementation.

The same non-supplementation medium (TCM -199) was used as a control for two different culture media supplements as follows: (1) TCM -199 medium supplemented with 20 iu/ml PMSG + 10 iu /ml hCG + 1 ug/ml 17 β-estradiol (E_2); (2) TCM -199 medium supplemented with 10% fetal bovine serum (FBS) + 20. iu/ml PMSG + 10 iu /ml hCG + 1 μ g/ml E_2.

In addition, RPMI-1640 medium alone (non-supplementation medium) and RPMI-1640 medium enriched with the same previous hormone supplements with or without FBS were tested on sheep oocytes (COCs or CDOs) to be serve as a maturation medium. Each treatment consisted of 8 replicates. The non-supplemented media (controls) or the media enriched with hormone supplements with or without FBS were sterilized using 0.22 µm Millipore filter.

For all experiments, 10-15 oocytes of COCs or CDO s were transferred separately into a 50 ul drop of each type of culture media (control medium or medium plus hormone supplements with or without FBS), covered with sterile mineral oil in a polystyrene culture dish (3.5 mm x 10 mm) which had been previously kept for about 2h in a CO_2 incubator before the oocytes were added. The oocytes (COCsor CDOs) were cultured for 26-29h at 39^{o}C in an atmosphere of 5% CO_2 in air with 95% humidity.

Following the culture period, the degree of cumulus expansion of COCs was determined. The degree of cumulus expansion was assessed on the following scale: 0 - no expansion; 1 - few expansion of cumulus layers or cumulus cells were non-homogeneously spread and clustered cells were still observed; 2- moderate expansion of cumulus layers; and 3 - full expansion of cumulus layers.

While in the CDOs, the oocytes were classified into two types according to the homogeneity of the cytoplasm, either homo-or heterogeneous cytoplasm.

All the in vitro matured oocytes (COCs) were used for accelerating the rate of nuclear maturation, irrespective of the degree of expansion (Chauhan *et al*., 1998; Bolamba *et al*., 2006).

3-Assessment of the nuclear maturation by cytogenetic analysis: For examining the rate of nuclear maturation (the proportion of oocytes whose nuclei reached metaphase II), the cumulus cells of COCs were removed by vortexing. The cumulus-free COCs and CDOs with homogenous cytoplasm were then fixed in solutions of acetic acetic : ethanol (1:3 v/v) in culture dishes (35 x10mm) for at least 48h at 4^{o}C. Fixed oocytes were transferred to glass slides; silicon gel was used to maintain a coverslip in contact with the oocytes. The slides were immersed in 1% aceto-orcein stain for 30 min. Then, slides were washed three times in ascending concentrations of ethanol to remove the surplus orcein dye as follows: 5 sec. in 70% ethanol, then 1 and 3 min. in absolute ethanol (Khalil, 2003). Oocytes were examined under a light microscope (1000 x magnification) and classified as being at one of the following stages: germinal vesicle stage (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII). Oocytes with no visible or abnormal chromatin configuration were classified as degenerate (Beker *et al*., 2000).

Statistical analysis: Data on the effect of hormones and type of media on nuclear maturation rates were analyzed by ANOVA using SAS program (SAS, 1996). Fisher's least significant difference (LSD) at 5% level ($P<0.05$) was used to test the differences between means of treatments. Data for the effect of oocyte quality on nuclear maturation rate were analyzed using Chi-square test (Snedecor & Cochran, 1989).

RESULTS

Effect of adding hormone combinations (PMSG+hCG+E_2) alone or with FBS to culture medium (TCM-199) on the nuclear maturation rate of sheep oocytes:

Effect on the COCs: The effect of hormonal supplementation (PMSG+hCG+E_2) with or without FBS on *in vitro* maturation rate of COCs of the sheep is illustrated in Table 1. The proportions of oocytes which their nuclei reaching MII in the control, hormones supplemented group and hormone combination plus FBS supplemented group were 3.50, 14.75 and 41.25, respectively.

The present results clearly indicated that supplementing maturation medium with hormone combinations (PMS+hCG+E_2) alone or with hormones combined with FBS improved markedly the oocytes

maturation rate. However, this improvement in the oocytes nuclear maturation was only significant (P<0.05) in hormone combination plus FBS supplemented group compared to the control group (41.25 vs. 3.50, respectively).

The results showed that the proportion of full expansion of cumulus cells of COCs cultured in TCM-199 medium supplemented with hormone combinations (PMSG+hCG+E2) or with hormones combined with FBS increased more than those cultured in TCM-199 medium alone (43.40 or 19.53 vs. 10.40, respectively). This increase was highly significant (P<0.01) in the hormones supplemented group and significant (P<0.05) in hormones and FBS supplemented group compared to the control group. Moreover, cumulus expansion was found to be more activated (P<0.05) with hormones addition to TCM-199 medium than with hormones plus FBS addition (43.40 vs. 19.53, respectively).

Effect on denuded oocytes: The *in vitro* maturation rates of denuded sheep oocytes matured in the basic medium (TCM-199) supplemented with hormone combinations (PMSG+hCG+E_2) or with hormones combined with FBS are presented in Table 2. The proportions of oocytes undergoing *in vitro* nuclear maturation and reaching MII were 5.99, 1.52 and 0.00 for oocytes groups that matured in TCM-199 medium or TCM-199 supplemented with hormone combination (PMSG+hCG+E_2) or TCM-199 supplemented with hormones plus FBS, respectively. These results showed that the addition of hormones or hormones plus FBS to TCM-199 medium decreased the nuclear maturation rate of denuded oocytes. This decrease was significant (P<0.05) in hormones supplemented group and highly significant in the group supplemented with hormones plus FBS compared to TCM-199 medium alone.

Effect of adding hormone combinations (PMSG+hCG+E_2) with or without FBS to RPMI-1640 medium on the *in vitro* maturation rate of sheep oocytes

The effect on COCs: The nuclear maturation rates of sheep COCs matured in RPMI-1640 medium supplemented with hormone combinations (PMSG+hCG+E_2) with or without FBS are shown in Table 3. The present results revealed that the addition of hormones (PMSG+hCG+E_2) combined with FBS to RPMI-1640 medium improved oocytes maturation rate as indicated by the higher mean percentage of oocytes reaching MII. This improvement in the oocytes nuclear maturation was significant (P<0.05) compared to the control group (7.22 vs. 4.69, respectively). However, there were no oocytes reaching MII in hormones (PMSG+hCG+E_2) supplemented group.

n the present study, it is evident that supplementation of hormone combinations (PMSG +hCG+E_2) with FBS to RPMI-1640 medium progressively enhanced (P<0.05) the full expansion of cumulus cells compared to RPMI-1640 medium alone (56.94 vs. 49.32, respectively). However, enrichment of the same medium with hormones alone induced a reverse trend (P<0.01) compared to the culture medium alone (24.33 vs. 49.32, respectively).

The effect on denuded oocytes: As shown in Table 4, there were no oocytes reaching MII for groups cultured in each of RPMI-1640 medium alone, and RPMI-1640 medium enriched with hormones (PMSG+hCG+E_2) plus FBS. However, the addition of hormones (PMSG+hCG+E_2) alone to RPMI-1640 medium significantly (P<0.05) improved oocytes maturation rate (as indicated by the mean percentage of oocytes reaching MII) compared to RPMI-1640 medium alone (3.30 vs. 0.00, respectively).

Effect of type of culture media on the maturation rate of sheep oocytes:

Effect on COCs: The effect of type of culture media on the maturation rate of sheep COCs is presented in Table 5. The results show that the proportion of oocytes reaching MII significantly increased (P<0.05) in the group matured in TCM-199 medium than that found in the group matured in RPMI-1640 medium (19.84 vs. 3.98, respectively). However, cumulus cell expansion was found to be more activated in RPMI-1640 medium (P<0.05) than that observed in TCM-199 medium (43.59 vs. 24.50, respectively).

Effect on denuded oocytes: The effect of type of culture media on the maturation rate of denuded sheep oocytes is shown in Table 6. Cytogenetical examination revealed that the proportion of oocytes reaching MII was comparatively elevated (P<0.05) in the group matured in TCM-199 medium than the group matured in RPMI-1640 medium (2.50 vs. 1.07, respectively).

Effect of oocytes quality on *in vitro* nuclear maturation rate of sheep oocytes: The results (table 7a,b) showed that the COCs recorded higher maturation rate (P< 0.01) compared to the denuded oocytes in TCM-199 supplemented groups (23.98 vs 2.57%). Also, the proportion of COCs reaching MII increased than that of denuded oocytes in RPMI-1640 supplemented groups (2.59 vs. 1.36%, for COCs and denuded oocytes, respectively). However, this increase was not significant.

Table 1: Effect of adding hormone combinations with or without FBS to TCM-199 medium on the maturation rate of COCs of the sheep.

Treatment	No. of COCs	Degrees of cumulus cells expansion				Nuclear maturation of COCs						
		0	1	2	3	GV	GVBD	MI	AI	TI	MII	Deg.
		No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)
M_1 (Control)	73	13 (16.20)^{a}	45 (59.00)^{a}	10 (14.40)^{c}	5 (10.40)^{c}	11 (9.17)^{a}	36 (46.80)^{a}	18 (34.50)^{a}	2 (2.40)^{b}	3 (3.60)^{c}	3 (3.50)^{b}	0 (0.00)^{b}
M_1 + H	85	1 (1.60)^{b}	11 (13.25)^{b}	30 (41.67)^{a}	43 (43.40)^{a}	8 (6.47)^{b}	32 (31.10)^{b}	26 (38.10)^{a}	1 (1.00)^{c}	6 (7.70)^{b}	11 (14.75)^{ab}	1 (1.00)^{a}
M_1 + H + FBS	88	2 (2.93)^{b}	41 (42.50)^{ab}	25 (34.90)^{b}	20 (19.53)^{b}	0 (0.00)^{c}	8 (14.40)^{c}	14 (21.87)^{b}	4 (5.17)^{a}	17 (17.30)^{a}	45 (41.25)^{a}	0 (0.00)^{b}

Values in the same column with different superscripts differ significantly (P <0.05).
Table represents mean numbers of oocytes (%) in each stage of maturation (M %).
M_1 = TCM-199 medium, H=PMSG+hCG+E_2, and FBS = fetal bovine serum.
COCs = Excellent + good oocytes.
GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI= Anaphase I,
TI = Telophase I, MII = Metaphase II, and Degenerated.

Table 2: Effect of adding hormone combination with or without FBS to TCM-199 medium on the maturation rate of denuded sheep oocytes.

Treatment	No. of Denuded oocytes	Homogeneity of cytoplasm		Nuclear maturation of COCs						
		Homo-genous	Hetero-geneous	GV	GVBD	MI	AI	TI	MII	Deg.
				No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)
M_1 (Control)	133	130	3	62 (49.74)^{a}	22 (15.88)^{b}	29 (21.66)^{a}	2 (1.47)^{b}	7 (5.26)^{a}	8 (5.99)^{a}	0 (0.00)
M_1 + H	135	132	3	42 (40.22)^{b}	36 (28.96)^{ab}	36 (26.27)^{a}	1 (1.00)^{c}	6 (7.70)^{b}	11 (14.75)^{ab}	0 (0.00)
M_1 + H +FBS	133	130	3	40 (33.44)^{b}	66 (48.45)^{a}	23 (17.74)^{b}	1 (0.39)^{c}	0 (0.00)^{b}	0 (0.00)^{c}	0 (0.00)

Values in the same column with different superscripts differ significantly (P <0.05).
Table represents mean numbers of oocytes (%) in each stage of maturation (M %).
M1 = TCM-199 medium, H=PMSG+hCG+E2, and FBS = fetal bovine serum.
GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI= Anaphase I, TI = Telophase I,
MII = Metaphase II, and Deg. = Degenerated.

Table 3: Effect of adding hormone combinations to RPMI-1640 medium on the maturation rate of COCs of the sheep.

Treatment	No. of COCs	Degrees of cumulus cells expansion				Nuclear maturation of COCs						
		0	1	2	3	GV	GVBD	MI	AI	TI	MII	Deg.
		No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)
M_2 (Control)	66	0 (0.00)^{b}	10 (20.68)^{c}	20 (30.00)^{b}	36 (49.32)^{b}	1 (3.13)^{b}	29 (45.11)^{cd}	25 (33.86)^{b}	4 (6.36)^{b}	3 (4.69)^{b}	2 (4.69)^{b}	2 (2.78)^{b}
M_2 + H	65	0 (0.00)^{b}	8 (12.73)^{d}	40 (62.95)^{a}	17 (24.33)^{d}	1 (1.11)^{c}	33 (48.87)^{c}	23 (34.10)^{b}	2 (4.72)^{b}	3 (7.04)^{a}	0 (0.00)^{f}	3 (4.17)^{c}
M_2 + H + FBS	62	1 (1.39)^{b}	4 (5.28)^{e}	26 (36.39)^{ab}	31 (56.94)^{a}	1 (1.39)^{c}	28 (45.04)^{cd}	28 (41.59)^{a}	1 (2.38)^{c}	0 (0.00)^{c}	3 (7.22)^{c}	1 (2.38)^{e}

Values in the same column with different superscripts differ significantly (P <0.05).
Table represents mean numbers of oocytes (%) in each stage of maturation (M %).
M2 = RPMI-1640 medium FBS = fetal bovine serum, and H=PMSG+hCG+E2
GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI= Anaphase I, TI = Telophase I, MII = Metaphase II, and Deg. = Degenerated.

Table 4: Effect of adding hormone combinations to RPMI-1640 medium on the maturation rate of denuded sheep oocytes.

Treatment	No. of denuded oocytes	Homogeneity of cytoplasm		Nuclear maturation of COCs						
		Homo-genous	Hetero-geneous	GV	GVBD	MI	AI	TI	MII	Deg.
				No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)
M_2 (Control)	120	117	3	3 (2.11)^{d}	61 (48.14)^{c}	35 (35.74)^{a}	1 (0.54)^{c}	0 (0.00)^{b}	0 (0.00)^{b}	17 (13.47)^{c}
M_2 + H	109	106	3	0 (0.00)^{e}	45 (49.19)^{c}	41 (39.28)^{a}	3 (1.11)^{b}	1 (1.28)^{b}	4 (3.3)^{a}	12 (5.83)^{d}
M_2 + H + FBS	65	64	1	6 (7.20)^{b}	31 (47.96)^{c}	25 (39.28)^{a}	0 (0.00)^{d}	0 (0.00)^{d}	0 (0.00)^{d}	2 (5.56)^{d}

Values in the same column with different superscripts differ significantly (P <0.05).
Table represents mean numbers of oocytes (%) in each stage of maturation (M %).
M2 = RPMI-1640 medium medium, FBS = fetal bovine serum and H=PMSG+hCG+E2
GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI= Anaphase I, TI = Telophase I, MII = Metaphase II, and Deg. = Degenerated.

Table 5: Effect of type of culture media on the maturation rate of COCs of the sheep.

Type of medium	No. of COCs	Degrees of cumulus cells expansion				Nuclear maturation of COCs						
		0	1	2	3	GV	GVBD	MI	AI	TI	MII	Deg.
		No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)
TCM - 199	246	16 (2.96)[a]	97 (38.26)[a]	65 (30.42)[b]	68 (24.50)[b]	19 (5.26)[a]	76 (30.76)[b]	58 (31.51)[b]	7 (2.92)[b]	26 (9.51)[a]	59 (19.84)[a]	1 (0.33)[b]
RPMI - 1640	193	1 (0.44)[b]	22 (12.92)[b]	86 (43.08)[a]	84 (43.59)[a]	3 (1.88)[b]	90 (46.42)[a]	76 (36.42)[a]	7 (4.48)[a]	6 (3.70)[b]	5 (3.98)[b]	6 (3.11)[a]

Values in the same column with different superscripts differ significantly (P <0.05).
Table represents mean numbers of oocytes (%) in each stage of maturation (M %).
COCs = Excelent + good oocytes.
GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI= Anaphase I,
TI = Telophase I, MII = Metaphase II, and Deg. = Degenerated.

Table 6: Effect of type of culture media on the maturation rate of denuded sheep oocytes**.**

Type of medium	No. of denuded oocytes	Homogeneity of cytoplasm		Nuclear maturation of COCs						
		Homo-genous	Hetero-geneous	GV	GVBD	MI	AI	TI	MII	Deg.
				No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)
TCM - 199	389	380	9	144 (41.18)[a]	124 (31.01)[b]	88 (21.92)[b]	7 (1.67)[a]	7 (1.76)[a]	10 (2.50)[a]	0 (0.00)[b]
RPMI - 1640	294	287	7	9 (3.10)[b]	137 (48.48)[a]	101 (48.13)[a]	4 (0.57)[b]	1 (0.44)[b]	4 (1.07)[b]	31 (8.27)[a]

Values in the same column with different superscripts differ significantly (P <0.05).
Table represents mean numbers of oocytes (%) in each stage of maturation (M %).
GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI= Anaphase I,
TI = Telophase I, MII = Metaphase II, and Deg. = Degenerated.

Table7 (a): Effect of oocytes quality (COCs & denuded) on the nuclear maturation rate of sheep oocytes cultured in TCM-199 medium.

Medium	Class	No. of oocytes	Nuclear maturation													
			GV		GVBD		MI		AI		TI		MII		Deg.	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
TCM - 199	COCs	246	19	7.72**	76	30.89	58	23.58	7	2.85	26	10.57**	59	23.98**	1	0.14
	Denuded	380	144	37.02	124	31.88	88	22.62	7	1.80	7	1.80	10	2.57	0	0.00

** Significant at P < 0.01.

Table7 (b): Effect of oocytes quality (COCs & denuded) on the nuclear maturation rate of sheep oocytes cultured in RPMI-1640 medium.

Medium	Class	No. of oocytes	Nuclear maturation													
			GV		GVBD		MI		AI		TI		MII		Deg.	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
RPMI – 1640	COCs	193	3	1.55	90	46.63	76	39.38	7	3.63	6	3.11	5	2.59	6	3.11
	Denuded	287	9	3.06	137	46.60	101	34.35	4	1.36	1	0.34	4	1.36	31	10.54**

** Significant at P < 0.01.

DISCUSSION

The present results showed that the supplementation of hormone combinations (PMSG+hCG+F_2) or hormones plus FBS to the culture media (TCM-199or RPMI-1640) improved maturation rate of sheep COCs compared to the control media (without additives). Our findings were supported by the reports of some studies on the *in vitro* maturation of goat oocytes. These studies have supplemented the maturation medium with FSH, LH and 17-esradiol and 20% FCS (Mogas *et al.* 1995) or 10% FCS (Pawshe *et al.* 1996). In the experiment conducted by Mogas *et al.* (1995), the supplementation of maturation medium with FCS + hormones led to 55% maturation rate. Pawshe *et al.* (1996) reported 50 and 50-65% of oocytes matured in the media supplemented with FCS and FCS + hormones, respectively. Their results showed that there was no significant difference between the two concentrations of FCS.

The present data are also consistent with those reported by Mogas *et al.* (1997 a-b) who achieved a high *in vitro* maturation rate of goat oocytes in the presence of hormonal supplementation (FSH+LH+E_2) plus estrous goat serum (EGS). Also, our results are in agreement with those cited by Totey *et al.* (1992) in buffalo and Kuwer *et al.* (1999) in cattle. In another study, Keskintepe *et al.* (1994) reported that the cleavage rate of caprine oocytes previously matured in TCM-199 medium enriched with 10% FCS and hormonal supplements (FSH+LH+E_2) was significantly higher than in hormone-free medium.

The present results also demonstrated that addition of hormones alone (gonadotropins and 17-estradiol) without FBS, improved sheep oocytes maturation rate. As observed in the present results, cattle and buffalo oocytes matured *in vitro* in the presence of gonadatropins and estradiol resulted in high maturation rates compared to maturation protocols in which no hormones were used (Saeki *et al.* 1990; Totey *et al.,* 1992). Also, in other studies, Moor and Trounson (1977); Ledda *et al.* (1997) and Szöllösi *et al.* (1988) reported high fertilization rates of sheep oocytes previously matured in the culture medium in the presence of hormonal supplementation (LH+FSH+E_2). In addition, Attia (2001) indicated that the supplementation of gonadotropins (LH and FSH) and 17-estradiol (E_2) to the maturation medium enhances the fertilizability and developmental efficiency of ovine oocytes, which has been verified by the significant elevation that existed in fertilization and cleavage rates of sheep oocytes cultured in hormonal-supplemented medium as compared to the control (72 and 74.07% vs. 50 and 54.54%, respectively). Our findings were also similar to the observation of Pawshe *et al.* (1996) who reported that estradiol and gonadotropins usually cause synergistic enhancement of nuclear maturation of caprine oocytes.

Moor and Trounson (1977) and Pawshe *et al.* (1996) showed that gonadotropins are the primary regulators of nuclear maturation in mammalian oocytes *in vitro*, and one of the functions of its preovulatory surge is to suppress the granulose cell factors that inhibit meiosis. On the other hand, estradiol has been found to improve the completion of maturational changes (Moor & Warnes, 1978) and also supported the synthesis of presumed male pronuclear growth factor (Fukui & Ono, 1989). However, Wahid *et al.* (1991) reported that higher rate of nuclear maturation can be achieved in sheep oocytes that were cultured in TCM-199 medium without hormonal supplementation.

Cumulus cell expansion was found in the present study to be activated by the addition of hormone combinations (PMSG+hCG+E_2) or hormone combinations plus FBS to the culture medium (TCM-199) or to the tested medium RPMI-1640, respectively. The present findings were almost similar to those reported by Braun (1988) who found that FCS but not BSA was able to support FSH-induced cumulus expansion of sheep oocytes. Comparable results were also found by Younis *et al.* (1991) in goats. They reported that the addition of LH or FSH to the culture medium enhanced cumulus expansion of goat oocytes

than those cultured in hormone-free medium. Morover, Younis *et al.* (1992) cultured goat oocytes in TCM-199 medium supplemented with 20% goat serum with addition/ml of (a) 5µg FSH, (b) 100µg LH, (c) 0.5µg TSH, or (d) no hormone (control), in 4 IVM trials. They obtained cumulus expansion rates of 100, 100, 83 and 42%, respectively.

The present results were also supported by reports of Sanbuissho and Threlfall (1988) and Chen *et al.* (1994) who suggested that FSH has a beneficial effect in the presence of FCS or fetal bovine serum and enhances cumulus expansion of bovine oocytes. In addition, Armstrong and Xia (1993) indicated that there was an effective role of FSH and LH in inducing cumulus expansion of cow oocytes. In buffalo, Barile *et al.* (1990) showed that 69.4% of the oocytes reached full cumulus expansion after maturation in the media supplemented with gonadotropin hormones and FCS. Chauhan *et al.* (1996) found that a higher percentage of buffalo oocytes cultured in the medium supplemented with FSH reached the maximum cumulus expansion and maturation compared to those cultured in medium without FSH. Also, Gupta *et al.* (2001) found that the addition of PMSG to the maturation media enhanced cumulus expansion of buffalo oocytes compared to those cultured in the media without PMSG. Buccione *et al.* (1990) explained that the presence of gonadotropins in the maturation media increases the level of intracellular cAMP, the activity of the hyaluronic acid synthesis enzyme system, and induced cumulus expansion in intact complexes.

In the present results it was showed that TCM-199 medium was more efficacious for *in vitro* maturation of sheep oocytes than RPMI-1640 medium. The proportion of COCs or denuded oocytes reaching MII was significantly increased (P<0.05) in the groups that were cultured in TCM-199 medium than those cultured in RPMI-1640 medium (19.84 or 2.5 vs. 3.98 or 1.07, respectively). Our results are in agreement with those reported by Gliedt *et al.* (1996), who showed that TCM-199 medium was superior to RPMI-1640 medium in promoting IVM of bovine oocytes. The results are also consistent with those reported by Bavister *et al.* (1992), who indicated that TCM-199, has given higher maturation rates of in vitro maturation, and fertilization for bovine oocytes than Ham's F-12 or Waymouth medium. Also, a higher rate of maturation in buffalo (Totey *et al.*, 1993) and goats (Pawshe *et al.,* 1996), oocytes was achieved in TCM-199 than with Ham's F-10 medium. Sahoo *et al.* (1998) reported that nearly 85.7% of bovine oocytes reached MII when they matured in TCM-199 medium compared to 60.3% of the oocytes which cultured in minimum essential medium. In other circumstances, in sheep Rexroad and Powell (1988) reported that TCM-199 supported more cleavage of *in vitro* fertilized oocytes than did Ham's F-10. Similarly, Attia (2001) found that in sheep oocytes the proportion of *in vitro* fertilized as well as the subsequently cleaved oocytes which previously matured in TCM-199 medium was significantly higher (P<0.01) than those matured in Ham's F-10 medium (70; 81.42% vs. 42.85; 36.66%, respectively). Furthermore, high cleavage rate of buffalo oocytes was achieved when the oocytes were previously matured in TCM-199 compared to those found in Ham's F-10 or MEM or FertiCult medium (Hegab et al., 2009).

Krisher and Bavister (1998) reported that the differences between different culture media in oocytes IVM may be due to the composition of the medium. Maturation media supplemented with essential and non-essential amino acids supported maturation and development after fertilization more than that supplemented with essential amino acids or glutamine alone. Furthermore, in a previous study Bae and Foote (1975) found that the addition of glutamine to the media had a beneficial effect by providing both energy and ammonia nitrogen to the maturing oocytes of the rabbit. Therefore, the higher maturation rate *in vitro* of sheep oocytes which was achieved with TCM-199 medium than RPMI-1640 medium in the present work might be attributed to some factors in its composition such as essential and non-essential amino acids, glutamine and insulin which stimulates DNA and RNA synthesis and enhances cell division in both media (Rexroad & Powell; 1988, Gordon, 2003, Bilodeau- Goeseels, 2006, Gilchrist &Thompson, 2007).

The present results demonstrated that COCs recorded higher maturation rate (P<0.01) compared to denuded oocytes in TCM-199 supplemented groups (23.98 vs. 2.57, respectively). Also, the proportion of COCs reaching MII increased more than that of denuded oocytes in RPMI-1640 supplemented groups (2.59% vs. 1.36%, respectively). These findings are in agreement with those reported on foreign sheep breeds by Shirazi *et al.* (2007) who found that the percentage of MII of COCs groups was significantly higher (P<0.05) than that of denuded oocyte groups (82.2 vs. 4.8, respectively). Also, the present results were consistent with those obtained in bovine by Leibfried and First (1979) and Kim *et al.* (1997). Leibfried and First (1979) observed that 71% of oocytes with cumulus cells could be matured *in vitro* compared to 44% of denuded

oocytes. Kim *et al.* (1997) found that the maturation rate of cumulus intact bovine oocytes reached 86.2% compared to 54.3% of denuded oocytes. The present findings were also supported by Das *et al.* (1997) who found that the maturation of denuded buffalo oocytes was significantly lower ($P<0.05$) compared to that of cumulus oocytes complexes (COCs). In addition, Datta and Goswami (1999) reported that nearly 70% of the good quality buffalo oocytes reached MII compared to 22% of the poor quality oocytes. Similar findings were also reported by other investigators on *in vitro* maturation of mice and porcine oocytes (Fagbohun & Downs, 1991; Coskun & Lin, 1994).

Moreover, several have studies demonstrated that the mammalian oocytes surrounded with cumulus cells (COCs) have significantly higher in vitro maturation rate and also higher in vitro fertilization and developmental rates than the denuded oocytes. (Nandi *et al.*, 1998; Chauhan *et al.,* 1998; Bilodeau-Goeseels & Panich 2002; and Lonergan *et al.* 2003). On the other hand, some studies explained the importance of cumulus cells in the oocytes. For example, Moor and Seamark (1986) and Mori et al. (2000) suggested that the coupling of cumulus cells and oocyte is important for the uptake of nutrients by oocytes during maturation in culture medium and to facilitate the transport of signals into and out of the oocyte. Furthermore, Cox *et al*., (1993), Chauhan *et al*., (1997b) and Ali and Sirard (2002) reported that the presence of cumulus cells during IVM of animal oocytes may provide an energy source or may produce factor (s) or hormones capable of regulating maturation.

Therefore the higher nuclear maturation rate which was achieved by COCs compared to denuded oocytes in the present study might be due to the presence of cumulus cells surrounding the oocytes.

In conclusion the present study showed that the addition of hormone combinations (PMSG + hCG + E2) with FBS to culture media (TCM- 199 or RPMI- 1640) could significantly improve the IVM of sheep oocytes especially COCs). Also, TCM – 199 medium is more effective for in vitro maturation of sheep oocytes than RPMI- 1640 medium. Moreover, for optimum oocyte nuclear maturation, the use of COCs is essential.

ACKNOWLEDGEMENTS

With profound gratitude and appreciation, we thank Prof. Omar A. Salama, Chief Researcher of Animal Reproduction Physiology, sheep and Goat Research Department, Animal Production Research Institute, Ministry of Agriculture and Prof. Mohamed Essam M. El-Nahass, Cell Biology Dept., National Research Center for generously providing some facilities in this work.

REFERENCES

Ali, A. and Sirard, M.A. (2002): Effect of the absence or presence of various protein supplements on further development of bovine oocytes during in vitro maturation. Biol. Reprod., 66: 901-905.

Armstrong, D. T. and Xia, P. (1993): Differential mitogenic action of insuline-like growth factors-I and FSH on bovine cumulus cells and granulose cells. Theriogenology, 39: 181.

Arunakumari, G.; Vagdevi, R.; Rao, B.S.; Naik, B.R.; Naidu, K.S.; Kumar, R.V.S. and Rao, V.H. (2007): Effect of hormones and growth factors on in vitro development of sheep preantral follicles. Small Rumin. Res., 70:93-100.

Attia, K.H.E. (2001): Studies on in vitro fertilization of small ruminant oocytes. Ph. D. Thesis, Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Egypt.

Bae, I. H. and Foote, R. H. (1975): Effects of hormones on the maturation of rabbit oocytes recovered from follicles of various sizes. J. Reprod. Fertil., 42: 357- 360.

Barile, V.L.; Dell'Aquila, M.E.; Cinone, M. and Minoia, P. (1990): In vitro maturation and fertilization of follicular oocytes in cattle. J. Boll. Soc. Ital. Biol. Sper., 66: 899-906.

Bavister, B.D.; Rose-Hellekant, T.A. and Pinyopummintr, T. (1992): Development of in vitro matured/in vitro fertilized bovine embryos into morulae and blastocysts in defined culture media. Theriogenology, 37: 127-146.

Beker, A.R.; Izadyar, F.; Colenbrander, B. and Bevers, M.M. (2000): Effect of growth hormone releasing hormone (GHRH) and vasoactive intestinal peptide (VIP) on in vitro bovine oocyte maturation. Theriogenology, 53: 1771-1782.

Bilodeau-Goeseels, S. (2006): Effects of culture media and energy sources on the inhibition of nuclear maturation in bovine oocytes. Theriogenology, 66: 297-306.

Bilodeau-Goeseels, S. and Panich, P. (2002): Effects of oocyte quality on development and

transcriptional activity in early bovine embryos. Anim. Reprod. Sci., 71: 143-155.

Binor, Z. and Wolf, D.P. (1979): In vitro maturation and penetration of mouse primary oocytes after removal of the zona pellucida. J. Reprod. Fertil., 56: 309-314.

Bolamba, D.; Russ, K.D.; Harper, S.A.; Sandler, J.L. and Durrant, B.S. (2006): Effects of epidermal growth factor and hormones on granulosa expansion and nuclear maturation of dog oocytes in vitro. Theriogenology, 65: 1037-1047.

Brackett, B.G.; Bousquet, D.; Bois, M.L.; Donawick, W.J.; Evans, J.F. and Dressel, M.A. (1982). Normal development following in vitro fertilization in the cow. Biol. Reprod., 27: 147-158.

Brackett, B.G., Younis, A.I. and Fayrer-Hosken, R.A. (1989): Enhanced viability after in vitro fertilization of bovine oocytes matured in vitro with high concentrations of luteinizing hormone. Fertil. Steril., 52: 319-324.

Braun, J. (1988): Influence of protein supplement and culture conditions on cumulus-cell expansion and nuclear maturation of sheep follicular oocytes. Theriogenology, 29:228.

Buccione, R.; Schroeder, A.C. and Eppig, J.J. (1990): Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol. Reprod., 43: 543-547.

Cecconi, S., Mauro, A., Capacchietti, G., Berardinelli, P., Bernabo, N., Di Vincenzo, A. R., Mattioli, M. and Barboni, B. (2008): Meiotic maturation of incompetent prepubertal sheep oocytes is induced by paracrine factor(s) released by gonadotropin-stimulated oocytes –cumulus cell complexes and involves mitogen-activated protein kinase activation. Endocrinology 149 (1): 100-107.

Chauhan, M.S.; Katiyar, P.K. and Madan, M.L. (1997 a): in vitro production of blastocyst in goats, sheep and buffaloes. Indian J. Anim. Sci., 67: 394-396.

Chauhan, M.S.; Katiyar, P.K.; Madan, M.L.; Manik, R.S. and Sigla, S.K. (1996): Influence of follicle stimulating hormone on maturation and development of water buffalo (Bubalus bubalus) oocytes up to blastocysts stage. Indian J. Dairy Sci., 49: 685-688.

Chauhan, M.S.; Palta, P.; Das, S.K.; Katiyar, P.K. and Madan, M.L. (1997 b): Replacement of serum and hormone additives with follicular fluid in the IVM medium: Effects on maturation, fertilization and subsequent development of buffalo oocytes in vitro. Theriogenology, 48: 461-469.

Chauhan, M.S.; Singla, S.K.; Palta, P.; Manik, R.S. and Madan, M.L. (1998): In vitro maturation and fertilization, and subsequent development of buffalo (Bubalus bublais) embryos: effect of oocyte quality and type of serum. J. Reprod. Fertil. Dev., 10: 173-177.

Chen L.; Russell, P.T. and Larsen, W.J. (1994): Sequential effects of follicle-stimulating hormone and luteinizing hormone on mouse cumulus expansion in vitro. Biol. Reprod., 51: 290-295.

Chian, R.C.; Niwa, K. and Sirard, M.A. (1994): Effect of cumulus cells on male pronuclear formation and subsequent early development of bovine oocytes in vitro. Theriogenology, 41: 1499-1508.

Coskun, S. and Lin, Y.C. (1994): Effect of transforming growth factors and activin-A on in vitro porcine oocytes maturation. Mol. Reprod. Dev., 38: 153-159.

Cox, J.F.; Hormázabal, J. and Santa María, A. (1993): Effect of the cumulus on in vitro fertilization of bovine matured oocytes. Theriogenology, 40: 1259-1267.

Das, S.K.; Chauhan, M.S.; Palta, P. and Tomer, O.S. (1997): Influence of cumulus cells on in vitro maturation of denuded buffalo oocytes. Vet. Rec., 141: 522-523.

Datta, T.K. and Goswami, S.L. (1999): Effect of quality of buffalo oocytes on their maturation rate in vitro. Indian J. Anim. Sci., 69: 23-26.

De Felici, M. and Siracusa, G. (1982): Spontaneous hardening of the zona pellucida of mouse oocytes during in vitro culture. Gamete. Res., 6: 107-113.

Fagbohun, C.F. and Downs, S.M. (1991): Metabolic coupling and ligand-stimulated meiotic maturation in the mouse oocyte-cumulus cell complex. Biol. Reprod., 45: 851-859.

Fukui, Y. and Ono, H. (1989): Effects of sera, hormones and granulosa cells added to culture medium for in vitro maturation, fertilization, cleavage and development of bovine oocytes. J. Report. Fertil., 86: 501-506.

Fukushima, M. and Fukui, Y. (1985): Efects of gonadotropins and steroids on the subsequent

fertilizability of extrafollicular bovine oocytes cultured in vitro. Anim. Reprod. Sci., 9: 323-332.

Ge, L.; Han, D.; Lan, G.C.; Zhou, P.; Liu, Y.; Zhang, X.; Sui, H.S. and Tan; J.H.(2008): Factors affecting the in vitro action of cumulus cells on the maturing mouse oocytes. Mol. Reprod. Dev., 75:136-142.

Geshi, M.; Takenouchi, N.; Yamauchi, N. and Nagai, T. (2000): Effects of sodium pyruvate in non-serum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells. Biol. Reprod., 63: 1730-1734.

Gilchrist, R.B. and Thompson, J. G. (2007): Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro. Theriogenology 67: 6-15.

Gliedt, D.W.; Rosenkrans, C.F.; Rorie, JR.R.W.; Munyon, A.L.; Pierson, J.N.; Miller, G.F. and Rakes, J.M. (1996): Effect of media, serum, oviductal cells, and hormones during maturation on bovine embryo development in vitro. J. Dairy Sci., 79: 536-542

Gordon, I. (2003): Laboratory Production of Cattle Embryos, second edition. In: Biotechnology in Agriculture Series, No.27. CAB International, Wallingford, Oxon OX10 8DE, UK.

Gupta, P.S.; Nandi, S.; Ravindranatha, B.M. and Sarma, P.V. (2001): Effect of commercially available PMSG on maturation, fertilization and embryo development of buffalo oocytes in vitro. Reprod. Fertil. Dev., 13: 355-360.

Hegab, A.O., Montasser, A. E., Hammam, A. M., Abu El-Naga, E. M. A. and Zaabel, S. M. (2009): Improving in vitro maturation and cleavage rates of buffalo oocytes. Anim. Reprod. , 6 (2): 416-421.

Hegab, A.O.; Montasser, A.E.; Hammam, A.M.; Abu El-Naga, E.M.A. and Zabel, S.M. (2009): Improving in vitro maturation and cleavage rates of buffalo oocytes Anim. Reprod., 6 (2): 416-421.

Henderson, K. M., McNeilly, A.S. and Swanston, I.A. (1982): Gonadotropin and steroid concentrations in bovine follicular fluid and their relationship to follicle size. J. Reprod. Fertil., 65: 467-473.

Hsu, C.J.; Holmes, S.D. and Hammond, J.M. (1987): Ovarian epidermal growth factor-like activity. Concentrations in porcine follicular fluid during follicular enlargement. Biochem. Biophys. Res. Commun., 147: 242-247.

Keskintepe, L.; Darwish, G.M.; Kenimer, A.T. and Brackett, B.G. (1994): Term development of caprine embryos derived from immature oocytes in vitro. Theriogenology, 42: 527-535.

Khalil, W.K.B. (2003): Effect of Preovulatory LH surge on meiotic progression and mRNA composition during oocytes maturation. Ph.D. Thesis, Georg-August University, Goettingen, Germany.

Kharche, S.D.; Goel, A.K.; Jindal, S.K. and Sinha, N.K. (2006): Technical note: In vitro maturation of caprine oocytes in different concentration of estrous goat serum. Small Rumin. Res., 64: 186-189.

Kim, K.S.; Minami, N.; Yamada, M. and Utsumi, K. (1997): Follicular cells affect the fertilizability and developmental competency of bovine oocytes in vitro. J. Reprod. Fertil. Dev., 9: 763-766.

Krisher, R.L. and Bavister, B. D. (1998): Responses of oocytes and embryos to the culture environment. Theriogenology, 49: 103–114.

Kuwer, A.; Lemme, E. and Niemann, H. (1999): Developmental capacity of cumulus oocytes complexes collected from prepubertal cattle with and without gonadotropin stimulation employing ultrasound-guided follicular aspiration. Theriogenology, 51: 323 (Abs).

Ledda, S.; Bogliolo, L.; Calvia, P.; Leoni, G. and Naitana, S. (1997): Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. J. Reprod. Fertil. , 109: 73-78.

Leibfried, L. and First, N.L. (1979): Characterization of bovine follicular oocytes and their ability to mature in vitro. J. Anim. Sci., 48: 76-86.

Lonergan, P.; Rizos, D.; Gutiérrez-Adán, A.; Fair, T. and Boland, M.P. (2003): Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. Reprod. Domest. Anim., 38: 259-267.

Madan, M.L.; Chauhan, M.S.; Singla, S.K. and Manik, R.S. (1994): Pregnancies established from water buffalo (Bubalus bubalis) blastocysts derived from in vitro matured, in vitro fertilized and co-cultured with cumulus and oviductal cells. Theriogenology, 42: 591-600.

Magnusson, C. (1980): Role of cumulus cells for rat oocytes maturation and metabolism. Gamete.

Res., 3: 133-140.

Mogas, T.; Izquierdo, M.D.; Palomo, M.J. and Paramio, M.T. (1995): Effect of hormones, serum source and culture system on the IVM and IVF of prepubertal goat oocytes and subsequent embryo development. Theriogenology, 34: 284.

Mogas, T.; Palomo, M.J.; Izquierdo, M.D. and Paramio, M.T. (1997 a): Developmental capacity of in vitro matured and fertilized oocytes from prepubertal and adult goats. Theriogenology, 47: 1189-1203.

Mogas, T.; Palomo, M.J.; Izquierdo, M.D. and Paramio, M.T. (1997 b): Morphological events during in vitro fertilization of prepubertal goat oocytes matured in vitro. Theriogenology, 48: 815-829.

Moor, R.M. and Seamark, R.F. (1986): Cell signaling, permeability and microvasculatory changes during antral follicles development in mammals. J. Dairy Sci., 69: 927-943

Moor, R.M. and Trounson, A.O. (1977): Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J. Reprod. Fertil., 49:101-109.

Moor, R.M. and Warnes, G.M. (1978): Effect of oocytes maturation in mammals. In: Crighton, D.B.; Foxcroft, G.R., Haynes, N.B. and Lamming, G.E. (eds) Control of Ovulation. Butterworths, London, pp.159-176.

Mori, T.; Amano, T. and Shimizu, H. (2000): Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured in vitro. Biol. Reprod., 62: 913-919.

Nandi, S.; Chauhan, M.S. and Palta, P. (1998): Influence of cumulus cells and sperm concentration on cleavage rate and subsequent embryonic development of buffalo (Bubalus bubalis) oocytes matured and fertilized in vitro. Theriogenology, 50: 1251-1262.

Pawshe, C.H.; Palanisamy, A.; Taneja, M.; Jain, S.K. and Totey, S.M. (1996): Comparison of various maturation treatments on in vitro maturation of goat oocytes and their early embryonic development and cell numbers. Theriogenology, 46: 971-982.

Rexroad, C.E.Jr. and Powell, A.M. (1988): Co-culture of Ovine ova with oviductal cells in Medium-199. J. Anim. Sci., 66: 947-953.

Saeki, K.; Hoshi, M.; Leibfried-Rutledge, M.L. and First, N.L. (1990): In vitro fertilization and development of bovine oocytes matured with commercially available follicle stimulating hormone. Theriogenology, 34: 1035-1039.

Sahoo, T.K., Mohanty, D.N.; Mohanty, B. N, and Barik, A.K. (1998): attachment of spermatozo to in vitro matured bovine oocytes in TCM-199 and MEM media. Indian J. Exp Biol., 36: 367-370.

Sanbuissho, A. and Threlfall, W.R. (1988): The influence of serum and gonadotropins on bovine oocytes maturation in vitro. Theriogenology, 29: 301(Abs).

Sanbuissho, A. and Threlfall, W.R. (1989): The effects of estrous cow serum on the in vitro maturation and fertilization of the bovine follicular oocyte. Theriogenology, 31: 693-699.

SAS (1996): SAS/Stat. User's Guide Static's, Ver., 6.06 4th ed. SAS Institute Inc. Cary, NC.

Seydou, S.; Oladele, G.; Eugene, A. and Seyoum, G. (1999): The effects of serum source and hormone supplementation on goat oocytes maturation, fertilization and early embryo development during the non-breeding season. Society for the study of reproduction, 32nd Annual Meeting, July 31- August 3, 1999.

Shirazi, A.; Shams-Esfandabadi, N.; Hosseini, S.M. and Karimi, I. (2007): The presence of cumulus cells on nuclear maturation of sheep oocytes during in vitro maturation. Small Rumin. Res., 68: 291-295.

Sirard, M.A. (1989): Practical aspects of in vitro fertilization in cattle. J. Reprod. Fertil. Suppl., 38: 127-134.

Snedecor, G.W. and Cochran, W.G. (1989): Statistical methods. Iowa State University Press, USA.

Szöllösi, D.; Desmedt, V.; Crozet, N. and Brender, C. (1988): In vitro maturation of sheep ovarian oocytes. Reprod. Nutr. Dev., 28: 1047-1080.

Tajik, P. and Shams-Esfandabadi, N. (2003): In vitro maturation of caprine oocytes in different culture media. Small Rumin. Res., 47: 155-158.

Tamilmani, G.; Rao, B.S.; Vegdevi, R.; Amarnath, D.; Naik, B.R.; Mutharao, M. and Rao, V.H. (2005): Nuclear maturation of bovine oocytes in cultured preantral follicles. Small Rumin. Res., 60: 295-305.

Totey, S.M., Pawshe, C.H . and Singh, G.P. (1993): In vitro maturation and fertilization of buffalo oocytes (Bubalus bubalus): effects of media, hormones and sera. Theriogenology 9: 153-

171.

Totey, S.M., Singh, G., Taneja, M, Pawshe, C.H. and Talwar, G.P. (1992): In vitro maturation, fertilization and development of follicular oocytes from buffalo (Bubalus bubalus). J. Reprod. Fertil., 95: 595- 607.

Tsafriri, A., Cao, X., Ashkenazi, H., Mottola, S.; Popliker M. and Pomerantz, S.H. (2005): Resumption of oocyte meiosis in mammals: on models, meiosis activating sterols, steroids and EGF-like factors. Mol. Cell. Endocrinol., 234 (1-2): 37- 45.

-Vanderhyden, B.C. and Armstrong, D.T. (1989): Role of cumulus cells and serum on the in vitro maturation, fertilization, and subsequent development of rat oocytes. Biol. Reprod., 40 (4): 720 - 728.

Wahid, H.; Gordon, I.; Sharif, H.; Lonergan, P.; Monaghan, P. and Gallagher, M. (1991): Development of ovine blastocysts following maturation, fertilization and culture of oocytes in vitro. Proceedings of the Seventh Meeting of the European Embryo Transfer Association (Cambridge), 214.

Webb, R.J.; Bains. H.; Cruttwell, C. and Caroll, J. (2002): Gap-junctional communication in mouse cumulus-oocyte complexe: implications for the mechanism of meiotic maturation. Reproduction, 123: 41-52.

Yadav, B.R.; Katiyar, P.K.; Chauhen; M.S. and Maden, M.L. (1997): Chromosome configuration during in vitro maturation of goat, sheep and buffalo oocytes. Theriogenology, 47: 943- 951.

Yamauchi, N. and Nagai, T. (1999): Male pronuclear formation in denuded porcine oocytes after in vitro maturation in the presence of cysteamine. Biol. Reprod., 61: 828-833.

Younis, A.I.; Keskintepe, L.; Mackie. K. and Brackett, B.G. (1992): In vitro maturation and fertilization of Toggenburg goat oocytes. Theriogenology, 37: 330 (Abs).

Younis, A.I.; Zuelke, K.A.; Harper, K.M.; Oliveria, M.A. and Brackett, B.G. (1991): In vitro fertilization of goat oocytes. Biol. Reprod., 44: 1177-1182.