Effect of Protein Additives on in vitro Maturation of Egyptian Sheep Oocytes with Reference to Seasonal Variation Effects on Yield and Quality of Oocytes

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Abstract: The present study was designed to investigate the effect of supplementing of protein additives (PA) in forms of 10% ovine amniotic fluid (OAF), 10% fetal bovine serum (FBS) and 10% sheep serum (SS), individually to culture media (TCM-199 or RPMI-1640) on in vitro nuclear maturation (IVM) of sheep oocytes. In addition, the effect of seasonal variations on the yield and quality of recovered sheep oocytes was also evaluated. Sheep ovaries were collected from local slaughterhouse. Cumulus-oocytes complexes (COCs) and denuded oocytes (DOs) were aspirated and matured in culture media (TCM-199 or RPMI -1640) for 26-29h at 39°C under 5% CO_2 in air and 95% humidity. The results showed that the supplementation of protein additives (PA) in forms of OAF, FBS or SS to culture media (TCM-199 or RPMI-1640) are required for IVM of sheep oocytes. The supplementing of PA to culture media was more efficacious in TCM-199 than RPMI-1640 for IVM of oocytes. The COCs were more response for PA than denuded oocytes. The spring was the best season for recovery of COCs which have a worthy competence to be matured in vitro. In conclusion, the study demonstrated the importance of PA with the suitable culture media during the proper season for IVM of sheep oocytes as an abundant source for production of transgenics and cloning. [Journal of American Science 2010;6(10):588-599]. (ISSN: 1545-1003).

Key words: Protein additives, nuclear maturation, sheep, oocytes, season, quality.

1. Introduction:

In vitro production of embryos is a multi – step process: oocytes maturation, fertilization and embryo culture. Oocyte maturation is the first and the most critical step towards successful in vitro embryo production. In vitro maturation (IVM) of oocytes provide an excellent opportunity for cheap and abundant embryos for carrying out basic research and for the application of emerging biotechnologies like cloning and transgenic (Li *et al.*, 2006).

Several workers have studied different aspects of IVM in mammalian oocytes (Roa *et al.*, 2002 and Kharche *et al.*, 2005). The maturation media with the selection of protein supplements and hormones play an important role for IVM in and subsequent for IVF and in vitro development (Motlagh *et al.*, 2008). In most studies on IVM of animal oocytes, the basic medium is supplemented with different kinds of sera. (Motlagh *et al.*, 2008 and Hegab *et al.*, 2009). The importance of sera may due to its contents of hormones, trace nutrients and proteins such as globulin and futuin (Hsu *et al.*, 1987). The addition of serum to the culture medium provides a source of albumin that balances the osmolarity and acts as a free radical scavenger (Thompson, 2000).

Also, in some experiments, the protein additives of the same species were utilized for in vitro maturation of their occytes. Examples of such protein additives were estrous goat serum for caprine occytes (Keskintepe *et al.*, 1994), water buffalo follicular fluid for buffalo oocytes (Tajik *et al.*, 2000), porcine follicular fluid for porcine oocytes (Funahashi *et al.*, 1997), and equine follicular fluid for equine oocytes (Hinrichs *et al.*, 1995).

Sheep oocytes have also been studied for different aspects of maturation (Roa *et al.*, 2002). In all experiments, maturation media was supplemented with FCS (Ghasemzadeh- Nava and Tajik, 2000), estrous sheep serum (ESS) (Ghasemzadeh- Nava and Tajk, 2000), human serum (Thomson *et al.*, 1998) and mare serum (Motlagh *et al.*, 2008).

Ovine amniotic fluid (OAF) has not been previously knowed to be used as a protein additive to culture media for IVM of sheep oocytes. Moreover, little informations are available about the use of sheep serum (SS) and fetal bovine serum (FBS) for IVM of Egyptian sheep oocytes. On the other hand, the success of IVM in livestock species depends on the quality and quantity of oocytes recovered per ovary from the slaughtered animals.

Seasonal effect was found to have an important consideration in this respect, especially in buffalo (Kadoom, 1995 and Das *et al.,* 1996), mare (Bruck *et al.,* 1996 and Colleoni *et al.,* 2004) and cattle (Silva *et al.,* 2006). However, such effect in sheep has not been recognized during previous studies.

Therefore, the present study was designed (i) to investigate the effect of addition of OAF, SS and FBS individually to culture media (TCM-199 or RPMI-1640) on in vitro nuclear maturation rate of sheep oocytes; also (ii) to evaluate the effect of seasonal variations on the yield and quality of recovered follicular sheep oocytes.

2. Materials and Methods:

2.1. Chemicals

Chemicals used during this study were purchased from sigma chemicals Co. (St. Louis, Mo, USA) unless otherwise indicated. Solutions expressed as percents were prepared as volume-to-volume (v/v) dilutions. All media used for IVM were incubated at 38.5-39°C and 5% CO_2 with maximum humidity for 4h before use. Concerning ovine amniotic fluid (OAF) and sheep serum (SS), they were prepared according the methods of Ocana- Quero *et al.* (1994) and Rao *et al.* (2002), respectively as follows:

a- Preparation of OAF:

Uteri were removed from pregnant ewes at a local slaughterhouse and transported to the laboratory in an ice box within 2h. Gestational stages were estimated by the length of the embryos to be 4 to 6wk (3.6 to 4cm). After opening the uteri and exposing the amniotic membrane, sterile amniotic fluid was aspirated with a 50 ml syringe equipped with an 18 ga needle. The amniotic fluid was centrifuged at 500 g for 10 min to remove the cellular component. The supernatant was heat- inactivated at 56°C for 30 min, filtered using 0.22 µm millipore filter, alliquated and stored at -20°C until being used for the culture of oocytes (Ocana-Quero *et al.,* 1994).

b- Preparation of SS

Blood samples were obtained from ewes during slaughtering (exsanguinations). These samples were collected in sterile conical tubes and left to clot for 1-2h at room temperature (~25°C). After blood clotting, tubes were transferred to a refrigerator (4-5°C) and the serum was allowed to separate. The serum was carefully collected and centrifuged at 1000g for 10 min. The serum was heat- inactivated, filtered, alliquated and stored at -20°C until use (Rao *et al.,* 2002).

Considering fetal bovine serum (FBS), it was purchased from sigma chemicals Co., heat-inactivated, allocated and stored at -20°C until use.

2.2. Collection of ovaries

Ovaries of sheep were obtained at an abattoir about 15-20 min after slaughering. They were transported within 2-3 h to the laboratory in 0.9% saline supplemented with 50 µg/ml gentamycin sulfate at 30 to 35°C.

2.3. Experiments

2.3.1. Experiment 1

During this experiment, the effect of addition of OAF, SS and FBS individually to culture media (TCM-199 or RPMI-1640) on in vitro maturation of sheep oocytes was studied. Oocytes from all visible antral follicles (2 to 6 mm in a diameter) in collected ovaries were aspirated with a 20 ga hypodermic needle attached to a 5 ml disposable syring containing 1 ml of aspiration medium. The aspiration medium consisted of Dulbecco's phosphate buffer saline (D-PBS) supplemented with 0.03 g/ml bovine serum albumin and 50 ug/ml gentamycin sulfate (Chauhan *et al.,* 1997).

Cumulus –oocytes complexes (COCs) (with an unexpanded mass cumulus cells and homogenous cytoplasm) and denuded oocytes (DOs) (with homogenous cytoplasm) were recovered under a stereomicroscope. Both the COCs and DOs were individually washed once with aspiration medium and twice in basic culture medium TCM -199 or RPMI-1640. These media (TCM-199 or RPMI-1640) enriched with 50 µg/ml gentamycin sulfate and without any hormone or serum supplementation. The same non-supplemented medium (TCM-199 or RPMI-1640) was used as control for three different culture media supplements as follows : (1) TCM-199 or RPMI-1640 medium supplemented with 10% (OAF); (2) TCM-199 or RPMI-1640 medium supplemented with 10% (SS); (3) TCM-199 or RPMI-1640 medium supplemented with 10% (FBS).

Each treatment was consisted of about 8 replicates. The non- supplemented media (controls) or the media enriched with protein supplements were sterilized by using 0.22 µm Millipore filter. For all experiments, 10-15 oocytes of COCs or DOs were transferred separately into a 50µl drop of each type of culture media (control medium or medium plus protein supplements), and 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.

Oocytes (COCs or DOs) were cultured for 26-29h at 39°C in an atmosphere of 5% CO_2 in air with 95% humidity.

Following culture period, the degrees of cumulus expansion of COCs were determined. The criteria used for assessing the degrees of cumulus expansion were as follows: Degree O: no expansion; Degree 1- denoted few expansion of cumulus layers or cumulus cells were non – homogeneously spread and clustered cells were still observed; Degree 2- was a moderate expansion of cumulus layers and Degree 3- was the full expansion of cumulus layers. In addition, DOs were classified into two types according to the homogeneity of the cytoplasm, either homo- or heterogenous cytoplasm. All in vitro matured occytes (COCs) were used for assisting the rate of nuclear maturation, irrespective of the degree of expansion (Bolamba *et al.*, 2006).

2.3.1.1. Assessment of the nuclear maturation by cytogenetic analysis

For examining the rate of nuclear maturation (the proportion of oocytes which their nuclei reached metaphase II), cumulus cells of COCs were removed by vortexing. The cumulus- free COCs and DOs with homogenous cytoplasm were then fixed individually in acetic: ethanol (1: 3 v/v) in culture dishes (35 x 10 mm) for at least 45h at 4°C. After that, the oocytes (COCs and DOs) were stained separately for 30 min with 1% (w/v) orcein in 45% (v/v) acetic acid (Rao *et al.*, 2002). 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 (M II). Oocytes with no visible or abnormal chromatin configuration were classified as degenerated (Beker *et al.*, 2000).

2.3.2. Experiment 2

During this experiment, the effects of breeding and non-breeding seasons on oocyte yield and quality in the sheep ovaries were studied. Ovaries from unknown reproductive status or age were collected and transported to the laboratory as before, during winter, spring, summer and autumn seasons. Oocytes were collected and classified into four categories based on their cumulus investment and granularity of ooplasm as follows: Grade A (Excellent oocytes) : corresponded to immature COCs which were completely invested with several layers of dense cumulus cells and homogenous cytoplasm; Grade B (good oocytes) : COCs which had fewer layers (2-4 layers) of compact cumulus investment with homogenous cytoplasm ; Grade C (denuded oocytes): oocytes which had no layers of cumulus cells but they had a homogenous cytoplasm; Grad D: that were defined as those with one or more of the following characteristics: very expanded cumulus, fragmented nucleus, misshaped, partially absent or vacuolated cytoplasm, empty zona pellucida (Wang *et al.*, 1998).

2.4. Statistical analysis

The obtained data for the effect of supplementation protein additives to culture media (TCM-199 or RPMI-1640) on in vitro nuclear maturation rate of sheep oocytes were statistically analyzed by ANOVA using SAS program (SAS, 2001). Fisher's least significant difference (LSD) at 5% significant level ($P<0.05$) was used to test the differences between means of treatments. Data for the effect of protein additives on IVM rate of sheep oocytes based on the type of media and oocytes quality were analyzed using Chi-square test (Snedecor and Cochran, 1989).

3. Results:

3.1. Effect of supplementation of protein additives (PA) in forms of OAF, FBS or SS to TCM-199 medium on IVM rate of sheep oocytes

3.1.1. The effect on COCs

Present results (Table 1) indicated that supplementation of the maturation medium (TCM - 199) with protein additives OAF or FBS or SS individually improved oocytes maturation rate, as indicated by the mean percentage of oocytes developing nuclear MII. This improvement in the oocytes nuclear maturation was significant ($P<0.05$) in OAF supplemented group, and highly significant ($P<0.01$) in FBS or SS supplemented groups compared to the control group (9.60; 17.87 and 20.00 vs. 0.0 %, respectively).

Table (1) revealed also that the proportion of oocytes reaching MII was comparatively increased ($P<0.05$) in either FBS or SS supplemented groups than that found in OAF supplemented group. While, the difference between FBS supplemented group and SS supplemented group for the proportion of oocytes reaching MII was not significant.

It was found that the proportion of full expansion of cumulus cells in oocytes that were cultured in the basic medium (TCM-199) enriched with OAF or with SS was higher than those cultured in TCM-199 medium alone (43.10 and 48.83 vs. 40.50%, respectively). In addition, the proportion of full expansion of cumulus cells in

FBS supplemented group (40.33) was approximately similar to that observed in the control group (40.50). In conclusion, it could be noticed that cumulus cell expansion was more pronounced with OAF or SS than with FBS. Whereas, the difference in the proportion of full expanded cumulus cells between OAF or FBS supplemented groups and the control group was not significant.

Statistical analysis showed that the proportion of full expansion of cumulus cells was significantly elevated ($P<0.05$) in SS supplemented group when compared with control, OAF or FBS supplemented one.

3.1.2. The effect on the denuded oocytes

Cytogenetical examination (Table 2) revealed that the proportion of oocytes reaching MII were absent in the control and SS supplemented groups. However, the addition of OAF but not FBS to TCM-199 medium significantly improved ($P<0.05$) the maturation rate of denuded oocytes compared to control group (1.79 and 0.48 vs 0.0%, respectively)

3.2. Effect of adding different protein additives (PA) in forms of OAF or FBS or SS to culture medium (RPMI-1640) on *in vitro* maturation rate of sheep oocytes:

3.2.1. The effect on COCs

As shown in Table (3), supplementing maturation medium with protein additives (OAF or SS) improved oocytes maturation rate. This improvement in the IVM of the oocyte was highly increased significantly ($P<0.001$ or $P<0.01$) in OAF or SS supplemented groups compared to the control group (11.11 or 10.00 vs 4.69%, respectively). However, the addition of FBS to RPMI-1640 medium significantly decreased ($P<0.05$) oocytes nuclear maturation rate compared to RPMI-1640 medium alone (2.53 vs. 4.69%, respectively).

Concerning the incidence of cumulus cell expansion, it was found that the proportions of full expanded cumulus cells of COCs in FBS supplemented group were approximately similar with those found in the control group (48.71 and 49.32%, respectively). Whereas the proportions of full expanded cells lowered in OAF and SS supplemented groups than those of the control (33.15 and 33.48 vs 49.32%, respectively).

3.2.2. The effect on denuded oocytes

As shown in Table (4), the proportion of oocytes reaching MII was absent for oocytes groups cultured in both of RPMI-1640 medium alone (control) and RPMI-1640 medium supplemented with protein additives groups. On the other hand, the addition of OAF to RPMI-1640 medium significantly ($P<0.01$) improved the proportion of denuded oocytes reaching AI (as matured oocytes) compared to the control medium (1.67 vs. 0.54%, respectively).

3.3. Effect of PA on IVM rate of sheep ocytes based on the type of culture media

As shoen in Table (5 a and b) showed that the supplementation of PA to TCM-199 medium was the most efficacious for IVM of sheep oocytes (COCs or DOs) than the supplementation of PA to RPMI -1640. The proportion of COCs reaching MII (Table 5a) increased in groups that were matured in TCM-199 medium plus PA (OAF, FBS or SS) than that were cultured in RPMI-1640 medium plus PA (13.51, 14.29 and 17.72 vs. 6.12, 3.08 and 2.78%, respectively). This increase was significant ($P<0.05$) in both FBS and SS supplemented groups.

The proportion of DOs reaching MII (Table, 5b) was comparatively increased in groups that were matured in TCM-199 medium plus PA (OAF, FBS and SS) than that were matured in RPMI-1640 medium plus PA (1.38,0.64 and 0.0 vs – 0.0, 0.0 and 0.0%, respectively).

3.4. Effect of PA on IVM rate of sheep oocytes based on oocytes quality

As shown in Table (6a and b), the effect of PA on the improvement of IVM rate of sheep oocytes were more obvious in COCs than denuded oocytes. The proportion of COCs reaching MII (Table, 6a) Significantly ($P<0.01$) increased than denuded oocytes when these oocytes were cultured in TCM-199 medium plus PA (13.51; 14.29 and 17.72 vs 1.38, 0.64 and 0.0%, respectively). Also, the proportion of COCs reaching MII (Table 6b) increased than denuded oocytes when these oocytes were matured in RPMI-1640 plus PA (6.12 , 3.08 and 2.78 vs 0.0, 0.0 and 0.0%, respectively). These increases were only significant in OAF supplemented group.

3.5. Seasonal effect on yield and quality of sheep oocytes

Table (7) revealed that the average numbers of recovered oocytes per ovary were 3.45. 4.31, 3.68 and 3.39 during winter, spring, summer and autumn seasons, respectively. It was evident that more oocytes (4.31) per ovary have been aspirated during spring than those collected during other seasons. On the other hand, it was found that the

lowest average number of collected oocytes per ovary was during autumn (3.39). While, the average number of harvested oocytes per ovary during summer was slightly higher (3.68) than those observed during winter (3.45). Statistical analysis showed that there was a significant difference (P<0.05) between the average number of collected oocytes per ovary during spring and those collected during other seasons.

Concerning the three studied classes of sheep oocytes, irrespective of degenerated oocytes it was observed that the mean value of recovered excellent oocytes increased during spring than those collected during other seasons; winter, summer and autumn (0.45 vs. 0.31, 0.34 and 0.26, respectively). This increase was higher (P<0.05) than those found during winter or during autumn. While, the difference among winter, summer and autumn in the mean percentage of recovered excellent oocytes was not significant.

Also, in the present study, it was found that the mean value of recovered good oocytes was higher (P<0.05) in spring than those collected during winter, summer and autumn (1.04 vs. 0.95, 0.97 and 0.73%, respectively). While, the difference among spring, winter and summer in the mean percentage of recovered good oocytes was not significant. Moreover, the mean value of recovered COCs (excellent +good oocytes) significantly (P<0.05 or P<0.01) increased during spring than those collected during winter, summer and autumn (1.49 vs. 1.26, 1.31 and 0.99%, respectively). Also, the mean percentage of harvested COCs significantly elevated (P<0.05) during winter and summer than that found during autumn (3.45 or 3.68 vs. 3.39%, respectively).

Concerning the denuded oocytes, the present results showed that the mean value of recovered denuded oocytes significantly (P<0.01 or P<0.05) increased during spring than those collected during other seasons; winter, summer and autumn (2.08 vs. 1.64, 1.63 and 1.94, respectively). Also, the mean value of denuded oocytes obtained during autumn was comparatively elevated (P<0.05) than those harvested during winter or during summer.

4. Discussion:

4.1. The effect of adding protein additives to culture media (TCM-199) or (RPMI-1640) on the nuclear maturation rate of sheep oocytes

Results illustrated in Tables (1, 2, 3 and 4) revealed that the supplementation of protein additives (OAF, FBS or SS) to the culture media (TCM-199 or RPMI-1640) provided better maturation rates of sheep oocytes than the control ones (TCM-199 or RPMI-1640 alone). In some treatments when SS or FBS were added to the culture media, they surpassed OAF as maturation promotive factors for sheep oocytes. However, in other treatments, supplementing culture media with OAF induced better results in nuclear maturation rate compared to the addition of FBS or SS. Similarly, Ghasemzadeh-Nava and Tajik (2000) compared the effect of FBS and estrus sheep serum (ESS) on IVM of sheep oocytes and concluded that ESS could support the *in vitro* maturation of the oocytes slightly better. Values were 70, 68 and 61% maturation rates for 10, 15 and 20% of FBS, respectively. Also, the addition of FCS, ESS or LS (Lamb serum) to the culture media (TCM-199 or Ham's F-10) provided better maturation rate of ovine oocytes (Attia, 2001) than BSA. However, ESS surpassed FCS and LS as maturation promotive factor. Furthermore, Rao *et al.* (2002) recorded higher maturation rate of sheep oocytes cultured in media supplemented with ESS as compared to those cultured in the same media supplemented with bovine embryonic fluid, ovine follicular fluid, granulosa cell culture and without serum (86 vs. 77, 76, 82 and 58%, respectively).

Sarseifi (2007) reported that the addition of human menopausal serum, ESS and EGS to maturation medium can enhance the IVM and IVF of sheep oocytes rather than ovine and bovine follicular fluid. Also, in goats, Seydou *et al.* (1999) reported that the maturation rate of caprine oocytes was significantly higher in the presence of fetal bovine serum (FBS) compared to goat serum (GS) (100 vs. 43%; P<0.05). These authors were also concluded that FBS is a superior serum source than GS for *in vitro* maturation of goat oocytes. While, Tajik and Shams-Esfandabadi (2003) found that the addition of ESS, FBS or EGS to TCM-199 medium improved the in vitro maturation rate of caprine oocytes compared to the control medium and no significant differences were observed between the different sera. In contrast, Zheng (2007) found that the addition of sera was not necessary for IVM of rhesus monkey oocytes.

Concerning the influence of OAF on IVM of ovine oocytes, the improvement of IVM rate of oocytes which was observed in the present study due to the use of OAF was also supported by the report of Ocaña-Quero *et al.* (1994) on cattle oocytes which concluded that the IVM rate of cattle oocytes was significantly increased (P <0.001) when oocytes were cultured in medium supplemented with bovine amniotic fluid (bAF) than those cultured in the same medium supplemented with bovine follicular fluid (bFF) (77 vs. 52%, respectively).

As shown in Tables (3 and 7), the addition of SS to the culture media (TCM-199) did not improve the IVM of denuded oocytes. Also, supplementing RPMI-1640 medium with FBS was ineffective in improving the maturation of COCs of sheep oocytes. Moreover, the addition of OAF or FBS or SS to RPMI-1640 did not improve the IVM rate of denuded oocytes. These findings were similar with that reported by El-Maghraby (2004) who found that the addition of BSA and EES to TCM-199 medium did not improve the nuclear maturation rate of sheep oocytes compared to the control medium. Our results were also supported by the report of Zheng and Sirard (1992) who found that the addition of BSA to the maturation media inhibited the maturation of porcine oocyte.

Moreover, Pawshe *et* al. (1996) demonstrated that the addition of estrous goat serum (EGS) or fetal calf serum (FCS) to TCM-199 medium was ineffective in improving the maturation of goat oocytes. In addition, Ali and Sirard (2002) found that the presence of BSA alone in the culture medium possibly had a toxic effect and delayed the maturation process of bovine oocytes.

4.2. The effect of protein additives on cumulus cell expansion

In the present study, cumulus cell expansion was found to be activated with the PA, especially SS or OAF when added to the culture medium (TCM-199). These findings were approximately similar with those reported by Braun (1988) who found that FCS but not BSA was able to support cumulus expansion of sheep oocytes. Also, Chen et al. (1994) suggested that addition of FCS or FBS to culture media led to enhance cumulus expansion of bovine oocytes. In addition, Barile et al. (1990) showed that 69.4% of buffalo oocytes reached full cumulus expansion after maturation in media supplemented with FCS.

4.3. Effect of PA on IVM rate of sheep oocytes based on the type of culture media

The present investigation showed that the addition of PA to TCM-199 medium was more efficacious for *in vitro* maturation of sheep oocytes than addition of PA to RPMI-1640 medium. The proportion of COCs or denuded oocytes reaching MII was comparatively increased in the groups that were cultured in TCM-199 medium supplemented with PA than those cultured in RPMI-1640 medium supplemented with PA (19.84 or 2.5 vs. 3.7 or 1.07, respectively). Similarly, Attia (2001) found that the maturation rate of ovine oocytes cultured in TCM-199 medium plus 10% FCS was significantly higher ($p<0.01$) than those matured in Ham's F-10 medium plus 10% FCS (83.33 vs. 70%, respectively). Moreover, Hegab et al. (2009) revealed that the supplementation of maturation media with FCS led to higher maturation rates (75.6% on average) of buffalo oocytes, without any significant variation between different media, than BSA (71.3% on average). However, those authors found that the supplementation of Ferti cult medium with BSA resulted in a significant increase in the IVM rate of buffalo oocytes (80%) than its addition to other media (TCM-199, Ham's F-10 or MEM). In consistence, a higher rate of maturation in goats (Pawshe et al., 1996) and buffalo (Totey et al., 1993) oocytes achieved in TCM -199 than Ham's F-10 medium.

In other circumstances, Rexroad and Powell (1988) reported that TCM-199 plus FCS supported more cleavage of *in vitro* fertilized sheep oocytes than did Ham's F-10 plus FCS. They concluded that the improved cleavage index could be related to additional factors in TCM-199 such as insulin which stimulates DNA and RNA synthesis and enhances cell division. Also, 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 alone.

Therefore, the higher maturation rate *in vitro* of sheep oocytes which achieved with TCM-199 medium than RPMI-1640 medium in the present work may be attributed to the differences in their ionic and energy sources concentrations in both media (Gordon, 2003).

4.4. Effect of PA on IVM rate of sheep oocytes based on oocytes quality

Addition of PA for culturing of COCs caused higher maturation rate ($P<0.05$) compared to use of PA for culturing of denuded oocytes in either TCM-199 (15.3 vs. 1.4, respectively) or in RPMI-1640 supplemented groups (5.82 vs. 0.53, respectively) medium.

The importance of PA may due to its contents of hormones, trace nutrients, globulin and futuin that promote the maturation of oocytes (Madan *et al.,* 1994). The presence of cumulus cells surrounding the oocytes play an important role for facilitating the transport of nutrients, signals and other promoting factors for maturation into and out of the oocytes (Byskov *et al.,* 1997). One of the routes

by which the factors (e.g. meiosis activating components, glutathione or its substrates glutamine and cysteine, regulatory molecules of less than 1KDa, low molecular weight substrates such as Ca^{+2} nucleotides an amino acids) are transmitted from cumulus cells to the oocyte is the gap junctional communication "GJC" (Webb *et al.*, 2002). Therefore the higher nuclear maturation rate which achieved by COCs compared to the poor quality oocytes (denuded) may due to the presence of cumulus cells surrounding the oocytes.

Similar results were detected by El-Maghraby (2004) who revealed that, sheep COCs (from class A and B) recorded higher maturation rate ($P<0.05$) compared to denuded oocytes (class C). The proportions of oocytes reaching MII in the three classes A, B and C were 86.7, 84.0 and 59.7, respectively. Moreover, Shirazi *et al.* (2007) found that the percentage of MII of sheep oocytes of COCs groups was significantly higher ($P<0.05$) than that of denuded groups (82.2 vs. 4.8, respectively). Also, the present results were in consistence with those obtained in bovine by Kim *et al.* (1997) who found that the maturation rate of cumulus intact bovine oocytes reached 86.2% compared to 54.3% of denuded oocytes. Present findings were also supported by Datta and Goswami (1999) who reported that nearly 70% of the good quality buffalo oocytes reached MII compared to 22% of the poor quality oocytes. In addition, Das *et al.*, (1997) found that the maturation of denuded buffalo oocytes was significantly lower ($P<0.05$) compared to that of cumulus oocytes complexes (COCs).

4.5. Seasonal effect on the recovery rate and quality of sheep oocytes

The present results revealed that more categories COCs and the total number of recovered oocytes per ovary were aspirated during spring than those collected during winter, summer and autumn seasons (1.49; 4.31 vs. 1.26; 3.45 or 1.31; 3.68 and 0.99; 3.39, respectively). This may attributed to the presence of more follicles during the breeding season. These findings are similar to those reported on sheep by Attia (2001), who found that the reduction in the proportion of good culturabel oocytes (class A and B) was evident among those recovered during summer and autumn (16.46%, 24.19% and 15.76%, 23.57%, respectively) than those harvested during winter and spring (50.83%, 57.77% and 28.90%, 28.88%, respectively). It could be suggest that oocytes were compromised during development and differentiation when climatic factor are not ideal for reproduction.

The present findings were also supported by Datta and Goswami (1998) who observed that, the average number, as well as, the proportion of good quality oocytes that were retrieved from buffalo ovaries during cool months (<25°C) was significantly higher than the corresponding values obtained during moderately hot (25-30°C) and hot (>30°C) months. They suggested that oocytes are compromised during development and differentiation when climatic factor are not ideal for reproduction. Also, Zohier *et al* (2007) found that the proportion of collected number of good buffalo oocytes significantly increased during spring and winter than those collected during summer and autumn (71 and 74.6 vs. 50 and 56.9%, respectively). In addition, Brück *et al.* (1996) revealed that the rate of oocytes recovery in mare was significantly higher in May/June (57.3%) than in August / September (44%). Also, more category (I) and total oocytes per ovary in camel were recovered during the breeding season than non breeding season (Abdoon 2001). This author attributed this difference to the presence of more follicles during the breeding season.

During our study, the average number of COCs categories and total oocytes per ovary were slightly raised during summer than those collected during winter (1.31; 3.68 vs. 1.26; 3.45, respectively). These findings are similar to those reported by Gou *et al.* (2009) who observed that proportion of sheep COCs was significantly ($P<0.05$) decreased in winter compared to summer and autumn (51.4 vs. 84.9 and 83.6%, respectively). Also these findings are in agreement with those reported by Kadoom (1995) who found that, the total recovery rate of oocytes from buffalo ovaries was higher in summer season than in other seasons. The same findings were also obtained by Seydou *et al.* (1999) in goat.

5. Conclusions

In conclusion, the present study demonstrated that the supplementation of protein additives (PA) in forms OAF, FBS or SS to culture media (TCM-199 or RPMI-1640) are required for IVM of sheep oocytes. The supplementing of PA to culture media was more efficacious in TCM-199 than RPMI-1640 for IVM of oocytes. The COCs were more response for PA than denuded oocytes. The spring was the best season for recovery of COCs which have a worthy competence to be matured in vitro.

Table (1): Effect of adding different protein additives 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)	71	4 (5.83)^{c}	15 (20.73)^{b}	24 (32.90)^{a}	28 (40.50)^{b}	5 (7.63)^{b}	33 (44.90)^{a}	27 (40.83)^{a}	3 (2.90)^{b}	2 (2.37)^{d}	0 (0.00)^{c}	1 (1.40)^{b}
M_1+OAF	**74**	4 (5.90)^{c}	15 (24.67)^{a}	20 (26.33)^{b}	35 (43.1)^{b}	7 (8.40)^{a}	26 (44.47)^{a}	25 (30.70)^{bc}	0 (0.00)^{c}	6 (6.83)^{c}	10 (9.60)^{b}	0 (0.00)^{c}
M_1+ FBS	**77**	12 (17.70)^{a}	12 (13.10)^{c}	26 (28.83)^{b}	27 (40.33)^{b}	1 (2.80)^{d}	14 (20.23)^{c}	27 (33.57)^{b}	5 (8.87)^{a}	9 (7.90)^{b}	11 (17.87)^{a}	10 (8.80)^{a}
M_1+ SS	**79**	13 (12.83)^{b}	12 (12.57)^{d}	19 (25.70)^{b}	35 (48.93)^{a}	2 (3.20)^{c}	24 (28.33)^{b}	23 (29.00)^{c}	0 (0.00)^{c}	7 (10.50)^{a}	14 (20.00)^{a}	9 (9.00)^{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 %).
- M_2 = RPMI-1640 medium, OAF = ovine amniotic fluid, FBS = fetal bovine serum, SS = sheep serum.
- GV = Germinal vesicle. GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI = Anaphase I, TI=Telophase, MII=Metaphase II, and Deg. =Degenerate

Table (2): Effect of different protein additives to TCM-199 medium on the maturation rate of denuded oocytes.

Treatment	No. of denuded oocytes	Homogeneity of cytoplasm		Nuclear maturation of homogenous denuded oocytes						
		Homo-genous	Heter-genous	GV	GVBD	MI	AI	TI	MII	Deg.
				No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)	No. (M %)
M_1 (Control)	**127**	**126**	**1**	53 (43.59)^{b}	42 (32.63)^{b}	20 (16.37)^{a}	6 (3.41)^{a}	0 (0.00)^{b}	0 (0.00)^{b}	5 (4.01)^{b}
M_1+OAF	**145**	**145**	**0**	70 (39.73)^{b}	48 (38.25)^{a}	22 (17.90)^{a}	0 (0.00)^{b}	0 (0.00)^{b}	2 (1.79)^{a}	3 (2.34)^{c}
M_1+ FBS	**160**	**157**	**3**	90 (53.89)^{a}	35 (26.12)^{c}	27 (14.49)^{ab}	0 (0.00)^{b}	1 (0.48)^{a}	1 (0.48)^{ab}	3 (4.55)^{b}
M_1 + SS	**186**	182	**4**	107 (53.71)^{a}	48 (27.41)^{bc}	18 (11.62)^{b}	1 (0.85)^{b}	0 (0.00)^{b}	0 (0.00)^{b}	8 (6.41)^{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 %).
- M_1 = TCM-199 medium, OAF = ovine amniotic fluid, FBS = fetal bovine serum, and SS = sheep 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 different protein supplements 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 (4.09)^{b}	2 (4.69)^{d}	2 (2.78)^{d}
M_2+OAF	**49**	2 (8.10)^{a}	12 (24.48)^{b}	17 (34.28)^{b}	18 (33.15)^{o}	3 (7.50)^{a}	23 (43.29)^{d}	6 (10.16)^{c}	4 (8.89)^{a}	0 (0.00)^{c}	3 (11.11)^{a}	10 (19.05)^{a}
M_2+ FBS	**65**	6 (9.19)^{a}	8 (10.31)^{d}	22 (31.79)^{b}	29 (48.7 l)^{b}	0 (0.00)^{d}	35 (58.33)^{b}	22 (29.78)^{b}	1 (1.10)^{cd}	4 (7.16)^{a}	2 (2.53)^{c}	1 (1.10)^{f}
M_2 + SS	**36**	1 (l0.00)^{a}	13 (43.38)^{a}	8 (13.14)^{b}	14 (33.48)^{c}	0 (0.00)^{d}	23 (69.29)^{a}	6 (12.14)^{c}	0 (0.00)^{d}	0 (0.00)c	1 (10.00)^{b}	6 (8.57)^{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_2 = RPMI-1640 medium, OAF = ovine amniotic fluid, FBS = fetal bovine serum, SS = sheep serum.
- GV = Germinal vesicle. GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI = Anaphase I, TI=Telophase, MII=Metaphase II, and Deg. = Degenerated.

Table (4): Effect of adding different protein supplements to RPM1-1640 medium on the nuclear maturation rate of denuded oocytes

Treatment	No. of denuded oocytes	Homogeneity of cytoplasm		Nuclear maturation of homogenous denuded oocytes						
		Homo-genous	Hetero-genous	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 (2.1l)^{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+OAF	76	74	2	0 (0.00)^{e}	53 (73.76)^{a}	3 (3.4 l)c	1 (1.67)a	0 (0.00)b	0 (0.00)b	17 (21.17)^{b}
M_2 + FBS	82	78	4	3 (4.76)^{c}	41 (53.03)^{c}	8 (11.04)^{b}	0 (0.00)^{d}	0 (0.00)^{b}	0 (0.00)^{b}	26 (31.17)^{a}
M_2 + SS	73	69	4	9 (13.09)^{a}	39 (60.40)^{b}	5 (5.54)c	0 (0.00)^{d}	0 (0.00)^{b}	0 (0.00)^{b}	16 (20.97)^{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_2 = RPMI-1640 medium, OAF= ovine amniotic fluid, FBS - fetal bovine serum, SS = sheep serum.
- GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, MI= Metaphase I, AI= Anaphase I, TI = Telophase 1, MII = Metaphase II, and Deg. = Degenerated

Table (5.a): Effect of PA on IVM rate of COCs of sheep oocytes based on the type of culture media.

Protein source	Type of media	No. of cultured oocytes	Maturation stages													
			GV		GVBD		MI		AI		TI		MII		Deg.	
			N	%	n	%	n	%	n	%	n	%	n	%	n	%
OAF	TCM	74	7	9.46	26	35.14	25	33.78**	0.00	0.0	6	8.11*	10	13.51	0	0.0
	RPMI	79	3	6.12	23	46.94	6	12.24	4	8.16*	0.00	0.0	3	6.12	10	20.41**
FBS	TCM	77	1	1.29	14	18.18	27	35.06	5	6.49	9	11.69	11	14.29*	10	12.99*
	RPMI	65	0.00	0.0	35	53.85**	22	33.85	1	1.54	4	6.15	2	3.08	1	1.54
SS	TCM	79	2	2.53	24	30.38**	23	29.11	0.00	0.0	7	8.86	14	17.72*	9	11.39
	RPMI	36	0.00	0.0	23	63.89**	6	16.67	0.00	0.0	0.00	0.0	1	2.78	6	16.67

* Significant at P<0.05; ** Significant at P<0.01.

Table (5.b): Effect of PA on IVM rate of denuded sheep oocytes based on the type of culture media.

Protein source	Type of media	No. of cultured oocytes	Maturation stages													
			GV		GVBD		MI		AI		TI		MII		Deg.	
			n	%	n	%	n	%	n	%	n	%	n	%	n	%
OAF	TCM	145	70	48.28**	48	33.10	22	15.17*	0.00	0.0	0.00	0.0	2	1.38	3	2.07**
	RPMI	74	0.00	0.0	53	71.62**	3	4.05	1	1.35	0.00	0.0	0.00	0.0	17	22.97**
FBS	TCM	157	90	57.32**	35	22.29	27	17.19	0.00	0.0	1	0.64	1	0.64	3	1.91
	RPMI	78	3	3.85	41	52.56**	8	10.26	0.00	0.0	0.00	0.0	0.00	0.0	26	33.33**
SS	TCM	182	107	58.79**	48	26.37	18	9.89	1	0.55	0.00	0.0	0.00	0.0	8	4.39
	RPMI	69	9	13.04	39	56.52**	5	7.25	0.00	0.0	0.00	0.0	0.00	0.0	16	23.19**

* Significant at P<0.05; ** Significant at P<0.01.

Table (6.a): Effect of PA on IVM rate of sheep oocytes based on oocyte quality: The oocytes (COCs or DOs) were cultured in TCM-199.

Protein source	Oocyte quality	No. of cultured oocytes	Maturation stages													
			GV		GVBD		MI		AI		TI		MII		Deg.	
			n	%	n	%	n	%	n	%	n	%	n	%	n	%
OAF	COC	74	7	9.46	26	35.14	25	33.78**	0.0	0.0	6	8.11**	10	13.51**	0.0	0.0

	Denuded	145	70	48.28**	48	33.10	22	15.17	0.0	0.0	0.0	0.0	2	1.38	3	2.07
FBS	COC	77	1	1.29	14	18.18	27	35.06**	5	6.49**	9	11.69**	11	14.29**	10	12.99**
	Denuded	157	90	57.32**	35	22.29	27	17.19	0.0	0.0	1	0.64	1	0.64	3	1.91
SS	COC	79	2	2.53	24	30.38	23	29.11**	0.0	0.0	7	8.86**	14	17.72**	9	11.39**
	Denuded	182	107	58.79**	48	26.37	18	9.89	1	0.55	0.0	0.0	0.0	0.0	8	4.39

** Significant at P<0.01

Table (6.b): Effect of PA on IVM rate of sheep oocytes based on oocyte quality: The oocytes (COCs or DOs) were cultured in RPMI-1640.

Protein source	Oocyte quality	No. of cultured oocytes	Maturation stages													
			GV		GVBD		MI		AI		TI		MII		Deg.	
			n	%	n	%	n	%	n	%	n	%	n	%	n	%
OAF	COC	49	3	6.12*	23	46.94	6	12.24	4	8.16	0.0	0.0	3	6.12*	10	20.41
	Denuded	74	0.0	0.0	53	71.62**	3	4.05	1	1.35	0.0	0.0	0.0	0.0	17	22.97
FBS	COC	65	0.0	0.0	35	53.85	22	33.85**	1	1.54	4	6.15*	2	3.08	1	1.54
	Denuded	78	3	3.85	41	52.56	8	10.26	0.0	0.0	0.0	0.0	0.0	0.0	26	33.33**
SS	COC	36	0.0	0.0	23	63.89	6	16.67	0.0	0.0	0.0	0.0	1	2.78	6	16.67
	Denuded	69	9	13.04*	39	56.52	5	7.25	0.0	0.0	0.0	0.0	0.0	0.0	16	23.19

* Significant at <0.05; ** Significant at P<0.01

Table (7): Seasonal effect on the recovery rate and quality of sheep oocytes.

Season	No. of ovaries	No. of oocytes		Oocytes quality													
		Total No.	RR (mean)	Excellent			Good			COCs (excellent + good)			Denuded			Degenerated		
				No.	RR (mean)	%	No.	RR (mean)	%	No.	RR (mean)	%	No.	RR (mean)	%	No.	RR (mean)	%
Winter	155	529	$(3.45)^b$	43	$(0.31)^b$	8.13	151	$(0.95)^a$	28.54	194	$(1.26)^b$	36.67	251	$(1.64)^c$	47.45	84	$(0.55)^b$	15.88
Spring	137	589	$(4.31)^a$	59	$(0.45)^a$	10.02	145	$(1.04)^a$	24.62	204	$(1.49)^a$	34.63	285	$(2.08)^a$	48.39	100	$(0.74)^a$	16.98
Summer	148	488	$(3.68)^b$	38	$(0.34)^{ab}$	7.79	119	$(0.97)^a$	24.39	157	$(1.31)^b$	32.17	213	$(1.63)^c$	43.65	118	$(0.74)^a$	24.18
Autumn	228	720	$(3.39)^b$	56	$(0.26)^b$	7.87	164	$(0.73)^b$	22.78	220	$(0.99)^c$	30.56	411	$(1.94)^b$	57.08	89	$(0.47)^c$	12.36

- Values in the same column with different superscripts differ significantly (P <0.05).
- Table represents mean numbers of collected oocytes.
- RR=recovery rate (no. of oocytes/no. of ovaries), %= no. of oocytes/total no. of oocytes/10

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