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Antioxidant Effect of Green Tea Leaves Extract on *in vitro* Production of Sheep Embryos

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Abstract.- This study was carried out to investigate the nuclear maturation and embryo development after *in vitro* fertilization of sheep cumulus oocyte complexes (COCs), either in green tea leaves extract (GTE) supplemented tissue culture medium (TCM-199) at different concentrations (0, 0.3, 0.6 and 1.2 mg ml^{-1}). Oocytes of a control group were matured in a maturation medium without GTE (0 mg GTE ml^{-1}). After maturation, half of oocytes were fixed and stained to evaluate the nuclear maturation. The rest of oocytes were fertilized *in vitro* with fresh semen then cultured for 9 days for assessment of the oocytes developmental capacity. The results showed that supplementation of GTE resulted in a significantly ($p \leq 0.05$) increasing of oocytes developed to metaphase II (M II) when compared to control group, exception group V that no significant difference compared to group I. Group II showed higher significant M II than other treatments groups. Addition of GTE to IVM medium resulted in significantly improvement of blastocyst formation with 0.3 mg ml^{-1} concentration than 0.6, 1.2 mg ml^{-1}, and control group. It was concluded that GTE at concentrations of 0.3 mg ml^{-1} IVM medium improvement the *in vitro* maturation and embryo development of sheep COC's to blastocyst stage. Also, addition of GTE at 0.6 mg ml^{-1} to IVM medium had little benefit in increasing the maturation rate and blastocyst formation.

Key words: Antioxidant, green tea leaves extract, *in vitro* fertilization, sheep embryos, *in vitro* embryo production.

INTRODUCTION

The *in vitro* embryo production (IVEP) system includes three major steps, namely *in vitro* maturation (IVM) of the primary oocytes, *in vitro* fertilization (IVF) of the matured oocytes and *in vitro* culture (IVC) of presumptive embryos, until transferred or cryopreserved for future use (Gandolfi *et al.*, 2005; Zhu *et al.*, 2007). Therefore, IVEP needs to be optimized to produce healthy and viable lamb yield. For the success of IVEP, IVM is one of the essential steps in the IVF process. Optimizing the culture medium to produce an *in vitro* environment similar to *in vivo* (Yuan *et al.*, 2003) is an important step toward achieving this goal. Several researchers have studied the IVM in mammalian oocytes (Rao *et al.*, 2002; Kharche *et al.*, 2005). Maturation of the oocytes includes two aspects: nuclear and cytoplasm maturation (Sun and Nagai, 2003). IVM oocytes provide an excellent opportunity for cheap and abundant embryo for carrying out basic research and for the application of emerging biotechnologies like cloning and transgenic (Nadi *et al.*, 2002). A major factor affecting *in vitro* mammalian embryo production is increased oxidative stress (Gaspmini *et al.*, 2000). Oxidative stress is resulted from increase of reactive oxygen species (ROS), which named free radicals (Miesel *et al.*, 1993). ROS, such as hydrogen peroxide (H_2O_2), superoxide anions (O_2-), and hydroxyl radicals (OH), are produced by aerobic organisms, oocytes and embryos, external environment and culture media surrounding embryos during IVF (Guerin *et al.*, 2001; Martin-Romero *et al.*, 2008). This can influence on the early embryo development of mouse, hamster, and bovine embryos (Blondin *et al.*, 1997; Harvey *et al.*, 2002; Watson *et al.*, 1994), sperm motility and axonemal protein phosphorylation (Aitken *et al.*, 1993), and block of *in vitro* two cell embryos (Nasr-Esfahani and Johnson, 1992). Increase of ROS in the culture environment leading to increase of oxidative stress, which can be affecting the cell membrane (Aitken *et al.*, 1989), DNA integrity (Halliwell and Aruoma, 1991), apoptosis (Yang *et*

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al., 1998), gene expression and transcriptional factors (Sikka, 2003). The success rates of IVF are depending on the antioxidant supplementation to neutralize the effect of ROS on oocytes and embryos quality (Agarwal *et al.*, 2008). Also, Vahedi *et al.* (2009) showed that addition of some useful materials such as growth factors, estradiol, gonadotropins, and antioxidant (Balasubramanian and Rhob, 2007) are necessary for improvement of oocytes IVM. The antioxidants supplementation to the culture medium can be divided into two large groups: ones that are non-enzymatic or metabolic with low molecular weight, such as L-ascorbic acid (vitamin C), cysteine, α-tocopherol, and β-mercaptoethanol and ones with an enzymatic action, such as superoxide dismutase, glutathione peroxidase and catalase (Nordberg and Arnér, 2001). Within the body, oocytes and embryos can be protected from oxidative stress by free radical and enzymatic scavenging antioxidant that exist within the follicular and oviductal fluid (Gupta *et al.*, 2010; Wang *et al.*, 2002). Medicinal plants used in the synthesis and production of new drugs and they play an important role in drug discovery operations (Amer *et al.*, 2013). Tea is one of the most popular beverages consumed worldwide and from the plant *Camellia sinensis*, is consumed in different parts of the world as green or black tea (Cabrera *et al.*, 2006). Tea components possess antioxidant effects (Mukhtar *et al.*, 1992). During the *in vivo* culture, embryos can face the detrimental effects of oxidative stress by antioxidants produced by the embryo in addition to the ones present in the follicular and oviductal fluid (Gardiner and Reed, 1995). However, during IVC, the embryonic physiological antioxidants production is not enough to prevent oxidative stress (Ali *et al.*, 2003), so exogenous antioxidant supplements may be necessary.

There are no reports regarding use of green tea leaves extract (GTE) as a source of exogenous antioxidant in culture media during *in vitro* production of sheep embryos system. Thus, this study was carried out to investigate the effects of GTE supplementation during IVM on the *in vitro* oocytes maturation and developmental competence of sheep oocytes to blastocyst stage after IVF.

MATERIALS AND METHODS

Chemicals and plastics

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise noted. Polystyrene plastic culture dishes (35x 10mm, 60x 10 mm) and 0.22 µm millipore membrane filters syringe were purchased from Nunclon, Nalge Nunc International, Roskide, Denmark.

Preparation of green tea water extracts

GTE was prepared for once time in the beginning of the experiments according to Haghparast *et al.* (2011). Briefly, 5 g of green tea leaves were added to 50 ml of distilled water and heated at 40–45°C for 1 h on magnetic stirrer. The mixture was cooled to room temperature and was filtered through a Whitman No. 1 filter paper. The mixture solution was concentrated under vacuum using Buchi Rotavapor-R evaporator. Soluble solid content was applied as GTE in the experiments.

Experimental design

Two thousands and four hundred forty seven sheep oocytes were used to investigate the effect and optimum concentration of GTE on IVM and developmental competence of sheep oocytes following IVF. One thousand and three hundred forty six oocytes were used for nuclear maturation assay and one thousand and one hundred one oocytes were used for IVF. Excellent and good collected cumulus oocytes complexes (COCs) were divided into four groups according to GTE present or absent in the maturation medium as follows: group I, basic maturation medium; group II, basic maturation medium + 0.3 mg GTE ml^{-1}; group III, basic maturation medium + 0.6 mg GTE ml^{-1}; and group IV), basic maturation medium + 1.2 mg GTE ml^{-1}. The basic maturation medium comprised TCM-199 with Earle's (Sigma, m4530) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY), 40 I.U ml^{-1} equine chronic gonadotropin (eCG, Folligon, Intervet International BV, Boxmee, Holland), 100 I.U ml^{-1} penicillin sodium and 50 µg ml^{-1} gentamycin sulfate. Group I served as the control group and

groups II, III, and IV were considered as treatment groups.

Oocytes collection and in vitro maturation

Ovaries were collected from slaughtered sheep females at a local abattoir and transported to the laboratory within 1-3 h in 0.9% (weight/vol) NaCl at 37ºC supplemented with antibiotics. Cumulus Oocyte-Complexes (COCs) were obtained by antral follicles (2 to 8 mm diameter) aspiration with an 20-gauge needle connected to a 20 ml syringe and prefilled with collection oocytes medium that consists of TCM-199 hank's and 4 mg bovine serum albumin (BSA) ml^{-1}. Ten to fifteen selected oocytes with a homogenous and evenly granulated cytoplasm and three to more layers of cumulus cells were washed four times in maturation medium and placed into 50 µl droplet of maturation medium with or without GTE under paraffin oil. Culture dishes were incubated in CO_2 incubator at 38.5 °C under an atmosphere of 5% CO_2 in air, and high humidity, for 24 h.

Assessment of the nuclear maturation by cytogenetic analysis

To evaluate the maturation rate at the end of IVM, half of matured oocytes after removal cumulus cells were transferred onto a glass slide and covered with cover slip. The slides were immersed in a 3:1 fresh fixative solution of ethanol/acetic acid for a minimum of 24 h. Fixed oocytes were stained with 1% (w/v) aceto-orcein in 45% (v/v) acetic acid (Rao *et al.*, 2002). Oocytes were analyzed cytogenetically for different stages of meiosis under a phase-contrast microscope at high magnifications. Different meiotic stages were classification according to Polanski and Kubiak (1999).

Preparation of sperm and in vitro fertilization

For IVF, 35 µl of Bracket and Oliphant (B.O.) medium (Bracket and Oliphant, 1975) drops were made in petri dishes. Petri dishes were equilibrated for at least 2 h before use at 38.5°C under 5% CO_2 in humidified air in the incubator. Fresh semen was collected from sheep ram of proven fertility. For swim up, 80 µl of semen was kept under 1 ml of B.O. medium supplemented with 5 mg BSA/ml and 0.3 mg Heparin/ml in a 15 ml conical Falcon tube at 38.5ºC for up to 45 min. After swim up, the 700 to 800 µl of the supernatant was added to 3ml of BSA-B.O. medium, centrifuged twice at 1800 rpm for 5 min and the final pellet was re-suspended with BSA-B.O. medium. Sperm suspension (15 µl) containing 1.0×10^6 sperm/ml was addition to the IVF drops contained 10 to 15 matured oocytes. Matured oocytes were co-incubated with sperm for 18 – 20 h in 5% CO_2 with humidified air at 38.5°C.

In vitro *culture of embryos*

After fertilization cumulus cells were removed from presumptive zygotes by repeated pipetting in culture medium comprising SOF medium (Maraa *et al.*, 2013) + 10 % FBS + 0.03 % sodium pyruvate, 100 I.U ml^{-1} penicillin sodium and 50 µg ml^{-1} gentamycin sulfate. Fifteen to twenty denuded presumptive zygotes were transferred into fertilization medium droplets and cultured for 9 days at 38.5°C, 5 % CO_2, 5 % O_2 and 90 % N_2 with high humidity. Day of IVF was considered as 0 day.

Statistical analysis

Replicates of experiments were performed on different days with different batches of oocytes and semen. Statistical analyses for all data were carried out using analysis of variance (ANOVA) using SAS program (SAS, 1996). Statistical differences were considered significantly at $P \leq 0.05$ levels by using Duncan's Multiple Range Test procedure (Duncan, 1955). Results were expressed as mean ± standard error of mean (SEM).

RESULTS

Cytogenetic analysis

The effect of GTE concentration on the maturation rate of sheep COCs (the proportion of oocytes whose nuclei reached metaphase II (MII) is presented in Table I and Figure 1. The results showed that the percentage of oocytes developed into MII elevated significantly ($P \leq 0.05$) in the group II (29.22±0.67) compared with group I, III, and IV (8.84±0.67, 19.60±1.39, and 6.80 ± 0.84, respectively). Also, oocytes in group III showed highly significant difference when compared with group I and group IV for M II trait. Moreover, there

was no significant difference in the percentage mean of M II oocytes between group I and group IV (8.84±0.67 vs. 6.8±0.84, respectively). As for the metaphase I (MI), it follows the same trend as that of MII. On the other hand, there was no statistically significant difference between all groups concerning the oocytes in anaphase stage (Table I). Oocytes with germinal vesicle follow reverse trend compared to the MII stage, while oocytes with germinal vesicle break down showed high percentage mean in group II and III (41.92±0.92 and 38.3±2.58, respectively) and low percentage mean in group I and IV (34.9±0.9 and 34.46 ± 1.02, respectively) (Table I).

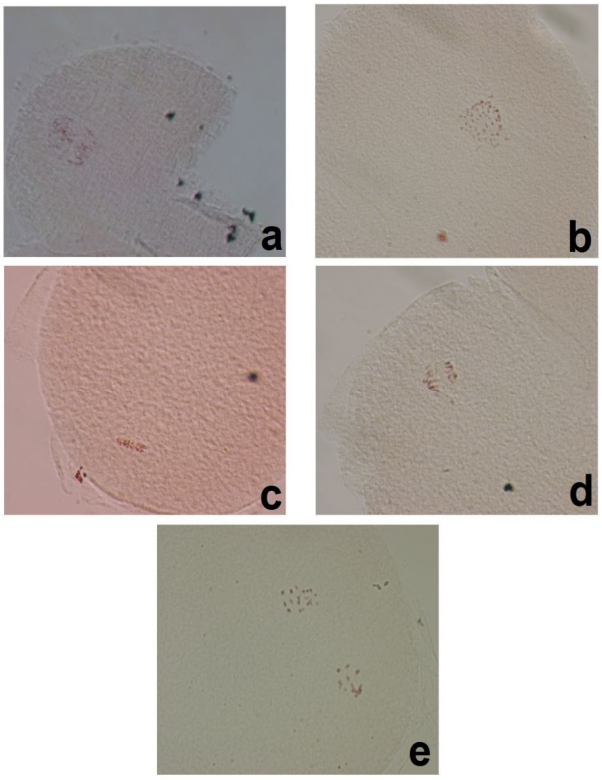

Fig. 1. Photomicrographs of stained sheep oocytes with aceto-orcein stain representing various nuclear stages during maturation. (a) Germinal vesicle (GV), (b) Germinal vesicle Break Down (GVBD), (c) Metaphase I (M I), (d) Anaphase I (anaph. I), and (f) Metaphase II (M II). (400 X)

Embryo development

To evaluate the effect of GTE on the development of sheep embryos, the developmental competences of oocytes matured in medium with or without GTE were statistically analyzed. Results presented in Table II showed that the increase in the development of oocytes in to blastocyst stage (Fig. 2) was statistically significant in group II (0.3 mg/ml) compared to group I, III and IV (0.0, 0.6 and 1.2 mg/ml, respectively). There was no statistically significant difference between mean percentages of group III (0.6 mg/ml) and group I (18.74 ± 0.71 and 15.78 ± 1.08, respectively). The oocytes matured in medium supplemented with 1.2 mg/ml GTE showed lower mean percentage (7.72 ± 1.19) than the other groups. Concerning morula stage (Fig. 1), Table II showed that the mean percentages of morula stage followed the same trend as blastocyst stage. Additionally, there was no significant difference between groups II and III where the mean percentage recorded were 47.66±2.04 and 42.06±1.57, respectively. Regarding cleavage of embryos, the results showed that the percentages were statistically significantly different between all groups. The mean percentage was as high as possible for oocytes matured in medium supplemented with 0.3 mg GTE/ml (73.82 ± 0.88) and was less possible for the group treated with 1.2 mg GTE/ml (36.66±1.62).

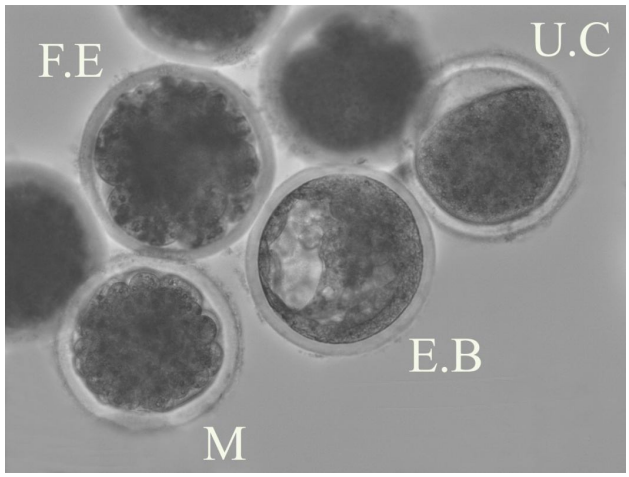

Fig. 2. Sheep embryos developmental stages; E.B, early blastocyst; F.E, Fragmented embryo; M, morula; U.C, un-cleavage Embryo.

Table I.- Effect of green tea extract (GTE) concentrations on the *in vitro* maturation of sheep oocytes (mean ± SEM).

GTE treatment groups	Nuclear maturation stages[§]					
	No. of examined oocytes	Germinal vesicle	GVBD	MI	Anaphase I	MII
0 mg/ml (Group I)	315	47.44 ± 1.2c	34.90 ± 0,90a	5.90 ± 1.27a	0.66 ± 0.41a	8.84 ± 0.67a
0.3 mg/ml (Group II)	361	11.76 ± 1.35a	41.92 ± 0.92b	15.70 ± 0.87c	1.18 ± 0.54a	29.22 ± 1.93c
0.6 mg/ml (Group III)	315	29.78 ± 1.31b	38.30 ± 2.58ab	11.38 ± 1.40b	0.96 ± 0.40a	19.60 ± 1.39b
1.2 mg/ml (Group V)	355	57.38 ± 0.63d	34.46 ± 1.02a	3.94 ± 0.48a	0.26 ± 0.26a	6.80 ± 0.84a

[a,b,c,and d] superscripts values within the same column are significantly different from each other at $p \leq 0.05$.
[§] Mean of cytogenetic analysis is reported as percentage from number of examined oocytes
GVBD, germinal vesicle break down; MI, Metaphase I; MII, Metaphase II.

Table II.- Effect of green tea extract (GTE) concentrations on the development of sheep embryos produced *in vitro* (mean±SEM).

GTE treatment groups	No. of embryos [§]					
	No. of inseminated oocytes	Cleveage embryos	Uncleveage embryos	Fragmented embryos	Morula	Blastocyst
0 mg/ml (Group I)	300	54.96 ± 0.94b	45.04 ± 0.94c	28.58 ± 1.50c	36.88 ± 1.43b	15.78 ± 1.08b
0.3 mg/ml (Group II)	270	73.82 ± 0.88d	26.18 ± 0.88a	7.28 ± 1.36a	47.66 ± 2.04c	30.90 ± 0.98c
0.6 mg/ml (Group III)	245	63.64 ± 1.47c	36.36 ± 1.47b	8.44 ± 1.32a	42.06±1.57bc	18.74 ± 0.71b
1.2 mg/ml (Group V)	286	36.66 ± 1.62a	63.58 ± 1.63d	13.96 ± 1.68d	23.80 ± 2.34d	7.72 ± 1.19a

[a,b,c,and d] Values with different superscripts within the same column are significantly different from each other at $p \leq 0.05$
[§] Number of embryos is reported as median for percentage from inseminated oocytes numbers
+ Number of fragmented embryos, morula and blastocyst is reported as median for percentage from cleavage embryos numbers.

DISCUSSION

The current study showed that the addition of low concentration of GTE as a source of antioxidant in the maturation medium resulted in significantly increased maturation rate of sheep oocytes and improved the rate of morula and blastocyst formation. Therefore, the discussion will include the effect of antioxidant on *in vitro* production of embryos. IVM oocytes have been used in some laboratories because their use makes it feasible to obtain a large number of oocytes from ovaries at relatively low cost (Roushandeh *et al.*, 2007). Mammalian oocytes collected from antral follicles can complete meiotic maturation in media *in vitro*. However, subsequent development of sheep oocytes matured and embryos cultured *in vitro* are quite lower than those matured and developed *in vivo* (Balasubramanian and Rhob, 2007; Abd-Allah, 2012). So, preparation of oocytes is one of the critical factors that determine the developmental competence of embryos produced by IVF (Kilyoung and Eunsong, 2007). *In vitro* cultured oocytes are known to have a lower developmental competence after IVF to *in vivo* derived oocytes (Moor and Dai, 2005). This is mainly due to medium that can not provide optimal conditions for the oocytes and *in vivo* conditions can not be mimicked totally under *in vitro* situations (Abd-Allah, 2012). Several factors influence the karyoplasmic and cytoplasmic maturation of oocytes *in vitro*, including various culture media, co-culturing with follicular cells, duration of maturation, type of media, and supplementations added to the maturation media, such as hormones, growth factors, serum, cells, follicular fluid and other substances (Hlker *et al.*,

2005; De Matos and Furnus, 2007; Dilip *et al.*, 2006). However, the developmental ability of oocytes matured in defined media still tends to be lower than oocytes matured in media supplemented with chemicals such as amino acids or antioxidants (Herrick *et al.*, 2004; Hong *et al.*, 2004). Oocytes and embryos produce endogenous ROS by various enzymatic actions during the metabolic process (Gardner and Lane, 2002; Harvey *et al.*, 2002; Gordon, 2003). There is evidence that ROS in *in vitro* oocytes maturation affect IVP of bovine embryos (Geshi *et al.*, 2000). These are formed when molecular O_2 is utilized as an electron acceptor during redox reaction in cells. They damage cell membranes, protein and DNA (Yuh *et al.*, 2010; Droge, 2002; Sudano *et al.*, 2010). Therefore, ROS must be inactivated continuously in order to maintain only the small amount necessary to maintain normal cell functions (Sudano *et al.*, 2010). Oxidative stress has a negative effect on *in vitro* maturation and embryonic development of oocytes, and several studies have been conducted in this subject (Guerin *et al.*, 2001; Matos *et al.*, 2002). Various studies were conducted to measure the effect of antioxidant on oocyte maturation and early embryo development (Guerin *et al.*, 2001; Urdaneta *et al.*, 2003; Feugang *et al.*, 2004). On the other hand, addition of antioxidant alone is not enough to protect ROS. Besides selection of antioxidant and its concentrations are very critical. The improving and increasing knowledge concerned with antioxidants and their mechanisms may contribute to development of embryos and evaluation methods of embryo/oocytes quality in *in vitro* culture system (Öztürkler *et al.*, 2010).

The addition of 0.3 mg GTE/ml IVM medium significantly improved ($p \leq 0.05$) the rate of blastocyst formation. This improvement in embryo development might be due to antioxidant effect of GTE which scavenges ROS during *in vitro* culture of embryos (Wang *et al.*, 2007) and promotes DNA synthesis of embryos and intracellular also glutathione (Funahashi, 2005). This improvement could be attributed to the protection of oocytes against oxidative stress during IVM by GTE (Wang *et al.*, 2007). Oxidative stress damage of cellular elements through the ROS is one of the important factors which cause damage to appropriate cell function (Del Corso *et al.*, 1994). It has been reported that other antioxidant such as β-mercaptoethanol, cysteine and cystine (Ali *et al.*, 2003), and cysteamine (Gaspmini *et al.*, 2000) added to IVM medium improve the rate of embryos development to the blastocyst stage.

The present study suggested that GTE supplementation beyond the optimum concentration ranges might have deleterious effects on the IVM events occurring in both the nucleus and the cytoplasm and on subsequent embryo development. Therefore, the observed variation in the developmental competence of gametes and embryos may be attributed to shift in the reduction oxidative status according to the concentration of antioxidant supplement (Natarajan *et al.*, 2010). Finally, the effects of ROS on oocytes and embryos development are not yet very clearly established and need more studies.

CONCLUSIONS

The addition of GTE to maturation medium as antioxidant with 0.3 mg ml^{-1} seems to improve the *in vitro* maturation rate of sheep oocytes and the morula and blastocyst formulation rate. Further studies are required to study the effect of GTE on development of sheep embryos.

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