j.Egypt.vet.med.Assoc 78, no 2. 269 – 285 (2018)

ANTIOXIDANT EFFECT OF GREEN TEA EXTRACT (CAMELLIA SINENSES) ON VIABILITY AND DEVELOPMENTAL COMPETENCE OF VITRIFIED CAMEL OOCYTES

By

Badr, M. R., Ebtihal, A. Ibrahim[2], and Rawash, Z. M[1].

[1]Artificial insemination and embryo transfer department, [2]Biology of reproduction department

Animal Reproduction Research Institute (ARRI) Giza, Egypt.

ABSTRACT

The cryopreservation of immature oocytes would generate a readily available, non-seasonal source of female gametes for research and reproduction. In domestic animals, the most promising results on oocyte cryopreservation have been reported in cattle, whereas few studies have been conducted on camel. The aim of the present study was to investigate the effect of green tea extract (GTE) on viability and developmental competence of camel oocytes vitrified at the germinal vesicle (GV) stage. Cumulus oocyte-complexes (COCs) obtained from mature camel ovaries were vitrified in media supplemented with different GTE concentrations (0, 0.5, 1, 1.5 mg/ml). Following vitrification and warming, viable COCs were matured in vitro for 42 h. Some COCs were denuded and stained+ with 1.0% aceto-orcein to evaluate the nuclear maturation, whereas the others were fertilized and cultured in vitro for 7 days to determine the developmental competence. Total antioxidant capacity and lipid peroxidation of the vitrified oocyte were assessed. The current results revealed that 1mg/ml GTE appeared to be the best concentration that increased significantly ($P \leq 0.05$) the morphological live survived oocytes(84.07±2.59) and significantly reduced the morphological abnormal oocyte; broken zona pellucida and abnormal cytoplasm (14.28 ±4.13 and 11.91±2.39, respectively). Furthermore, addition of 1mg /ml GTE to the vitrification media elevated significantly ($P \leq 0.05$) the fertilization rate (57.14 ±10.92), cleavage rate, morula and blastocyst development (39.15±2.08, 24.60±3.97 and 15.61±3.05, respectively) compared with control group. The current data emphasized the beneficial antioxidant effect of GTE. Green tea extract at a concentration of 1 mg/ml augmented the total antioxidant capacity and reduced the lipid peroxidation in the vitrified camel oocyte (0.482±0.04 mμ/ml and 9.33±1.77 nmol/ml, respectively) compared with the control oocytes (0.185±0.04 and 25.0±3.06, respectively). Additionally, GTE at a concentration of 1 mg/ml increased the MTT reduction

and the ATP content in the vitrified camel oocyte (3.9±0.55 and 882.00±16.67 fmol, respectively) compared with the control oocytes (2.4±0.25 and 607.33.0±44.24 fmol, respectively). In conclusion, addition of 1 mg/ ml GTE to the verification media of camel oocytes improved the developmental competence of camel oocytes possibly by protecting the embryos from oxidative stress. Further studies are required to study the effect of GTE on development of camel embryos.

Keywords:

Antioxidant, green tea extract, camel oocytes, in vitro culture, Embryo development.

INTRODUCTION

Recently, in spite of the increasing attention regarding the preservation of oocytes from several mammalian species sporadic reports of success have been published in humans and animals. Despite decades of research, oocyte cryopreservation remains a challenge in many species owing to the complex structure of the oocyte. Gamete and embryo preservation are planned as being necessary for conserving the genome of all species **(El-Sokary *et al*., 2013).** Although sperm and embryos of several species are readily cryopreserved, oocyte preservation has been less successful **(Ali *et al.,* 2003).** Conventional slow freezing frequently leads to intracellular ice crystallization and cell damage, so vitrification of oocytes is being applied increasingly. Vitrification of oocytes is a relatively simple and economical method of oocytes cryopreserving. While, successful oocyte cryopreservation has been achieved in many species; the progress is still limited in camels. Oocyte survival rate after cryopreservation is affected by both morphological and biophysical factors **(Ledda *et al.,* 2007).** A major factor affecting vitrification of oocytes is increased oxidative stress. Oxidative stress is the 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), may harm early embryo development **(Harvey *et al.,* 2002),** sperm motility and axonemal protein phosphorylation **(Aitken *et al.,* 1993),** the cell membrane **(Aitken *et al.,* 1989),** DNA integrity **(Halliwell and Aruoma, 1991),** apoptosis **(Yang *et al.,* 1998),** gene expression and transcriptional factors **(Sikka, 2003).** Although these facts, there is some evidence that ROS may be beneficial at some steps of reproduction to permit successful gamete interaction **(Miesel *et al.,* 1993)**. 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)**.Therefore, the success rates of in vitro embryo production

are depending on the antioxidant supplementation to counteract the effect of ROS on oocytes and embryos quality **(Agarwal *et al.,* 2008).** These antioxidants include enzymes such as superoxide dismutase (eliminate O_2) catalase and selenium dependent glutathione peroxidase (convert H_2O_2 into H_2O and O_2) as well as lipid and water soluble antioxidants such as vitamins C, E and uric acid **(Knapen *et al.,* 1999).** So, the selection of antioxidants and antioxidant doses for the supplementation of culture media can be challenging, as excessive antioxidant doses in the medium may cause negative impacts **(Camargo *et al.,* 2006).** Medicinal plants are used in the synthesis and production of new drugs and can play an important role in drug discovery operations **(Amer *et al.,* 2013).** Tea is one of the most popular beverages consumed worldwide originated from the plant Camellia sinensis. It 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). Hijazi *et al.* (2015)** reported that GTEs consist predominantly of secondary metabolites and have antioxidant activity that is 20 times more powerful than that of vitamin C. There are two major bioactive components of GT: polyphenols and flavonoids **(Awoniyi *et al.,* 2012).** There are four major polyphenols in GT that are classified as catechins:epicatechin {EC}, epigallocatechin {EGC}, epicatechin-3-gallate {ECG} and epigallocatechin-3-gallate {EGCG}. Catechins are the main astringent components in GT that make up 30 - 40 % of the water-soluble solids **(Rahmani *et al.,* 2015).** During the in vivo culture, embryos can overcome 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 in vitro conditions, the embryonic physiological antioxidants production is not enough to prevent oxidative stress **(Ali *et al.,* 2003),** so exogenous antioxidant supplements may be necessary. To our knowledge, there are no reports regarding use of green tea leaves extract (GTE) as a source of exogenous antioxidant in camel oocytes vitrification media.Thus, this study was carried out to investigate the effects of different concentrations of GTE supplementation during oocyte cryopreservation on the camel oocyte cryoresistance, in vitro oocytes maturation and developmental competence.

MATERIAL AND METHODS

Chemicals and media:

Unless otherwise stated, all chemicals and media were obtained from Sigma chemical Co. (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA), respectively.

Experimental design:

Excellent and good quality aspirated cumulus oocytes complexes (COCs) were divided into four groups according to GTE concentration in the vitrification medium as follows: group I, basic vitrification medium (control); group II, basic vitrification medium + 0.5 mg GTE /m1; group III,basic vitrification medium+1 mg GTE /m1and group IV, basic vitrification medium +1.5 mg GTE /m1. The basic vitrification medium comprised 15% v/v EG + 15% v/v DMSO +0.5M sucrose in TCM-199. Group I served as the control group and groups II, III, and IV were considered as treatment groups.

Preparation of green tea water extracts (GTE):

GTE was prepared for once time in the beginning of the experiments according to **Haghparast *et al.* (2011).** Briefly, 5 gm 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.

Oocyte collection:

Ovaries were collected from slaughtered she-camel at a local abattoir and transported to the laboratory within 1-3 h in 0.9% NaCl supplemented with antibiotics (penicillin-streptomycin) at 37ºC. Cumulus Oocyte-Complexes (COCs) were recovered from medium sized follicles (2 to 8 mm diameter) by aspiration with an 18-gauge needle connected to a 20 ml syringe and prefilled with collection oocytes medium that consists of Dulbecco's Phosphate Buffered Saline. The COCs were screened under a stereomicroscope and washed three times in Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen Inc., Burlington, ON, Canada), supplemented with 5% newborn calf serum (v/v; CS; Invitrogen Inc.). Grade-1 COCs were selected based on their morphological appearance (uniformly granulated cytoplasm surrounded by more than 3 layers of compact cumulus cells) for further experimentation.

Vitrification and warming procedures:

Vitrification of COCs was performed using 0.25 mL mini-straw (IMV, l'Aigle, France). All COCs were first equilibrated in vitrification solution 1 (VS1; TCM-199 + 7.5% v/v dimethyl sulfoxide (DMSO) + 7.5% v/v ethylene glycol (EG, v/v) + 20% v/v calf serum (CS, v/v) for 10 min at room temperature (22 to 25 °C). After equilibration, COCs were transferred through four 30 µL droplets of vitrification solution 2 (VS2) that contained either

green tea extract (0.5,1 and 1.5 mg/ml+15% v/v EG+15% v/v DMSO+0.5M sucrose in TCM-199 at 37°C within 1 min. For straw vitrification, group of 5 COCs was loaded in 0.25 mL mini-straw (IMV,l'Aigle, France) in a middle column of vitrifying solution separated by air bubbles. After sealing with polyvinyl alcohol powder, straws were pre-cooled in liquid nitrogen (LN2) vapour for at least 1 min, and then they were directly plunged into LN2 **(Nikiforaki *et al,* 2014)**. For warming, vitrified specimens were transferred to a water bath at 37 °C for 1 min. The contents of each straw were expelled into a tissue culture dish (Nunc, 6 mm) contains 1 mL of warming solution 1 (WSI; 1 M sucrose solution) in a BM at 37 °C. COCs were kept in this solution for 3 min before being transferred to decreasing concentrations of sucrose solutions in BM (0.5 M and 0.25 M) then to a BM for 3 min each at room temperature.

Evaluation of oocyte viability and IVM:

Following vitrification and warming oocyte viability was assessed morphologically under a stereomicroscope. Oocytes with spherical, symmetrical shape and no signs of degeneration were considered viable whereas oocytes with losing cumulus cells and/or ruptured zona pellucida, fragmented cytoplasm, or degenerative signs were classified as non-viable **(Sharma *et al.,* 2010)** and were discarded from the experiments. To confirm oocyte viability, portion of morphologically viable COCs were stained by trypan blue exclusion dye according to the method described by **(Sharma and Loganathasamy 2007)**. Trypan blue solution (0.05%) was prepared by dissolving trypan blue powder in phosphate buffer saline (PBS; pH = 7.0) and the staining procedures were performed at room temperature for 2 min. COCs that not taken the stain were classified as live and fully or partially stained COCs were recorded as dead.

In vitro oocyte maturation:

The vitrified-warmed COCs were washed four times in maturation medium (TCM-199 supplemented with 5% CS, 5 µg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 µg/mL FSH (Bioniche) and 0.05 µg/mL gentamicin) and placed into 50 µl droplet of maturation medium 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 42 h. **(Wani *et al.,* 2004).**

Assessment of the nuclear maturation:

To evaluate the maturation rate at the end of IVM, half of matured oocytes after removal of 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 under a phase-contrast microscope at high magnifications.

In vitro fertilization (IVF) and culture (IVC):

Frozen-thawed semen was washed through swim up method. For swim up, 50 µl of semen was kept under 1 ml of S-TALP medium supplemented with 6 mg BSA/ml and 0.3 mg Heparin/ml in a 15 ml conical Falcon tube at 38.5ºC for up to 30 min. After swim up, the 700 to 800 µl of the supernatant was added to 3ml of BSA- S-TALP medium, centrifuged twice at 1800 rpm for 5 min and the final pellet was re-suspended with BSA- S-TALP medium. Sperm suspension (15 µl) containing 2.0×106 sperm/ml was added to the IVF drops contained 10 to 15 matured oocytes. Matured oocytes were co incubated with sperm for 18 - 20 h in 5% CO2 with humidified air at 38.5°C **(Parrish *et al.,* 1988).**

The presumptive zygotes were denuded and washed three times through in vitro culture (IVC) medium (CR1aa medium with 5% v/v FCS, 1% MEM non-essential amino acids, 2% BME essential amino acids and 0.05 µg/mL gentamicin). Finally, the presumptive zygotes were transferred (15 to 20 zygotes) into 100 µL droplets of IVC-medium under mineral oil and incubated at 38.5 °C under 5% CO_2 and high humidity and cultured for 7 days. Day of IVF was considered as 0 day **(Sagirkaya *et al.,* 2006).**

Biochemical analysis:

The FRAP assay.

The total antioxidant capacity (TAC) was measured as ferric-reducing ability of follicular fluid and serum (FRAP), according to Benzie and Strain **(Benzie and Strain 1996)** by spectro-photometric quantification. The absorbance measurement was taken at 593 nm. All measurements were taken at room temperature.

MDA measurement:

Follicular fluid MDA measurement was done according to the protocol described by **Ohkawa *et al.* (1979).** MDA reacts with thiobarbituric acid (TBA) to give a red compound which has a maximum absorbance at 532 nm. The absorbance of the upper butanol phase was read at 532 nm against a blank and the results were calculated as µM MDA.

Assessment of mitochondrial function of vitrified oocytes:

The 3 - (4, 5 - dimethylthiazol-2-yl) -2, 5 - diphenyltetrazolium bromide (MTT) assay is a colorimetric assay for assessing cell metabolic activity. NAD (P) H-dependent cellular

oxidoreductase enzymes are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple color. The absorbance of this colored solution can be quantified by measuring at wave length 575 nm by a spectrophotometer according to Pattie and Christiaan (2002).

Analysis of ATP Content in Fresh or Vitrified Oocytes:

ATP content was determined quantitatively by measuring the luminescence generated in an ATP-dependent luciferin-luciferase bioluminescence assay (ATP Bioluminescence Assay Kit HS II, Roche Diagnostics GmbH, Mannheim, Germany), as described by **Zhao *et al.* (2011).**

Statistical analysis:

Statistical analyses for all data were carried out using analysis of variance (ANOVA**) using costat computer program (1986).** Statistical differences were considered significantly at $P \leq 0.05$ levels by using Duncan's Multiple Range Test. Results were expressed as mean ± standard error of mean (SEM). Three replicates of experiments were performed on different days with different batches of oocytes and semen.

RESULTS

As shown in (Tables 1, 2) GTE supplementation of vitrification medium improved overall survival of frozen-thawed oocytes. In this regard, within the GTE supplemented groups, 1mg/ml appeared to be the most efficient concentration and induced the best cryoprotection. The proportion of morphological survival of vitrified-warmed oocytes that vitrified with 1mg/ml was (73.81 ±6.31), which was significantly different ($P \leq 0.05$) with the related values of control group (41.90±4.24). Analysis of quality of cryopreserved oocytes indicated significant difference ($P \leq 0.05$) of morphological live survived oocytes that vitrified with 1mg/ml GTE (84.07±2.59) and significantly reduced the abnormal oocyte; broken zona pellucida and abnormal cytoplasm (14.28 ±4.13 and11.91±2.39, respectively), compared with the control group (64.05±5.54, 32.38±5.62 and 25.72±8.74, respectively).

However, the current results revealed that 1.5mg/ml GTE impaired drastically both total normal and viable oocytes (36.63±4.23 and 60.56±10.57, respectively and increased the abnormality in the vitrified camel oocytes.

Table (1): Effect of different concentrations of green tea extract (GTE) on post-warming survival rate of vitrified camel oocytes (mean ± SEM).

Treatment	No of oocyte	Morphologically normal	
		Total normal oocyte	Live normal oocyte
Control	38	16 (41.90±4.24) [b]	10 (64.05±5.54) [ab]
0.5 mg GTE ml-1	45	27 (62.46±4.51) [a]	21 (77.59±1.45)[ab]
1 mg GTE ml-1	42	31 (73.81 ±6.31)[a]	26 (84.07±2.59)[a]
1.5 mg GTE ml-1	41	15 (36.63±4.23)[b]	9 (60.56±10.57)[b]

a and b superscripts values within the same column are significantly different from each other at p≤0.05.

Table (2): Effect of different concentrations of green tea extract (GTE) on post-warming cryo injury of vitrified camel oocytes (mean ± SEM).

Treatment	No of oocytes	Morphologically abnormal	
		Broken zona pellucda	Abnormal cytoplasm
Control	38	12(32.38±5.62) [a]	10 (25.72±8.74) [ab]
0.5 mg GTE ml-1	45	7 (15.83±2.89) [b]	11 (23.67±6.17)[b]
1 mg GTE ml-1	42	6 (14.28 ±4.13)[b]	5 (11.91±2.39)[b]
1.5 mg GTE ml-1	41	8 (19.59±2.71)[ab]	18 (43.77±3.36)[a]

a and b superscripts values within the same column are significantly different from each other at p≤0.05.

Regarding the effect of GTE concentration on the in vitro maturation rate of vitrified camel COCs the proportion of oocytes whose nuclei reached metaphase II (MII) is presented in (Table 3). The results showed that addition of 1mg /ml GTE elevated significantly (P≤0.05) maturation rate (71.69±4.13) compared with control group (47.62±8.59). Meanwhile, the present result revealed that 1.5 mg /ml GTE impaired drastically oocytes in vitro maturation.

Table (3): Effect of different concentrations of green tea extract (GTE) on the in vitro maturation rate of vitrified camel oocytes (mean ± SEM).

Treatment	No of oocyte	Maturation rate
Control	**42**	**20 (47.62±8.59) [b]**
0.5 mg GTE ml-1	**41**	**22 (53.48±5.53)[ab]**
1 mg GTE ml-1	**46**	**33 (71.69±4.13) [a]**
1.5 mg GTE ml-1	**45**	**19 (41.87±3.31) [b]**

a and b superscripts values within the same column are significantly different from each other at p≤0.05.

As shown in (Table 4), GTE supplementation of vitrification medium improved overall survival of frozen-thawed oocytes as assessed by the ratios of in vitro fertilization rate. In this regard, within the GTE supplemented groups, 1mg/ml appeared to be the most efficient concentration and induced the best cryoprotection. Addition of 1mg /ml GTE to the vitrification media elevated significantly (P≤0.05) fertilization rate (57.14±10.92) compared with control group (25.71±5.02). However, 1.5 mg/ml GTE decreased significantly (P≤0.05) normal fertilization rate (21.98±4.16).

Table (4): Effect of different concentrations of green tea extract (GTE) on the in vitro fertilization rate of vitrified camel oocytes (mean ± SEM**).**

Treatment	No of oocyte	Penetration rate	Fertilization rate
Control	**38**	**26(68.57±2.18)[a]**	**10 (25.71±5.02)[b]**
0.5 mg GTE ml-1	**42**	**28 (66.67±8.59)[a]**	**18(42.85±12.39)[ab]**
1 mg GTE ml-1	**42**	**32 (76.19±6.31)[a]**	**24(57.14±10.92)[a]**
1.5 mg GTE ml-1	**41**	**28 (68.32±2.12)[a]**	**9 (21.98±4.16) [b]**

a and b superscripts values within the same column are significantly different from each other at p≤0.05.

As demonstrated in (Table 5), GTE supplementation of vitrification medium improved overall survival of frozen-thawed oocytes as assessed by the ratios of in vitro embryo production. In this regard, within the GTE supplemented groups, 1mg/ml appeared to be the most efficient concentration and induced the best cryoprotection. Addition of 1mg /ml GTE to the vitrification media elevated significantly (P≤0.05) cleavage rate, morula and blastocyst

development (39.15±2.08, 24.60±3.97 and 15.61±3.05, respectively) compared with control group (16.35±2.55, 4.60±2.31 and 2.22±2.20, respectively). Moreover, the current results confirmed that 1.5 mg/ml GTE lowered drastically in vitro embryo development to morula and blastocyst stages (4.76±2.38 and 2.38±2.38, respectively).

Table (5): Effect of different concentrations of green tea extract (GTE) on the in vitro embryo development of vitrified camel oocytes (mean ± SEM).

Treatment	No of oocyte	Cleavage rate	Morula	Blastocyst
Control	**43**	**7 (16.35±2.55) ^{b}**	**2 (4.60±2.31)^{b}**	**1 (2.22±2.20) ^{b}**
0.5 mg GTE ml-1	**42**	**12 (28.79±4.32)^{ab}**	**6 (14.29±4.13)^{ab}**	**3 (7.14±4.13)^{ab}**
1 mg GTE ml-1	**46**	**18 (39.15±2.08)^{a}**	**11 (24.60±3.97)^{a}**	**7 (15.61±3.05)^{a}**
1.5 mg GTE ml-1	**44**	**8 (18.75±6.58)^{b}**	**2 (4.76±2.38)^{b}**	**1 (2.38±2.38)^{b}**

a and b superscripts values within the same column are significantly different from each other at p≤0.05.

Data presented in (Table 6) indicated that GTE has a beneficial antioxidant effect. GTE at a concentration of 1 mg/ml augmented the total antioxidant capacity and reduced the lipid peroxidation (MDA) in the vitrified camel oocyte (0.482±0.04 mμ/ml and 9.33±1.77 nmol/ml, respectively) compared with the control oocytes (0.185±0.04 and25.0±3.06, respectively).

Table (6): Effect of different concentrations of green tea extract (GTE) on the antioxidant capacity and lipid peroxidation of vitrified camel oocytes (mean ± SEM).

Treatment	TAC mμ/ml	MDA nmol/ml
Control	**0.185±0.04 ^{c}**	**25.0±3.06^{a}**
0.5 mg GTE ml-1	**0.315±0.02 ^{b}**	**14.33±1.20 ^{b}**
1 mg GTE ml-1	**0.482±0.04 ^{a}**	**9.33±1.77 ^{b}**
1.5 mg GTE ml-1	**0.237±0.02 ^{bc}**	**23.67±3.18 ^{a}**

a and b superscripts values within the same column are significantly different from each other at p≤0.05. TAC: Total Antioxidant Capacity.

Table (7): Effect of different concentrations of green tea extract (GTE) on the mitochondrial function and ATP level of vitrified camel oocytes (mean ± SEM).

Treatment	MTT	ATP fmol
Control	2.40±0.25 ^{bc}	607.33±44.24 ^{bc}
0.5mg GTE ml-1	3.30±0.22 ^{ab}	762.33±78.69 ^{ab}
1mg GTE ml-1	3.90±0.55 ^{a}	882.00±16.67 ^{a}
1.5mg GTE ml-1	2.00±0.38 ^{c}	570.00±58.66 ^{c}

a,b and c superscripts values within the same column are significantly different from each other at p≤0.05.

Data presented in (Table 7) indicated that GTE has an advantageous effect on the mitochondrial function of the veitrified oocyte. GTE at a concentration of 1 mg/ml increased the MTT reduction and the ATP content in the vitrified camel oocyte (3.9±0.55 and 882.00±16.17 fmol, respectively) compared with the control oocytes (2.4±0.25 and 607.33.0± 44.24 fmol, respectively; P<0.05).

DISCUSSION

The current study showed that, the addition of 1 mg /ml of green tea extract to the vitrification medium resulted in a significant improvement in the cryotolerance of camel oocytes and augmented in vitro embryo development to the morula and blastocyst. The beneficial effect of GTE might be due to its antioxidant effect **(Wang *et al.*, 2007).** Little studies have dialed with vitrification of camel oocytes. To our knowledge, this is the first study in Egypt that demonstrates the beneficial effect of GTE supplementation to the vitrification media on camel embryo development. Although the vitrified camel oocytes can complete meiotic maturation in vitro, subsequent development are quite lower than fresh oocytes **(Herrick *et al.*, 2004).** So, preparation of oocytes is one of the critical factors that determine the developmental competence of embryos produced by IVF **(Kilyoung and Eunsong, 2007**).The current results indicated that the supplementation of 1mg/ml GTE to the verification media appeared to be the most efficient concentration that induced the best cryoprotection of camel oocyte. These results are in harmony with **(Wang *et al.*, 2007)** who recorded that supplementation of 15 µM GTPs (Green tea polyphenols) improved the developmental competence of bovine oocytes possibly by protecting the embryos from oxidative stress.The developmental ability

of oocytes matured in defined media still tends to be lower than oocytes matured in media supplemented with chemicals such as growth factors, estradiol, gonadotrophins, amino acids or antioxidants **(Hong *et al.,* 2004 and Vahedi *et al.,* 2009).** Oxidative stress (OS) is one of the main factors responsible for the lowered quality of in vitro produced bovine embryo **(Del Corso *et al.,* 1994 and Wang *et al.,* 2014).** Oocytes and embryos produce endogenous ROS by various enzymatic actions during metabolic processes (**Gardiner and Lane, 2002 and Harvey *et al.,* 2002).** Generation of ROS is a normal feature of basal aerobic metabolism that supports life. Low concentration of hydrogen peroxide, reactive nitrogen species (RNS) and ROS function are important signaling molecules within cells and also necessary for the physiological processes of reproduction such as ovulation **(Shkolnik *et al.,* 2011),** capacitation **(DeLamirande and Gagnon, 1995)** and corpus luteum formation and function **(Vu *et al.,* 2012).** However, excessive ROS production may damage cell membranes, protein and DNA **(Sudano *et al.,* 2010)** and diminish in vitro embryo development. **Guerin *et al.,* (2001)** suggested that ROS are able to diffuse and pass through cell membranes and alter most types of cell molecules such as lipids, proteins and nucleic acids and consequently, mitochondrial alterations, embryo cell-block,ATP depletion and apoptosis. Furthermore, lipid peroxidation causes deformation of cell structures, and this process is associated with anomalies in cell metabolite transport that can affect physiological function **(Volinsky and Kinnunen, 2013).** The current results were emphasized by these data, where excessive lipid peroxidation diminished embryos development. Therefore, ROS must be inactivated continuously in order to maintain only the small amount that necessary to maintain normal cell function **(Sudano *et al.,* 2010)**. The success rates of IVF depend on antioxidant supplementation to neutralize the effect of ROS on oocyte and embryo **(Agarwal *et al.,* 2008).** So, the supplementation of antioxidant at its best concentration is very critical against ROS. Various studies were conducted to measure the effect of antioxidant on oocyte maturation and early embryo development **(Feugang *et al.,* 2004)**. The current results revealed that addition of1mg GTE/ml to the vitrificationmediumsignificantlyimproved($P \leq 0.05$) the cyrotolerance and in vitro embryo production rates.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),** protects oocytes against oxidative stress and promotes DNA synthesis of embryos **(Funahashi, 2005).** The current results emphasized the antioxidant action of GTE through increasing the TAC and reducing the lipid peroxidation in the vitrified-warmed camel

oocytes. Concerning the mitochondrial function, the present results revealed that, the GTE in a concentration of 1.0 mg/ml improve the mitochondrial function and increases ATP content in vitrified camel oocytes. **Scott (2013)** explained that, the best-known function of mitochondria is the generation of ATP from food sources. Pyruvate, converted from glucose, is consumed by mitochondria to produce ATP. As mitochondria produce ATP, they release ROS locally that must be detoxified as they can induce oxidative damage to mitochondrial DNA (mtDNA). This damage results in mutations and deletions of mtDNA. The relative absence of repair enzymes for mtDNA may explain its sensitivity to oxidative stress-induced damage. The 10 - to 20-fold higher mutation rate in mitochondrial DNA compared with nuclear DNA is believed to be due to its proximity to ROS generation and the limited DNA repair capacity. As the organism, tissue, and cells age, exposure of the mitochondrial genome to ROS increases.This compromises the function of this organelle **(Das and Kohlschütter, 1996).** ATP synthase plays an important role in energy metabolism, acting as a key enzyme of ATP synthesis and hydrolysis in the oxidative phosphorylation pathway **(Cui *et al.,* 2006).** Decreased activity of mitochondrial ATP synthase depletes ATP content in the cell, which may lead to cellular dysfunction. The results of the present study suggested that GTE supplementation beyond the optimum concentration ranges might have deleterious effects on the developmental camel oocytes as it drastically lowered in vitro embryo development to morula and blastocyst stages. 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)**. These findings proved that GTE have two different actions: antioxidant action at lower concentrations and pro-oxidant action at higher concentrations **(Wang *et al.,* 2007)**.

CONCLUSION

The current work concluded that supplementation of 1 mg/ ml GTE to the verification media of camel oocytes improved the developmental competence of the oocytes possibly by protecting the embryos from oxidative stress. Further studies are required to study the effect of GTE on development of camel embryos.

REFERENCES

Agarwal, A.,Gupta, S.,Sekhon, L.and Shah, R. (2008): Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. Antioxid. Redox. Signal. 10: 1375 - 403.

Aitken, R.J., Clarkson, J.S. and Fishel, S. (1989): Generation of reactive oxygen species, lipid peroxidation and human sperm function. Biol. Reprod., 40: 183 -197.

Aitken, R.J., Buckingham, D. and Harkiss, D. (1993): Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. J. Reprod. Fertile. 97: 441 - 450.

Ali, A.A., Bilodeau, J.F. and Sirard, M.A. (2003): Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. Theriogenology, 59: 939-949.

Amer, O.S., Dkhil, M.A. and Al-Qurashy, S. (2013): Antischistosomal and hepatoprotective activity of Morus Alba leaves extract. Pakistan J. Zool., 45: 387-393.

Awoniyi, D.O., Aboua, Y.G.,Marnewick, J.and Brook, N.(2012): The effect of Rooibos (Aspalathus linearis), green tea supplementation on Epididymal sperm in oxidative stress-induced rats. Phytother. Res.26: 1231-1239.

Benzie IF and Strain JJ. (1996): The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal Biochem. 239:70 -76.

Cabrera, C., Artacho, R. and Gimenez, R. (2006): Beneficial effects of green tea: a review. J. Am. Coll. Nutr., 25: 79-99.

Camargo, L.S.A., Viana, J.H.M., Sa, W.F., Ferreira, A.M., Ramos, A.A. and Vale Filho, V.R. (2006): Factors influencing in vitro embryo production. Anim Reprod Sci. 3: 19-28.

Costat Computer Program Copyright (1986): Version 3.03 copyright Cottort Software.

Cui, X.S., Li, X.Y., Jeong, Y.J., Jun, J.H., Kim, N.H. (2006): Gene expression of Cox5a, 5b, or 6b1 and their roles in preimplantation mouse embryos. Biol Reprod 74:601 - 610.

Das, A.M., Kohlschutter, A. (1996): Decreased activity of the Mito - € chondrial ATP-synthase in fibroblasts from children with lateinfantile and juvenile neuronal ceroid lipofuscinosis. J Inherit Metab Dis 19:130 - 132.

DeLamirande, E. and Gagnon, C. (1995): Impact of ROS on spermatozoa: a balancing acts between beneficial and detrimental effects. Hum. Reprod. 10, 1:15-21.

Del Corso, A., Cappiello, M. and Mura, U. (1994): Thiol dependent oxidation of enzymes: the last chance against oxidative stress. Int. J. Biochem., 26: 745-750.

El-Sokary, M.M.M., Kandiel, K.G.H.M., Mahmoud, M.E.A., Abouel-Roos and Sosa, G.A.M. (2013): Evaluation of Viability and Nuclear Status in Vitrified Mature Buffalo Oocytes. Global Veterinaria, 10: 297-302.

Feugang, J.M., De roover, R., Moens, A., Leonard, S., Dessy, F. and Donnay, I. (2004): Addition of beta-mercaptoethanol or trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. Theriogenology, 161: 171-190.

Funahashi, H. (2005): Effect of beta-mercaptoethanol during in vitro fertilization procedures on sperm penetration into porcine oocytes and the early development in vitro. Reproduction, 130: 889 - 898.

Gardiner, D.K. and Reed, D.J. (1995): Synthesis of glutathione in the preimplantation mouse embryo. Arch. Biochem. Biophys. 318: 30-36.

Gardiner, D.K. and Lane, M. (2002): Development of viable mammalian embryo in vitro: evaluation of sequential media. In: Principal of cloning. Academic Press, New York, 187-208.

Gupta, S., Sekhon, L., Kim, Y. and Agarwal, A. (2010): The role of oxidative stress and antioxidants in assisted reproduction. Curr. Women's Hlth. Rev., 6: 227-238.

Guerin, P., El mouatassim, S. and Menezo, Y. (2001): Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum. Reprod, 7: 175-189.

Haghparast, S., Kashiri, H., Alipour, G. and Shabanpour, B. (2011): Evaluation of green tea (Camellia sinenses) extract and onion (Allium Cepa L.) juice effects on lipid degradation and sensory acceptance of Persian sturgeon (Acipenser persicus) fillets: A comparative study. J. Agric. Sci. Tech., 13: 855 - 868.

Halliwell, B. and Aruoma, O.I. (1991): DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. 281:9-19.

Harvey A.J.,Kind, K.L.and Thompson, J.G.(2002): Redox regulation of early embryo development. Repr Mod, 123: 479 - 486.

Herrick, J.R., Behboodi, E., Memili, E., Blash, S., Echelard, Y. and Krisher, R.L. (2004): Effect of macromolecule supplementation during in vitro maturation of goat oocytes on developmental potential. Mol. Reprod. Dev., 69: 338 -346.

Hijazi, M.M., Khatoon, N., Azmi, M.A., Rajput, M.T., Zaidi, S.I., Azmi, M.A., Perveen, R., Naqvi, S.N. and Rashid, M. (2015): Report: effect of Camellia Sinensis L.(green tea) extract on the body and testicular weight changes in adult Wistar rate. Pak. J. Pharm. Sci. 28:249-253.

Hong, J.R., Yong, H.Y., Lee, B.C., Hwang, W.S., Lim, J.M. and Lee, E.S. (2004): Effects of amino acids on maturation, fertilization and embryo development of pig follicular oocytes in two IVM media. Theriogenology, 62: 1473-1482.

Kilyoung, S. and Eunsong, L. (2007): Modification of maturation condition improves oocyte maturation and in vitro development of somatic cell nuclear transfer pig embryos. J. Vet. Sci., 8: 81–87.

Knapen,M.F.,Zusterzeel, P.L.,Peters, W.H. and Steegers, E.A.(1999): Glutathione and glutathione-related enzymes in reproduction. A review. Eur. J.Obstet. Gynecol. Reprod. Biol. 82:171-184.

Ledda, S., L. Bogliolo, S. Succu, F. Ariu, D. Bebbere, G.G. Leoni and S. Naitana (2007): Oocyte cryopreservation: oocytes assessment and strategies for improving survival. Reprod. Fertile. Dev., 19: 13-23

Miesel, R., Drzejczak, P.J. and Kurpisz, M. (1993): Oxidative stress during the interaction of gametes. Biol. Reprod., 49: 918-923.

Mukhtar, H., Wang, Z.Y., Katlya, K.S. and Agarwal, R. (1992): Tea components: ant mutagenic and ant carcinogenic effects. Prev. Med., 21: 351-360.

Natarajan, R., Shankar, M.B. and Munuswamy, D. (2010): Effect of α-tocopherol supplementation on in vitro maturation of sheep oocytes and in vitro development of preimplantation sheep embryos to the blastocyst stage. J. Assist. Reprod. Genet. 27: 483 - 490.

Nikiforaki,F.Vanden Meerschaut, F.C. Qian, I. De Croo, Y.Lu, T.(2014): Oocyte cryopreservation and in vitro culture affect calcium signaling during human fertilization. Hum. Reprod. 29: 29 - 40.

Ohkawa H,Ohishi N.and Yagi K.(1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 95:351-358.

Parrish, J. J.; Susko-Parrish, J.; Winer, M.A. and First, N.L. (1988): Capacitation of bovine sperm by heparin. Biol. Reprod.; 38:1171-1180.

Pattie, S.G. and Christian, L. (2012): Mitochondrial dysfunction is an early indicator of doxorubicin-induced apoptosis. Biochimica ET Biophysica Acta (BBA) - Molecular Basis of diseases, 1588, (1):94 -101.

Rao, B.S., Naidu, K.S., Amarnath, D., Vagdevi, R., Roa, A.S., Brahmaiah, K.V. and Roa, V.H. (2002): In vitro maturation of sheep oocytes in different media during breeding and non-breeding seasons. Small Rumin. Res., 43: 31-36.

Rahmani,A.H.,Al-Shabrmi,F.M.,Allemailem, K.S., Aly, S.M. and Khan,M.A.(2015): Implications of green tea and its constituents in the prevention of cancer via the modulation of cell signaling pathway. BioMed. Res. Int. 5:434 - 444.

Sagirkaya, M. Misirlioglu, A. Kaya, N.L.First, J.J. Parrish, E. and Memili (2006): Developmental and molecular correlates of bovine preimplantation embryo. Reprod. 131,5: 895-904.

Scott, C.(2013): The role of mitochondria from mature oocyte to viable blastocyst Obst. And Gynecol. Internat. 2013:1-10.

Sharma, K. Loganathasamy (2007): Effect of meiotic stages during in vitro maturation on the survival of vitrified-warmed buffalo oocytes.Vet.Res.Comm.31:881-893.

Sharma, P.K., Dubey, V. and Chandra (2010): Morphological changes, DNA damage and developmental competence of in vitro matured, vitrified-thawed buffalo (Bubalus bubalis) oocytes:a comparative study of two cryoprotectants and two cryodevices.Cryobiol. 60:315-321.

Shkolnik, K., Tadmor, A., Ben-Dor, S., Nevo, N., Galiani, D. and Dekel, N. (2011): Reactive oxygen species are indispensable in ovulation. Proc. Natl. Acad. Sci. U.S.A., 108: 1462-1467.

Sikka, S.C. (2003): Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. J.Androl, 25:5-18.

Sudano, M.J., Mattos, M.C.C., Fernandes, C.B., Mazieiro, R.R. and Landim-Alvarenga, F.C. (2010): In vitro production of bovine embryos using Sigma antioxidant supplement α-tocopherol and L-ascorbic acid. Anim. Reprod., 7-347-359.

Vahedi, V., Zeinoaldini, S., Kohram, H. and Farahavar, A. (2009): Retinoic acid effects on nuclear maturation of bovine oocytes in vitro. Afr. Biotechnol.8:39 - 47.

Volinsky, R. and Kinnunen, P.K. (2013): Oxidized phosphatidylcholines in membrane level cellular signaling: from biophysics to physiology and molecular pathology. FEBSJ. 280:2806 -2816.

Vu, H.V., Lee, S., Acosta, T.J., Yoshioka, S., Abe, H. and Okuda, K. (2012): Role of prostaglandin F2-α and hydrogen peroxide in the regulation of copper-zinc superoxide dismutase in bovine corpus luteam and luteal endometrial cells. Reprod. Biol. Endocrinol. 10:87-97.

Wang, Z.G., Yu, S.D. and Xu. Z.R. (2007): Effect of supplementation of green tea polyphenols on the developmental competence of bovine oocytes in vitro. Braz. J .Biol. Res. 40: 1079 -1085.

Wang, T.Y., Li, Q., Li, H., Zhu, J., and Cui, W., Jiao, G.Z. and Tan, J.H. (2014): Non frozen preservation protocols for mature mouse oocytes dramatically extend their developmental competence by reducing oxidative stress. Mol.Hum. Reprod. 20: 318 -329.

Wani, N.A,Misra, A.K. and Maurya, S.N. (2004): Maturation rates of vitrified-thawed immature buffalo (Bubalus bubalis) oocytes: effect of different types of cryoprotectants. Anim. Reprod. Sci., 84:327-335.

Yang, H.W., Hwang, K.J., Kwon, H.C., Kim, H.S., Choi, K.W. and Oh, **K.S. (1998):** Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum. Reprod. 13:998-1002.

Zhoa, X.M., Du, W.H., Wang, D., Hao, H.S., Liu, Y., Qin, T. and Zhu, H.B. (2011): Recovery of mitochondrial function and endogenous antioxidant systems in vitrified bovine oocytes culture.