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Antioxidant effect of Epigallocatechin gallate on bovine IVM and IVF

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Abstract
The present study examined the effect of epigallocatechin gallate (EGCG) during *In-vitro* maturation (IVM) and *In-vitro* fertilization (IVF) of bovine oocytes. Cumulus-oocyte complexes (COCs) were aspirated from the ovaries derived from slaughter house and cultured in Tissue culture medium-199 supplemented with 5, 10 or 15 µM of epigallocatechin gallate for 24 h. Oocytes of a control group were matured in a maturation medium without EGCG. After IVM, COCs were coincubated with frozen-thawed spermatozoa for 15–18 h. In comparison with the absence of EGCG, treatment with EGCG at 10 and 15 µM showed a significant increase in the proportion of cumulus cell expansion, 1st polar body and 2nd polar body. However, compared to control, the presence of 10 and 15 µM EGCG during IVM significantly ($P < 0.01$) increased the proportion of *In-vitro* maturation and fertilization rate. However, a further decrease to 5 µM EGCG reduced ($P < 0.01$) the *In-vitro* maturation and fertilization rate. The results suggest that at certain concentrations of EGCG (10 and 15 µM) in IVM medium has beneficial effects on *In-vitro* maturation and subsequent *In-vitro* fertilization of bovine oocytes.

Keywords: Oocyte, IVM, IVF, antioxidant, epigallocatechin gallate, green tea

1. Introduction
In-vitro embryo production (IVEP) technology has been successfully applied in a number of animal species with transferred embryos resulting in live offsprings. Incorporation of the various types of additives in tissue-culture media has been found to be useful to increase the rate of *In-vitro* maturation of domesticated livestock as well as wildlife oocytes [3]. *In-vitro* cultures of oocytes and embryos are maintained at higher concentrations of oxygen than the *in-vivo* environment, leading to an increased level of reactive oxygen species (ROS). *In-vivo*, the damaging effects of oxygen radicals are usually prevented or limited by endogenous antioxidants (or scavengers of free radicals). However, the level of antioxidants was lower than *in-vivo* during *In-vitro* culture of oocyte and embryo. Consequently, the addition of an antioxidant may be important. A new antioxidant, Green tea polyphenols have been found as an alternative for the *In-vitro* culture of oocyte and embryo. Green tea polyphenols are mainly epigallocatechin gallate (EGCG), epicatechingallate (ECG), epicatechin (EC) and epigallocatechin (EGC) etc. All these catechins have strong antioxidant activity [10, 11] and are effective in enhancing *in-vitro* maturation and fertilization rate [5, 9]. So, the present study was conducted with an aim to standardize the culture condition of bovine oocytes using epigallocatechin gallate as an antioxidant for *in-vitro* maturation and subsequent *in-vitro* fertilization of bovine oocytes.

2. Materials and Methods
The media and chemicals used to conduct the present study were procured from Sigma-Aldrich, USA.

2.1 Collection of ovary and oocytes: The cattle ovaries were collected from a local slaughter house in warmed (37 ^{0}C) normal saline solution (0.9%) containing Gentamicin (50 µg/ ml) in a thermos flask and brought to the laboratory within 2 h after the animals was slaughtered. The extraneous tissues were removed from the ovaries with the help of scissors. The ovaries were then washed 3-4 times in physiological saline solution containing Gentamicin (50 µg/ ml) prior to processing. The oocytes were retrieved using aspiration technique and slicing technique in

aspiration medium [4]. Only oocytes with at least three layers of compact cumulus cells and homogenous cytoplasm were selected for further processing.

2.2 *In-vitro* maturation: COCs were washed three times each in washing medium (TCM-199 supplemented with Fetal bovine serum (10%), sodium pyruvate (0.8 mM), L-glutamine (0.7 mM) and gentamicin sulphate (50µg/ml)) and in maturation medium [3] with the different concentration of EGCG @ 5, 10 or 15 µM and control without EGCG. COCs were then incubated in 50 µl droplets (8–10 oocytes per drop) of maturation medium. The droplets were covered with mineral oil and then incubated at 38.5 ˚C in 5% CO2 with 90-95 % humidity for 24 h.

2.3 *In-vitro* fertilization: The IVF was carried out as described by Wang *et al*. 2007 [9]. Briefly, matured COCs were washed thrice in washing TALP (Tyrode's Albumin Lactate Pyruvate Solution) and twice in fertilization TALP and then placed in 50µl droplets (10–12 COCs per droplet) of fertilization medium. Frozen bull semen was thawed and prepared by a swim-up procedure. Sperm cells were added to the fertilization drops at a concentration of 2 million per ml. Incubation was carried out at 38.5˚C in 5% CO2 with 90-95 per cent humidity for 15–18 h.

2.4 Evaluation of oocytes after IVM and IVF: IVM of oocytes was carried out with TCM-199 based maturation medium in the presence of 5, 10 or 15 µM EGCG and without treatment. The rates of maturation were counted after IVM of 24 h of culture and then matured oocytes were subjected to fertilization in standard fertilization medium. Fertilization rate is assessed after 18 h of co-culture. After 24 h of incubation in a CO2 incubator maintaining the temperature at 38.5^0C with 5% CO2 in the humidified air, oocytes were examined under the Phase contrast inverted Microscope at 40 × 10X zoom for assessment of *in-vitro* maturation. Maturation status was assessed based on: (a) The degree of expansion of cumulus cells, (b) Extrusion of 1st polar body. As a result of *in-vitro* maturation, compact cumulus cell mass changes into a disperse structure which leads to a volumetric expansion of the cumulus oocytes complexes. The degree of expansion of cumulus cell expansion was observed as (i) Full cumulus cell expansion: Expansion of the cumulus cell masses to at least 3X of its original diameter away from the zona pellucida (ZP), (ii) Moderate cumulus expansion: Expansion of the cumulus cell to at least twice of its original diameter away from the ZP, (iii) Slight or no expansion of the cumulus cell mass: Cumulus cells tightly adhered to the ZP.

Fertilization status was assessed based on extrusion of the 2nd polar body in the perivitelline space after 18 h of co-culture in a CO2 incubator at 38.5^0C with 5% CO2 in the humidified air, under the Phase contrast inverted Microscope at 40 × 10X zoom.

2.5 Statistical analysis: The statistical analysis was done by using SAS enterprise guide 4.3.

3. Results and Discussion

In the present study, 10 µM and 15 µM of Epigallocatechin Gallate was found best in both IVM and IVF than other groups (Table 1). Similar finding was also reported by Wang *et al.* [9] with Green tea polyphenols (GTP) that treatment with 10 and 15 µM GTP significantly enhanced *in-vitro* maturation rate and subsequent development to the blastocyst stage in bovine oocytes. This improvement is due to increase of intracellular glutathione (GSH) concentration after IVM of oocytes. However, a further increase in GTP concentration from 20 to 25 µM did not improve the fertilization competence or the proportion of oocytes reaching the blastocyst stages. Wang *et al*. [6] reported that 15 µM GTP (green tea polyphenols) during IVM and IVC improved pregnancy rates after Embryo Transfer, this improvement is due to the increase of relative transcript abundance (RA) of antioxidant enzyme genes, SOD1, CAT, and GPX, and the decrease in apoptosis index (AI) in bovine blastocysts. Further increase in concentration failed to improved rates. Roychoudhury *et al.* [1] demonstrated that at highest dose (200 µg/ ml) of green tea extract apopotosis is markledly increased than lower dose (0.1, 1, 10 and 100 µg/ml) which is due to the increase accumulation of caspase-3 and p53 apoptotic markers in granulosa cells of Porcine. Barakat *et al*. [5] found that GTE (green tea extract) at concentrations of 0.3 mg/ml in IVM medium enhanced the *in vitro* maturation and embryo development of sheep oocytes to blastocyst stage. Addition of GTE at 0.6 mg/ml and more to IVM medium had little benefit in increasing the maturation rate and blastocyst formation. Another constituent of Green tea polyphenols *i.e* Epigallocatechin-3-gallate was found to have similar effect as reported by Spinaci *et al*. [8] that at certain concentration it enhanced *in vitro* maturation and fertilization in pig. However, it also exerts a diphasic effect on fertilization rate that is improved at medium (0-25 µg/ml) dosages while it is inhibited at high (more than 25 µg/ml) concentrations [8]. Similar to Yavari *et al.* [7] that Epigallocatechin-3-gallate (EGCG) at 10 and 50 µM was apparently harmful for *in vitro* development of porcine parthenotes [7]. On fertilization, addition of Epigallocatechin-3-gallate at 25 µM and 50 µM in thawing extender exhibited a significantly (P<0.01) increase *in vitro* penetration rate and total fertilization efficiency in boar [2]. Above discussion is well supported by Sakagami *et al*. [12] report that GTP has two different actions: an antioxidant action at lower, and a pro-oxidant action at higher concentrations [12].

In the present study, oocytes treated with 10 µM and 15 µM EGCG had higher *in vitro* maturation and fertilization rates than control (Table 1). This improvement might have been partly because of the increase relative transcript abundance (RA) of antioxidant enzyme genes, SOD1, CAT, and GPX or/ and due to increase of intracellular glutathione (GSH) concentration. However, the precise reasons for this improvement are unclear, and need to be clarified in future investigations.

In conclusion, treatment with Epigallocatechin Gallate at 10 µM and 15 µM on *in-vitro* maturation medium significantly improved the rate of *in-vitro* maturation and subsequent fertilization. So, Epigallocatechin Gallate alone might have the similar effect of GTP and GTE at lower concentration on *in-vitro* culture of oocytes.

Table 1: *In-vitro* Maturation and fertilization rate of Bovine Oocyte based on Cumulus cell expansion, 1st Polar body extrusion and 2nd Polar body extrusion in TCM-199 Based medium containing different concentration of Epigallocatechin Gallate

Antioxidant	No. of oocytes used for IVM	Cumulus Cell Expansion		1st Polar body extrusion		2nd Polar body extrusion		
		No. of oocytes matured	IVM rate (Mean ± SE)	No. of oocytes matured	IVM rate (Mean ± SE)	No. of Oocytes use for IVF	No. of Fertilized Oocytes	IVF Rate Mean ± SE
EGCG (5 µM)	304 (15)	163	53.97^{c}±2.06	131	43.24^{c}±1.03	131	52	39.79^{b}±1.57
EGCG (10 µM)	299 (15)	221	74.10^{a}±1.96	171	57.27^{a}±1.31	171	94	55.34^{a}±2.24
EGCG (15 µM)	313 (15)	228	73.22^{a}±1.98	177	56.68^{a}±1.40	177	94	53.54^{a}±2.06
Control	298 (15)	184	56.39^{c}±1.76	139	45.26^{c}±1.73	139	55	41.82^{b}±2.20

Means with the different superscripts in a column differ significantly ($P< 0.01$)
Figure in the parenthesis indicate no. of trails

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