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Original Research Article

Dietary supplementation with green tea extract improves the antioxidant status and oocyte developmental competence in Italian Mediterranean buffaloes

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ABSTRACT

The aim of this work was to assess the antioxidant status and the developmental competence of oocytes recovered by ovum pick-up (OPU) in Italian Mediterranean buffaloes supplemented with green tea extracts (GTE) for 90 days. Buffalo cows (n = 16) were randomly assigned to a control group receiving no supplement and a treatment group, receiving GTE starting 90 days before OPU, carried out for five consecutive sessions. Blood samples were collected before the start of supplementation with GTE (T0) and at day 45 (T1) and day 90 (T2) of supplementation, to measure ferric reducing activity (FRAP), total antioxidant capacity (TAC), superoxide dismutase (SOD) and catalase (CAT). The antioxidant status of follicles was measured as TAC on the follicular fluid collected from the dominant follicle just prior OPU, coinciding with T2, and at the end of five repeated OPU sessions (T3). Another objective was to assess *in vitro* the protective effects of green tea extracts on hepatic cells exposed to methanol insult. Different concentrations of GTE (0.5 μM and 1 μM) were tested on cultured hepatic cells and viability, morphology and SOD activity were assessed at 24, 48 and 72 h.

Supplementation with GTE increased ($P < 0.05$) the number of total follicles (8.7 ± 0.5 vs 6.9 ± 0.5), the number and the percentage of Grade A + B cumulus-oocyte complexes (COCs) compared with the control (3.7 ± 0.4 vs 2.3 ± 0.3 and 57.5 ± 4.2 vs 40.4 ± 4.9 %, respectively). Oocyte developmental competence was improved in the GTE group as indicated by the higher ($P < 0.05$) percentages of Grade 1,2 blastocysts (44.8 vs 29.1 %). In the GTE group, plasma TAC was higher both at T1 and T2, while FRAP increased only at T2, with no differences in SOD and CAT. The TAC of follicular fluid was higher ($P < 0.05$) in the GTE compared to the control both at T2 and at T3 The *in vitro* experiment showed that co-treatment with methanol and 1 μM GTE increased ($p < 0.01$) cell viability at 24 h ($P < 0.01$), 48 h ($P < 0.05$) and 72 h ($P < 0.01$) compared with the methanol treatment co-treatment with 1 μM GTE prevented the decrease in SOD activity observed with methanol at 24 and 48 h of culture. In conclusion, the results of *in vivo* and *in vitro* experiments suggest that supplementation with GTE increases buffalo oocyte developmental competence, by improving oxidative status and liver function.

1. Introduction

The river buffalo (*Bubalus bubalis*) is widely distributed and is growing in importance globally as a source of meat and milk [1]. The importance of buffalo in the world economy is demonstrated by the constant growth of its population, which has gone from 107 million in 1970 to 201 million in 2017, with 20%growth rate between 2002 and 2017 [2].

The unreplaceable role as an animal protein producer particularly in tropical countries makes buffalo an important livestock resource in the

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current scenario, where the challenge is to feed an increasing world population and counteract climate changes and water scarcity. In addition, buffalo milk is highly nutritious and contains relatively high amounts of functional biomolecules [3]. Buffaloes are tendentially short-day breeders and reproductive seasonality affects the profitability of buffalo breeding, due to the discontinuity of milk supply. Although ovarian cyclic activity may occur throughout the year, seasonal anestrus and calving and milk production cycles are observed. When buffaloes are forced to conceive during the non-reproductive season, to meet the market demand, they may undergo a higher incidence of embryonic mortality, due to reduced luteal function and oocyte developmental competence [4–7], and long post-partum anestrus periods [8].

Buffalo farming sustainability greatly depends on genetic improvement and hence on advanced reproductive technologies. Due to the low response to conventional multiple ovulation and embryo transfer [9], Ovum pick-up in conjunction with *in vitro* embryo production is highly important for the utilization of female buffaloes for genetic improvement [10]. The major limiting factor is the low number of competent oocytes that are recovered per donor in this species, related to the scarce follicular reservoir [10,11], leading to high embryo production costs. As the number of follicles and oocytes can be only improved to a limited extent, strategies to improve the oocyte competence are needed, to counteract the low number of recruitable oocytes with higher embryo yields. The oocyte developmental competence, i.e., the capacity to undergo fertilization and embryo development, is gradually acquired during the last phase of follicular growth and is influenced by the follicular composition [12]. This may be affected by several factors, such as season [6], nutrition [13], metabolic disorders [14] and, hormonal treatments [15,16]. During months characterized by increasing day length, buffalo oocytes have a reduced capacity to undergo fertilization and embryonic development [17,18]. This is associated with metabolite changes and reduced antioxidant defense in the follicular environment [13]. Furthermore, it is known that nutrition affects liver function and consequently fertility in dairy cows [19]. The use of high-concentrate diets to maximize milk production [20–22]. Dairy buffaloes can also develop hepatic lipidosis between the time of parturition and the early stages of lactation [23]. Furthermore, a diet too rich in nutrients can interfere with the physiological antioxidant defense and lead to a condition of oxidative stress [24].

It is known that oxidative stress (OS) is associated with reproductive disorders and low fertility in livestock [25–29]. An excess of reactive oxygen species (ROS) provokes several cell damages, due to the oxidation of lipids, proteins, and DNA, as well as metabolic disruptions [30]. Cattle show an increased ROS production in response to negative energy balance [31–33], heat stress [34], and age [35], all of which are linked with reduced fertility. Increased OS was also reported in Egyptian buffaloes during summer [36], when reproductive activity was impaired [37].

Currently, the use of natural antioxidants has been proposed to improve fertility in ruminants [29]. Green tea (GT), a widely consumed beverage derived from the plant Camellia sinensis, has strong antioxidant properties, due to the high content of polyphenols [38,39]. The main polyphenols in green tea are catechins which act as potent scavengers of ROS including hydrogen peroxide, hydroxyl radicals, and nitric oxide [40,41]. The addition of green tea polyphenols during oocyte *in vitro* maturation improved glutathione oocyte levels and embryo development in cattle [42]. Furthermore, green tea polyphenols during *in vitro* maturation and culture influenced the expression of antioxidant genes and apoptosis in bovine embryos, and increased pregnancies [43]. Improved oocyte maturation and embryo development were also observed with green tea extract during *in vitro* maturation of sheep oocytes [44]. Similar effects of green tea polyphenols on oocytes and embryos *in vitro* were reported for buffaloes [45].

The novelty of the study was to evaluate for the first time the effect of dietary supplementation with green tea on oocyte developmental competence in livestock. This may open the way for the inclusion in the diet of natural polyphenols as a more physiological approach to modulating ovarian function in high-yielding dairy buffaloes. The hypothesis tested was that dietary supplementation with green tea extracts (GTE) would increase oocyte quality and developmental competence during the non-breeding season in buffaloes, by improving antioxidant status and liver function. Therefore, the aim of this work was to evaluate the antioxidant status and the developmental competence of oocytes recovered by ovum pick-up (OPU) in Italian Mediterranean buffaloes supplemented with GTE for 90 days. Another objective was to assess the effects of GTE on liver function, by using an *in vitro* model, i.e. cultured hepatic cells exposed to a chemical insult.

2. Experiment design

To assess the effect of oral green tea supplementation on oocyte competence, buffalo cows (n = 16) were randomly assigned to two groups, i.e., a control group receiving no supplement and a treatment group, receiving GTE starting 90 days before oocyte collection by OPU technique. Donors were stimulated, as described below, and underwent OPU every 6 days for 5 consecutive sessions. Blood samples were collected before the start of supplementation with green tea (T0) and at day 45 (T1) and day 90 (T2) of supplementation. The antioxidant status of blood was determined by measuring ferric-reducing activity (FRAP), total antioxidant capacity (TAC), superoxide dismutase (SOD), and catalase (CAT).

The antioxidant status of follicles was measured as TAC on the follicular fluid collected from the dominant follicle on synchronized donors just prior to OPU, coinciding with T2, and at the end of five repeated OPU sessions (T3). Then donors were stimulated as described below and underwent OPU every 5 days for five consecutive sessions.

An *in vitro* experiment was undertaken to evaluate the protective role of GTE on hepatic cells exposed to a chemical insult (methanol). Different concentrations of GTE (0.5 μM and 1 μM) were tested on cultured hepatic cells and viability, morphology, and superoxide dismutase (SOD) activity were assessed at 24, 48, and 72 h.

2.1. Reagents and media

Unless otherwise stated all chemicals and reagents were purchased from Merck Life Science S.r.l. (Milan, Italy). The GTE consisted of standardized powdered extract from Camellia sinensis (Green Tea P.E. 50 % Polyphenols, product code 01.1000211, Italfeed, Milan, Italy). Commercial kits were used to assess total antioxidant capacity, superoxide dismutase, and catalase (Abcam, Cambridge, United Kingdom).

2.1.1. In vivo experiment: animals

The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design and animal treatments (PG/2019/0070004 del July 02, 2019). The trial was carried out during the non-breeding season at a farm located in the Campania region of Southern Italy. Multiparous buffalo cows (n = 16) were used in the study. Age, parity, and milk production were (mean ± SE) 6.1 ± 0.1 years, 3.9 ± 0.1, and 2734 ± 1 kg, respectively. The cows were barn-housed throughout the study.

2.1.2. Dietary supplementation with green tea extract

Buffalo cows were randomly assigned to two groups: control (n = 8), cows received a standard total mixed ration; treated (n = 8), cows received a total mixed ration + 6 g/day GTE. The dose was chosen according to a previous work on dairy cows, in which the effect of GTE supplement was assessed on milk production [46]. Dietary supplementation with green tea extract was commenced at 20.0 ± 1.2 days before calving.

2.1.3. Blood and follicular fluid sampling

Blood samples were obtained by venipuncture of the lateral tail vein

[47] before the start of supplementation with green tea (T0) and at day 45 (T1) and day 90 (T2) of supplementation. Blood samples were centrifuged at 14.000×*g* and plasma was stored at −80 °C until required for analyses. Follicular fluid was collected from dominant follicles by transvaginal follicular aspiration in buffaloes synchronized as described below, at times T2 and two weeks after T3. Follicular fluid samples were transferred into 1.5 mL tubes and centrifuged (300×*g* for 10 min), then the supernatant was collected and stored at −80 until the moment of the analysis.

2.1.4. Synchronization and priming of donors and OPU

To collect follicular fluid from dominant follicles at T2 and T3, the estrous cycle was synchronized by administration of 100 μg GnRH on Day 0, 375 μg PGF2α on Day 7, and 100 μg GnRH on Day 9 (Ovsynch) as previously described [48]. The priming prior OPU consisted of dominant follicle removal and insertion of a progesterone (P4)-releasing intravaginal device (PRID Delta 1.55 g, Ceva Animal Health Ltd., Amersham, Buckinghamshire, United Kingdom) on day 0 (T2), administration of 40 mg of FSH (Folltropin, Vétoquinol S.A., Magny-Vernois, France) every 12 h for six times starting on day 2, with OPU carried after 28–32 h after the last FSH administration, as previously described [36].

After 90 days of supplementation, OPU was carried out as previously described [49]. A 5 MHz micro-convex probe was used to scan ovaries (Sonoace Pico, Medison, Seoul, Korea) and follicles were aspirated using an 18-gauge needle attached to a vaginal guide (WTA Ltda., Cravinhos/SP, Brazil) and −40 mmHg vacuum (K-MAR-5100, Cook IVF Co., Queensland, Australia). The aspiration line was rinsed with phosphate-buffered saline supplemented with 100 USP units/mL of heparin (Eparina Vister, Teva Italia s.r.l., Milan, Italy), 1 % fetal calf serum (FCS) and 1 % penicillin and streptomycin complex (20000 IU and 20000 μg ml^{-1}, respectively, Lonza, Milan, Italy). Oocytes were collected into 50 mL conical tubes (Falcon, Corning Science, Reynosa, México) maintained at 37 °C. All antral follicles with a diameter ≥2 mm were aspirated and classified according to size: small (<5.0 mm diameter), medium (5.0–10.0 mm diameter), and large (≥10.0 mm diameter). The aspirated solution was filtered through a 70 μm sterile nylon strainer (Corning, Life Science, Durham, NC, USA), and cumulus-oocyte complexes (COCs) were recovered, washed twice in Hepes 199 (H199; Gibco, Thermo Fisher Scientific, Waltham, USA) with 10 % FCS and classified according to their morphology as previously described [7]. Only grade A + B + C COCs were selected and transferred in 1.8 mL sterile Cryovials tubes (Simport Scientific, Beloeil, Canada) containing H199 supplemented with 10 % FCS, 0.2 mM sodium pyruvate, 100 μM β-mercaptoethanol, 0.5 μg mL^{-1} FSH, 5 μg mL^{-1} LH, 1 μg mL^{-1}17-β-estradiol and 50 μg mL^{-1} gentamycin (*in vitro* maturation medium, IVM medium). COCs were transported to the laboratory within 4–6 h in a portable incubator (WTA, Cravinhos, SP, Brazil) at 38.7 °C. For each OPU session, the recovery rate (percentage of total oocytes in relation to aspirated follicles), number of total COCs, and COCs suitable for *in vitro* embryo procedures (IVEP; grade A + B + C) were recorded. Cows underwent five consecutive OPU at 6-day intervals.

2.1.5. In vitro embryo production

In the laboratory, COCs-containing tubes were incubated at 38.7 °C for 22 h, and *in vitro* fertilization (IVF) was performed according to the method previously reported [36]. Frozen-thawed sperm from a bull suitable for IVF were separated by Bovipure density gradient (Nidacon, Mölndal, Sweden). After centrifugation, the pellet was resuspended to a final concentration of 2×10^6 mL^{-1} in the IVF medium (modified Tyrode's albumin lactate pyruvate supplemented with 0.2 mM mL^{-1} penicillamine, 0.1 mM mL^{-1} hypotaurine, and 0.01 mM mL^{-1} heparin). The IVF was carried out in 50 μL drops (5 COCs/drop) covered by mineral oil that were incubated at 38.7 °C under a controlled gas atmosphere of 5 % CO_2 in humidified air. After 20 h of gamete co-incubation, presumptive zygotes were denuded and cultured for 7 days in 20 μL drops (10 zygotes/drop) of synthetic oviduct fluid (SOF) supplemented with essential and non-essential amino acids and bovine serum albumin (BSA) in a modular chamber containing a gas atmosphere of 5 % CO_2, 5 % O_2, and 88 % N_2. At day 5 (day 0 = IVF day), cleavage rate was assessed and embryos were transferred into fresh drops of the same medium for a further 2 days. Embryo yield (tight morulae-blastocysts and superior quality blastocysts) was evaluated on day 7 of culture.

2.1.6. Measurement of antioxidant markers

Plasma ferric-reducing antioxidant power (FRAP) was determined using a commercial kit (MAK369) according to the manufacturer's instructions. Briefly, for each well, 190 μL of the reaction mixture, consisting of FRAP Assay Buffer, $FeCl_3$ Solution, and FRAP Probe were mixed with 10 μL of sample and the reducing power was calculated by monitoring the increase in absorbance at 594 nm for 1h at 37 °C. FRAP value was expressed as Fe^{2+} equivalents (μM, range = 10–250 x μg/mL of ascorbic).

Total antioxidant capacity (TAC) was measured in plasma and in follicular fluid using the TAC assay kit to examine the cumulative effect of all antioxidants present in samples (cat.no. ab65329; Abcam, Cambridge UK). Briefly, an amount of 100 μL of Cu^{2+} Working Solution was mixed with 100 μL of sample and incubated at room temperature for 90 min on an orbital shaker protected from light, according to the manufacturer's protocol. The samples were read at optical density (OD) 570 nm using a microplate reader (Glomax Multi Detection System Spectrophotofluorimeter, Promega Italia s.r.l., Milan, Italy). Data were expressed in nmol/μL.

Superoxide dismutase (SOD) activity in plasma and hepatic cells was assessed using a SOD colorimetric assay kit (cat.no. ab65354; Abcam, Cambridge, United Kingdom), following the manufacturer's instructions. The reduction of the water-soluble tetrazolium salt (WST-1) in a water-soluble formazan dye by superoxide anions provides the basis for this test. Since SOD inhibits formazan dye generation, SOD activity was determined using the inhibition percentage of the water-soluble formazan dye, which reflected the percent inhibition of the superoxide anions. The formazan dye was detected at OD 450 nm using a microplate reader (EnVision 2103 Multilabel Reader, PerkinElmer Life and Analytical Sciences, Shelton, USA). Data were expressed as a percentage of inhibition of SOD activity (inhibition rate %).

Catalase (CAT) activity in plasma was measured using a CAT assay kit (cat.no. ab83464; Abcam, - Cambridge, United Kingdom), according to the manufacturer's guidelines. Briefly, the H_2O_2 supplied to each well reacted with CAT in the samples to produce water and oxygen. After 10 min of incubation at 25 °C with Developer Mix, the unconverted H_2O_2 interacted with the OxiRed probe and was read at OD 570 nm on a microplate reader (EnVision 2103 Multilabel Reader, PerkinElmer Life and Analytical Sciences, Shelton, USA). Data were expressed as nmol/min/mL.

2.1.7. In vitro experiment: cell culture

HepaRG™ cells (Gibco, Thermofisher Scientific, Waltham, USA) were thawed at 37 °C in a water bath and resuspended in William's E Medium supplemented with GlutaMAX™ (Gibco, Thermofisher Scientific, Waltham, USA). After assessing viability (TC20™ Automated Cell Counter; Biorad, Hercules, USA) and number (>10^7 viable cells/vial), 0.072×10^6 cells were seeded in 96-well plates and 0.48×10^6 in 24-well plates, according to the manufacturer's instructions. All plates were maintained at 37 °C with 5 % CO_2 and saturated humidity. The medium was renewed after 24, 96, 144, 168 h. Cell monolayers were exposed to 3 % methanol in William's E Medium and either 0 (methanol), 0.5 μM, or 1 μM GTE were tested to assess the protection against hepatic damage. Cells cultured in the William's E Medium non-exposed to methanol were used as control.

Cell morphology was daily observed with ZOE Fluorescent Cell Imager (Biorad, Hercules, USA) as previously reported [50]. Cytotoxicity was determined using the Cell Proliferation Kit I (Roche,

Darmstadt, Germany) according to the manufacturer's instructions. This assay is based on the conversion of a soluble tetrazolium salt in formazan (a purple insoluble compound) by mitochondrial dehydrogenases in live cells. Briefly, 10 μL of the labeling reagent (0.5 mg/mL) was added to each well and kept at 37 °C. After 4 h, 100 μL of the solubilization buffer was added and plates were allowed to stand overnight at 37 °C. The optical absorbance was read at 570 nm using Glomax Multi Detection System spectrophotometer (Promega, Italia s.r.l., Milan, Italy). Results were expressed as a percentage of viable cells relative to the control (non-exposed to methanol). Duplicate experiments were performed. To prepare cells for measurement of SOD activity (see above) cells were centrifuged at 16.000×*g* for 10 min, washed with PBS, re-centrifuged, and stored at −80 °C until analysis.

2.1.8. Statistical analysis

Differences between treatment groups in the mean numbers of small, medium, large, and total aspirated follicles, as well as in the total COCs and grade A + B + C COCs, were analyzed by ANOVA for repeated measures test using SPSS IBM version 22.0 statistical software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA: IBM Corp; 2013). Differences in the percentage of blastocysts were analyzed by Chi-Square test. Differences between the control and GTE groups in plasma TAC, FRAP, SOD, and CAT, as well as follicular fluid TAC, were analyzed by the non-parametric Kruskal-Wallis test. For cultured hepatic cells, differences in cell viability and SOD activity were analyzed by ANOVA. Statistical significance was set at $P < 0.05$.

3. Results

Results for follicular and oocyte populations are shown in Table 1. Green tea supplementation increased ($P < 0.05$) the average number of total follicles and large follicles. Total COCs tended to be higher in the GTE group, but the difference was not significant ($P = 0.09$). Both the number and the percentage of superior-quality COCs (Grade A) were greater ($P < 0.01$) for the GTE group. The number and percentage of good quality COCs (Grade A + B COCs) were also higher in the GTE group compared with the control ($P < 0.05$ and $P < 0.01$, respectively). The mean number and the incidence of COCs suitable for IVEP (Grade A + B + C COCs) increased in the GTE group ($P < 0.05$). There were no apparent differences in the recovery rate between groups. Oocyte developmental competence was improved in the GTE group as indicated by the higher ($P < 0.05$) percentages of Grade 1,2 total transferable embryos (tight morulae and blastocyst) and Grade 1,2 blastocysts (Fig. 1).

Table 1
Follicular population (FL) and cumulus-oocyte complexes (COCs) of superior quality (Grade A), good quality (Grade A + B), and suitable for IVEP (Grade A + B + C) in the control and green tea extracts (GTE)-supplemented groups.

Groups	Control	GTE
	Mean ± SEM	Mean ± SEM
Small FL n (%)	2.4 ± 0.3 (37.5 ± 3.6)	2.7 ± 0.4 (30.6 ± 3.7)
Medium FL n (%)	2.3 ± 0.3 (31.7 ± 3.7)	2.9 ± 0.4 (32.6 ± 2.7)
Large FL n (%)	2.1 ± 0.4^{a} (30.9 ± 3.7)	3.1 ± 0.3^{b} (36.7 ± 3.5)
Total FL n	6.9 ± 0.5^{a}	8.7 ± 0.5^{b}
Total COCs n	5.0 ± 0.5	6.4 ± 0.6
Grade A COCs n (%)	0.8 ± 0.2^{A} (14.5 ± 3.1)^{A}	1.9 ± 0.3^{B} (28.3 ± 3.6)^{B}
Grade A + B COCs n (%)	2.3 ± 0.3^{a} (40.4 ± 4.9)^{A}	3.7 ± 0.4^{b} (57.5 ± 4.2)^{B}
Grade A + B + C COCs C n (%)	3.5 ± 0.4^{a} (67.7 ± 5.6)^{a}	5.3 ± 0.5^{b} (84.6 ± 3.6)^{b}
Recovery Rate %	68.4 ± 5.1	69.6 ± 4.5

$^{A, B}$ Values within rows with different superscripts are significantly different; $P < 0.01$.
$^{a, b}$ Values within rows with different superscripts are significantly different; $P < 0.05$.

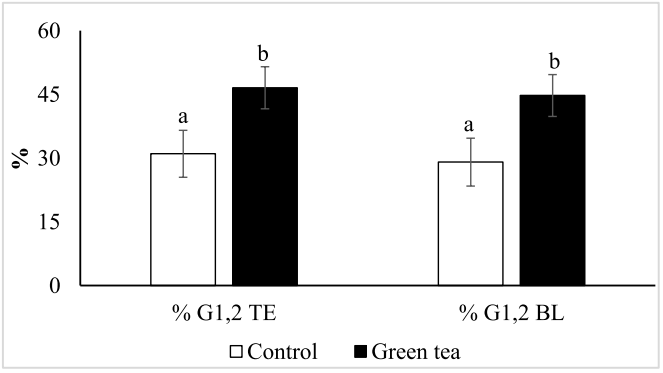

Fig. 1. Embryo yields in terms of Grade 1,2 total transferable embryos, i.e., tight morulae and blastocysts (G1,2 TE) and of Grade 1,2 blastocysts.
^{a,b} Values with different letters are significantly different; $P < 0.05$.

As shown in Fig. 2, the plasma TAC was higher in the GTE group both at T1 and T2. The FRAP showed an increase only at T2 in the GTE group. No differences were observed in SOD and CAT enzymatic activity between the two groups. The TAC of follicular fluid was higher in the GTE group compared to the control group both at T2 (beginning of OPU; 7.5 ± 0.1 vs 7.1 ± 0.1 nmol/μL, $P < 0.05$) and at T3 (end of OPU; 9.1 ± 0.4 vs 8.1 ± 0.3 nmol/μL, $P < 0.05$).

The TAC of follicular fluid was higher in the green tea extract compared to the control both at T2 (beginning of OPU; 7.5 ± 0.1 vs 7.1 ± 0.1 nmol/μL, $P < 0.05$) and at T3 (end of OPU; 9.1 ± 0.4 vs 8.1 ± 0.3 nmol/μL, $P < 0.05$).

As regards the results on the effects of GTE on the *in vitro* cultured hepatic cells, the exposure to methanol induced morphological changes in the cells resulting in monolayer fragmentation clearly visible at 72 h. Co-treatment with GTE prevented the effect of methanol (Fig. 3).

As shown in Fig. 4, cell viability was reduced by exposure to methanol compared with control at 48 h ($P < 0.01$) and 72 h ($P < 0.01$). Co-treatment with methanol and 1 μM GTE increased ($P < 0.01$) cell viability at 24 h ($P < 0.01$), 48 h ($P < 0.05$), and 72 h ($P < 0.01$) compared with the methanol treatment (Fig. 4).

Exposure to methanol decreased ($P < 0.01$) SOD activity in hepatic cells at 24 and 48 h of culture (Fig. 5). Co-treatment with methanol and 1 μM GTE prevented the decrease in SOD activity (Fig. 5).

4. Discussion

The hypothesis tested in the present study was that dietary supplementation with GTE, rich in polyphenols with antioxidant properties, would improve oocyte quality and developmental competence during the non-breeding season in Italian Mediterranean Buffaloes. It was found that GTE supplementation for 3 months prior to OPU influenced folliculogenesis and improved the antioxidant status and developmental competence of oocytes. Furthermore, a protective effect of GTE on hepatic cells was demonstrated *in vitro*.

A beneficial effect of GTE dietary supplementation on several reproductive parameters was observed in buffalo donors. Buffaloes that received GTE had a higher number of total follicles, mainly due to the increased incidence of large follicles. This was associated with higher numbers and percentages of both superior-quality oocytes (Grade A and B) and those considered suitable for IVEP (Grade A, B, and C). It was reported that buffalo oocytes recovered from large follicles show improved maturation and blastocyst development [51,52] Accordingly, oocytes recovered from buffaloes supplemented with GTE had increased embryo yields, both in terms of total transferable embryos and superior quality blastocysts. This was an important finding as lower oocyte recovery and reduced oocyte developmental competence are major causes of reproductive failure during the non-breeding season in buffaloes [6].

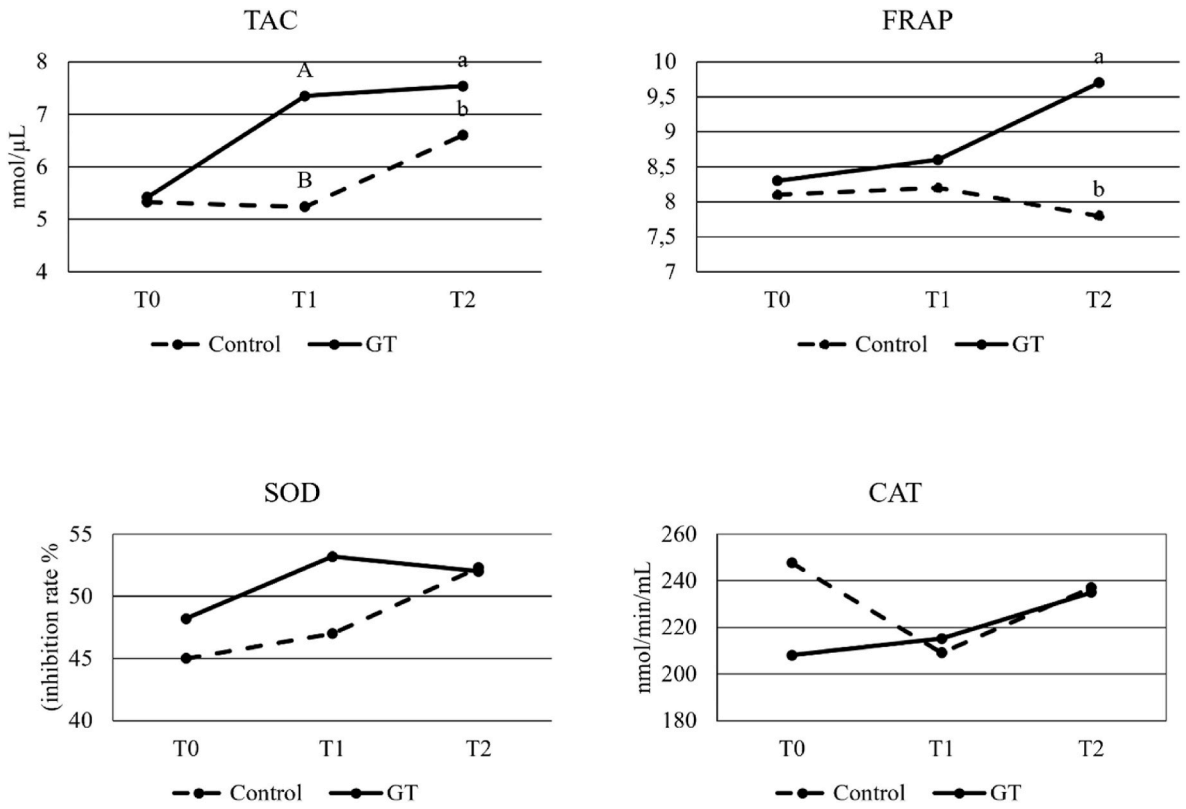

Fig. 2. Plasma total antioxidant activity (TAC), ferric reducing antioxidant power (FRAP), superoxide dismutase (SOD), and catalase (CAT) in the control and green tea-supplemented (GT) groups at the beginning of supplementation (T0), after 45 days (T1) and 90 days (T2).
[A, B] Values with different superscripts are significantly different; $P < 0.01$.
[a, b] Values with different superscripts are significantly different; $P < 0.05$.

A beneficial effect of green tea on oocyte competence was previously demonstrated by *in vitro* studies in many species, including buffalo [42–45]. Despite the evidence of positive effects *in vitro*, the influence of a GTE-enriched diet on fertility has not been evaluated in livestock. Previous *in vivo* studies with GTE showed that GTE increased blood antioxidative activity and vitamin E in steers [53] and improved the metabolic status and reproductive activity in rats with induced polycystic ovarian syndrome [54].

The beneficial effect of GTE on buffalo oocyte competence was likely due to its antioxidant activity. This interpretation is based on increased plasma total antioxidant capacity (TAC) and ferric reducing antioxidant power (FRAP) at 45 days (TAC) and 90 days (FRAP) of GTE supplementation and increased follicular fluid TAC at the time of oocyte recovery. This is an interesting finding as the decreased oocyte competence during the non-breeding season in buffaloes was associated with an overall reduction of antioxidant status measured by decreased glutathione and its precursors and catalase in follicular fluid [55]. Egyptian buffaloes showed increased plasma malondialdehyde, a marker of lipid peroxidation, and moderate decreases in antioxidative markers during the non-breeding season [36]. An increase in follicular fluid TAC during the non-breeding season in buffaloes was interpreted as a compensatory response to persistent oxidative stress [55]. The increase in TAC and FRAP in buffaloes supplemented with GTE may have been at least partly due to catechin and flavonol antioxidants present in green tea [56].

The *in vitro* study demonstrated that GTE has a protective effect on methanol-induced toxicity in hepatocytes. Indeed, the treatment with 1 µM GTE preserved both the morphology and viability of methanol-exposed hepatic cells. This protective effect was likely also due to the antioxidant activity of GTE given the increase in superoxide dismutase (SOD). Studies in laboratory animals showed that the protective effect of green tea on the liver was associated to reduced oxidative stress. In obese mice, green tea increased hepatic catalase and glutathione peroxidase activity and reduced steatosis [57], having a protective effect against fatty liver disease by decreasing hepatic TNF-α protein and inhibiting adipose *TNF-α* expression [57]. Furthermore, the oral administration of GTE was demonstrated to counteract the cadmium-induced toxicity in rats by increasing hepatic antioxidant activity [58]. The combined *in vivo* and *in vitro* findings in the present study suggest that the beneficial effects of GTE on oocyte competence were the result of a general improvement in oxidative status and liver function, which is reflected in an improved follicular environment.

In conclusion, this is the first *in vivo* study that demonstrated a beneficial effect of dietary supplementation with GTE on the ovarian function of buffaloes undergoing OPU during the non-breeding season. The GTE supplement increased the number of total follicles, large follicles, and good-quality COCs and improved oocyte developmental competence, as indicated by the higher embryo yields. The beneficial effect of GTE on buffalo oocyte competence was likely due to its antioxidant activity, as demonstrated by the improved oxidative status of both plasma and follicular environments in treated animals. Furthermore, an *in vitro* model was used to evaluate the effect of GTE on liver function. The preserved viability, morphology, and antioxidant ability of methanol-exposed hepatic cells in the presence of GTE demonstrated its protective effect.

Therefore, GTE integration is a feasible and physiological way to improve the efficiency of assisted reproduction during the non-breeding season in buffaloes. The increase in embryo yields may allow enrollment in the OPU + IVEP programs of highly productive buffaloes as donors, improving the benefits/costs ratio. In prospective studies, it will be worth investigating the effect of dietary supplementation with GTE on buffalo recipients, as well as on bull semen quality.

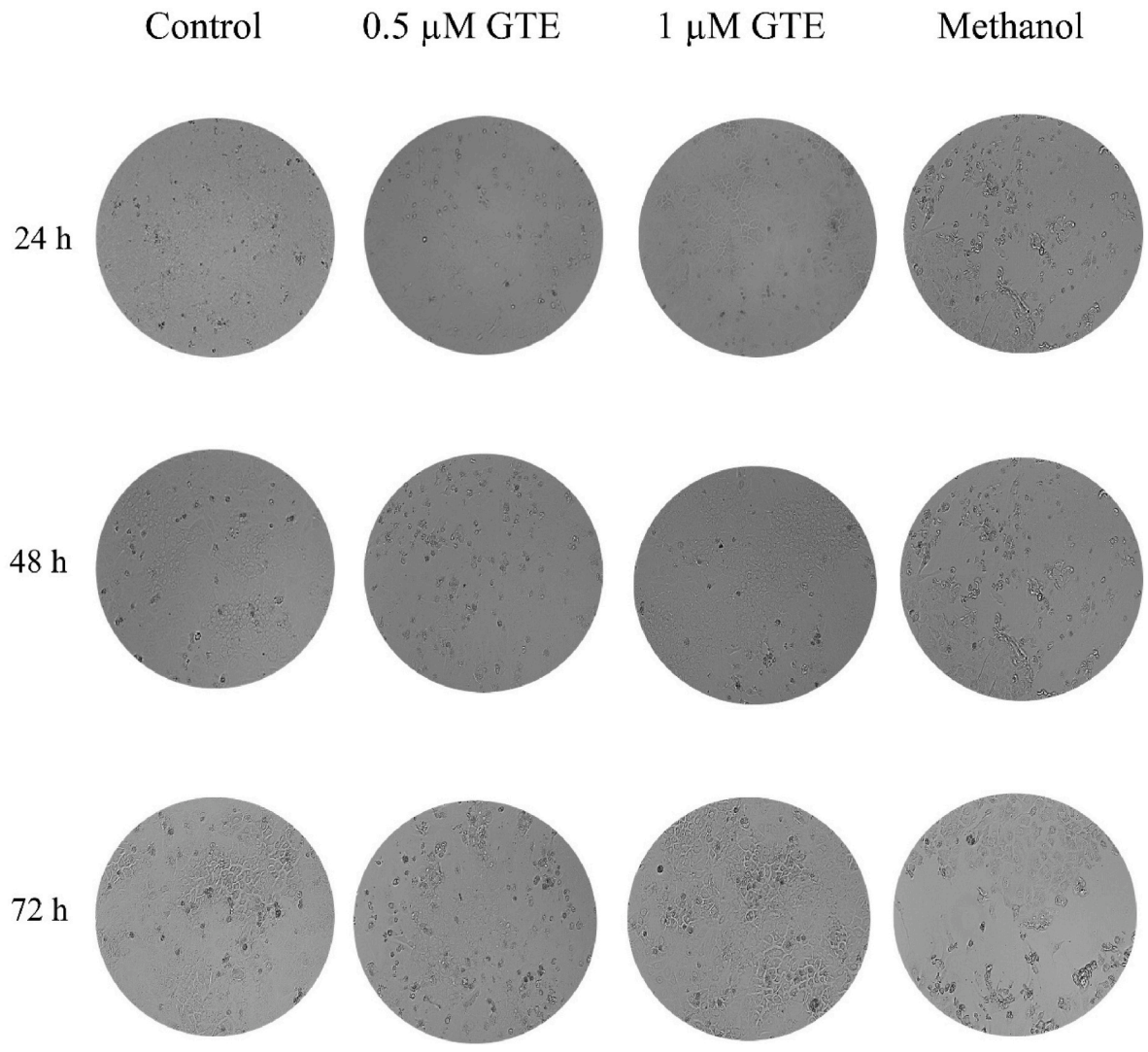

Fig. 3. Effect of different concentrations (0.5 and 1 µM) of green tea extract (GTE) on the morphology of hepatic cells insulted by methanol at 24 h, 48 h, and 72h. The control was not exposed to methanol insult.

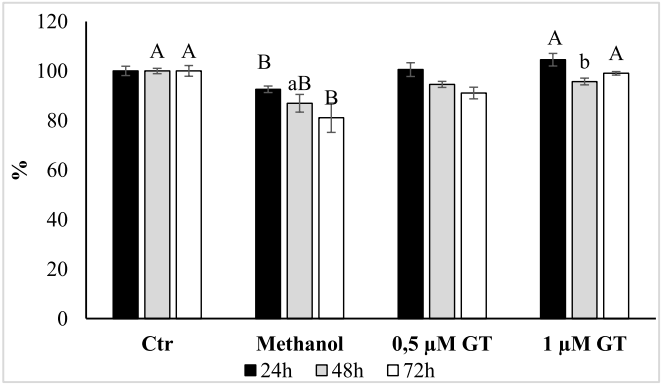

Fig. 4. Effect of different concentrations (0.5 and 1 µM) of green tea extract (GTE) on the viability of hepatic cells insulted by methanol at 24 h, 48 h and 72h. The control was not exposed to methanol insult. Data are expressed as means ± SEM

[A,B] Bars with different values within time are significantly different; $p < 0.01$

a,b Bars with different values within time are significantly different; $p < 0.05$.

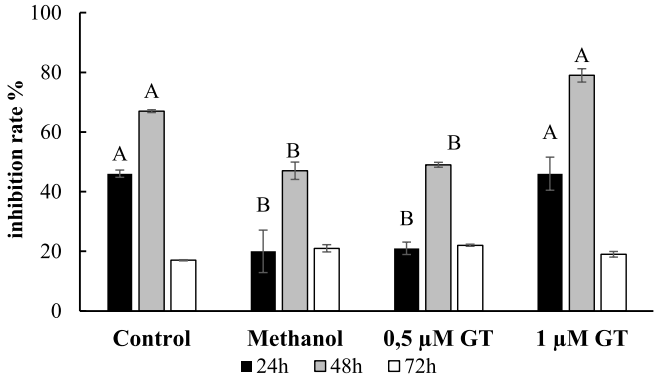

Fig. 5. Effect of different concentrations (0.5 and 1 µM) of green tea extract (GTE) on the SOD activity of hepatic cells insulted by methanol at 24 h, 48 h, and 72h. The control was not exposed to methanol insult. Data are expressed as means ± SEM.

[A,B] Bars with different values within time are significantly different; $P < 0.01$.

Declarations of competing interest

None.

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