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EFFECT OF DILUENT SUPPLEMENTATION WITH DIFFERENT LEVELS OF GREEN TEA ON ROOSTERS' SEMEN QUALITY DURING *IN VITRO* STORAGE

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ABSTRACT : This experiment was conducted to determine the effect of supplementation roosters' semen diluent with green tea infusion on semen quality during *in vitro* storage for up to 72 h. Sixty males (White Layers, 24 wk of age) divided in six groups of 10 cocks were used for experimentation. Semen samples were collected twice a week from all roosters during the whole period of experiment (12 weeks). Treatment groups were as follows: T1 : fresh semen (control group), T2 : semen diluted 1:2 with Lake diluent (LD) alone, and T3 – T6 : semen samples diluted 1:2 with LD supplemented with 6, 8, 10 or 12 ml of green tea infusion / 100 ml of diluent, respectively. Results indicated that the inclusion of green tea infusion in LD diluent resulted in a significant decrease ($p < 0.05$) in percentages of dead and abnormal spermatozoa and acrosomal abnormalities and significant increase ($p < 0.05$) in mass activity and individual motility in comparison with T1 and T2 groups when semen samples were evaluated directly after collection (0h) or after 24, 48 or 72 h of *in vitro* storage at 4 – 6 ºC. There were no significant differences ($p > 0.05$) between T3, T4 and T6 groups concerning all semen characteristics involved in this study. However, T5 group (10 ml green tea / 100 ml of diluent) recorded the lowest values of percentages of dead and abnormal spermatozoa and acrosomal abnormalities and highest values of mass activity and individual motility as compared with other treatments of green tea (T3, T4 and T6). In conclusion, the addition of green tea infusion into semen diluent could be used successfully for suppress the detrimental effects of lipid peroxidation that naturally occurred during *in vitro* storage of avian semen.
Key words: Green tea, Roosters semen, invitro storage

INTRODUCTION

Hypothermic storage of semen is used to reduce metabolism and maintain sperm viability over extended periods of time. Although extensive effort has been made to develop liquid storage procedures for holding avian sperm for 24 h or longer, semen quality and fertilizing ability is generally lower when hens are inseminated with semen stored more than 6 h (4, 27). The lipid composition of chicken semen is an important determinant of its quality and fertilizing capacity (6). Chicken spermatozoa are characterized by comparatively high levels of 20: 4 *n*-6 and 22: 4 *n*-6 fatty acids within their phospholipids (5). As a result of this high proportion of polyunsaturated fatty acids (PUFA) chicken semen is susceptible to lipid peroxidation, which could lead to sperm deterioration during storage (25). However, there is considerable evidence that the lipid composition of the sperm membrane is a major determinant of motility, cold sensitivity and overall viability during *in vitro* storage (11). A combination of optimal phospholipid fatty acid composition and antioxidative protection may therefore envisage as a major determinant of male fertility. Thus, the viability, motility and fertilizing ability of spermatozoa are highly dependent on the expression of an effective antioxidant capacity by these cells and in the surrounding seminal plasma.

Green tea is particularly rich in polyphenols, including catechins, theavlavins and thearubigins, which are thought to contribute to the health benefits of tea. Green tea polyphenols act as antioxidants *in vitro* by scavenging reactive oxygen and nitrogen species and chelating redox – active transition metal ions (10). Maron et al. (17) found that the consumption of the green tea extract resulted in a reduction in total cholesterol, LDL cholesterol, total lipoproteins and total lipids. However, recent studies have shown that green tea has far greater antioxidant protection than the well known polyphenols in antioxidant vitamins such as C and E (26). Green tea, with its all – important chemical compounds, has also shown many other benefits and potential uses. It reduces incidence of cancer, lowers blood cholesterol, inhibits increase of blood pressure, inhibits increase of blood sugar, kills bacteria and virus, fights cariogenic bacteria, maintains healthy fluid balance, relieves fatigue and stress, boosting immunity, and reducing DNA damage by oxidation (29). Sung et al. (24) found that total antioxidant capacity of plasma was significantly increased after taking green tea in amounts of 300 and 450 ml.

A positive increment according to green tea dosage was also observed. The power of green tea lies in its catechin polyphenols, particularly something called epigallocatechin gallate (EGCG). EGCG is 200 times more effective than vitamins C and E, three times more powerful than BHA, and twice as powerful as resveratrol (the antioxidant in red wine)(16). In addition, green tea provides polysaccharides, flavonoids, vitamin B complex, vitamin C (There's more vitamin C in one cup of green tea than in an orange), vitamin E, R- amino Butyric acid and fluoride in its natural state. Therefore, the present study was designed to examine whether the addition of green tea infusion to semen diluent can boost the resistance of spermatozoa .against lipid peroxidation which naturally occurred during *in vitro* storage of avian semen

MATERIALS AND METHODS

This study was undertaken at the Poultry Farm, Department of Animal Resources, College of Agriculture, University of Baghdad during the period from May 1, 2005 to August 1, 2005 to investigate whether the addition of green tea infusion to cockerels semen diluent can improve semen quality during *in vitro* storage for up to 72 h. Cockerels (White layer, 24 weeks of age) were allocated to six treatment groups with 10 birds in each group. They were raised in floor pens and fed a commercial diet containing 16.5 % crude protein and 2850 kcal metabolizable energy / kg of diet *ad libitum*. Semen samples were routinely collected two times a week from all cockerels by abdominal massage method (14) during the total period of experiment which lasted 12 weeks (24 – 36 weeks of age). Care was taken to avoid any contamination of the semen by faces or urates. Two replicate samples of semen, each consisting of pooled material from five cockerels, were obtained from each treatment group. Consequently, forty – eight replicates for each of six groups were used for the measurements. One of the treatment groups was left without any addition of substances (control group; T1). Semen samples of the second group were diluted 1:2 with Lake diluent (LD; 13) alone. Semen samples of the other four groups were diluted 1:2 with LD supplemented with 6, 8, 10 or 12 ml of green tea infusion / 100 ml of diluent, respectively (Chumee green tea, packed in the United Arab Emirates by Unilever Gulf FZE and arrangement with the Trade Marks proprietor). Green tea was prepared by infusing 2.5 g of dried green tea leaves for 2 min at 80 ºC in 150 ml of water (24). Aliquots of semen samples were taken at 0, 24, 48 or 72 h after *in vitro* storage at 4 – 6 ºC for further measurements of spermatozoa motility, viability and membrane and acrosome integrity. Spermatozoa motility (movement in a forward) was estimated on a percentage basis by using the microscopic method (23). Viability was evaluated by Fast green stain – Eosin B stain – glutamate extender (3). The proportion of morphologically abnormal spermatozoa was measured by using a Gentian violet – Eosin stain (1). Acrosomal abnormalities were determined according the procedure reported by Al – Daraji (2).

Changes in motility, viability and morphology of spermatozoa and acrosomes after *in vitro* storage for certain periods (0, 24, 48 or 72 h) were evaluated by analysis of variance. Differences among treatment groups' means were analyzed by Ducan's Multiple Range Test using the ANOVA procedure in Statistical Analysis System (22).

RESULTS AND DISCUSSION

The characteristics of the semen samples stored at 4–6ºC for different storage periods, in terms of percentages of dead and abnormal spermatozoa and acrosomal abnormalities are given in Tables (1, 2 and 3). Semen samples incubation for 0, 24, 48 or 72 h at 4 – 6 ºC in the presence of added green tea infusion (T3, T4, T5 and T6) were associated with a significant ($p < 0.05$) decrease in the percentages of dead and abnormal spermatozoa and acrosomal abnormalities as compared with T1 and T2 groups. However, there were no significant differences among T3, T4 and T6 groups, while T5 recorded the lowest values with relation to these three characteristics in comparison with all other treatments of green tea (T3, T4 and T6).

Results from the current study also confirmed that supplementation of roosters' semen diluent with green tea infusion (T3, T4, T5 and T6) resulted in significant ($p < 0.05$) increases in mass activity and individual motility when semen samples evaluated directly after semen collection (0 h) or after 24, 48 or 72 h of *in vitro* storage in comparison with T1 and T2 groups (Tables 4 and 5). Furthermore, there were no significant differences ($p > 0.05$) between T3, T4 and T6 during all storage periods as regards percentage of mass activity and individual motility of spermatozoa, with an exception for the individual motility at 48 h of storage period when T3 group (6 ml green tea / 100 ml of diluent) recorded the lowest individual motility as compared with T4 and T6 groups (Table 5). However, T5 group (10 ml green tea / 100 ml of diluent) showed the highest ($p < 0.05$) means for mass activity and individual motility during all storage periods (0, 24, 48 or 72 h) as compared with all other treatments of green tea (T3, T4, T5 and T6)(Tables 4 and 5).

Table 1. Effect of diluent supplementation with green tea on percentage of dead spermatozoa (Mean ± SE) during *in vitro* storage of roosters' semen.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	20.3 ± 2.3 a	45.6 ± 3.5 a	93.7 ± 4.1 a	100.0 ± 0.0a
T2	11.7 ± 2.1 b	40.3 ± 2.4 b	82.9 ± 1.9 b	99.7 ± 4.6 a
T3	6.1 ± 0.9 c	21.1 ± 3.6 c	40.1 ± 5.1 c	52.3 ± 2.7 b
T4	5.4 ± 0.9 c	20.0 ± 2.5 c	39.6 ± 3.4 c	47.2 ± 4.9 b
T5	1.2 ± 0.4 d	12.5 ± 1.7 d	26.7 ± 1.9 d	34.1 ± 3.6 c
T6	4.9 ± 0.2 c	18.9 ± 4.7 d	37.2 ± 2.5 c	48.0 ± 5.5 b

T1: Fresh semen, T2: Semen diluted with LD alone, T3 – T6: Semen diluted with LD supplemented with 6, 8, 10 or 12 ml of green tea infusion / 100 ml of diluent and LD: Lake diluent.
* Each value represents the mean of 48 measurements.
** Values in a column with different letters differ significantly ($p < 0.05$).

Table 2. Effect of diluent supplementation with green tea on percentage of abnormal spermatozoa (Mean ± SE) during *in vitro* storage of roosters' semen.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	26.7 ± 1.2 a	53.6 ± 2.5 a	96.1 ± 4.5 a	100.0 ± 0.0 a
T2	15.3 ± 0.6 b	44.2 ± 3.4 b	85.9 ± 3.7 b	98.2 ± 4.8 b
T3	8.8 ± 0.1 c	18.6 ± 2.9 c	39.6 ± 4.4 c	56.7 ± 3.9 c
T4	6.9 ± 1.1 c	17.3 ± 1.0 c	38.4 ± 3.9 c	54.9 ± 2.8 c
T5	3.5 ± 0.9 d	9.9 ± 2.1 d	24.6 ± 5.5 d	40.1 ± 1.9 d
T6	6.3 ± 0.5 c	16.9 ± 2.4 c	38.8 ± 3.2 c	55.1 ± 4.6 c

T1: Fresh semen, T2: Semen diluted with LD alone, T3 – T6: Semen diluted with LD supplemented with 6, 8, 10 or 12 ml of green tea infusion / 100 ml of diluent and LD: Lake diluent.
* Each value represents the mean of 48 measurements.
** Values in a column with different letters differ significantly ($p < 0.05$).

Table 3. Effect of diluent supplementation with green tea on percentage of acrosomal abnormalities (Mean ± SE) during *in vitro* storage of roosters' semen.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	25.7 ± 2.5 a	55.8 ± 2.5 a	84.7 ± 5.4 a	99.2 ± 4.3 a
T2	14.7 ± 2.0 b	41.1 ± 2.7 b	72.4 ± 3.9 b	87.8 ± 6.0 b
T3	8.9 ± 1.9 c	23.6 ± 1.9 c	40.9 ± 2.6 c	59.8 ± 3.5 c
T4	7.8 ± 1.6 c	22.5 ± 1.1 c	38.7 ± 3.0 c	58.6 ± 4.1 c
T5	1.9 ± 0.6 d	13.7 ± 1.5 d	22.9 ± 2.1 d	31.9 ± 2.7 d
T6	8.0 ± 1.9 c	22.9 ± 2.1 c	37.9 ± 2.5 c	57.4 ± 3.1 c

T1: Fresh semen, T2: Semen diluted with LD alone, T3 – T6: Semen diluted with LD supplemented with 6, 8, 10 or 12 ml of green tea infusion / 100 ml of diluent and LD: Lake diluent.
* Each value represents the mean of 48 measurements.
** Values in a column with different letters differ significantly ($p < 0.05$).

Table 4. Effect of diluent supplementation with green tea on mass activity of spermatozoa (Mean ± SE) during *in vitro* storage of roosters' semen.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	89.5 ± 3.8 d	55.1 ± 2.3 c	15.1 ± 1.9 d	0.0 ± 0.0 d
T2	92.4 ± 1.6 c	65.8 ± 3.5 c	20.7 ± 2.1 c	2.3 ± 0.4 c
T3	96.3 ± 2.0 b	71.6 ± 4.2 b	50.3 ± 2.5 b	37.6 ± 2.8 b
T4	95.9 ± 1.9 b	73.0 ± 3.5 b	49.5 ± 3.3 b	39.8 ± 2.9 b
T5	98.7 ± 4.8 a	81.8 ± 4.6 a	66.2 ± 3.4 a	50.1 ± 3.9 a
T6	95.6 ± 3.3 b	72.8 ± 2.9 b	51.8 ± 5.1 b	40.0 ± 2.6 b

T1: Fresh semen, T2: Semen diluted with LD alone, T3 – T6: Semen diluted with LD supplemented with 6, 8, 10 or 12 ml of green tea infusion / 100 ml of diluent and LD: Lake diluent.
* Each value represents the mean of 48 measurements.
** Values in a column with different letters differ significantly ($p < 0.05$).

Table 5. Effect of diluent supplementation with green tea on individual motility of spermatozoa (Mean ± SE) during *in vitro* storage of roosters' semen.

Treatments	Storage periods (hours)			
	0	24	48	72
T1	86.5 ± 3.6 d	46.3 ± 3.7 c	19.9 ± 1.2 e	0.0 ± 0.0 d
T2	92.6 ± 4.8 c	47.5 ± 4.9 c	25.8 ± 2.4 d	6.0 ± 0.6 c
T3	95.1 ± 5.1 b	71.2 ± 3.5 b	58.2 ± 3.6 c	38.9 ± 1.9 b
T4	95.8 ± 4.3 b	72.7 ± 4.9 b	64.6 ± 4.9 b	39.7 ± 2.3 b
T5	97.9 ± 6.2 a	85.9 ± 3.6 a	72.1 ± 3.9 a	52.0 ± 3.7 a
T6	96.0 ± 5.5 a	73.0 ± 6.1 b	63.9 ± 2.9 b	37.6 ± 4.2 b

T1: Fresh semen, T2: Semen diluted with LD alone, T3 – T6: Semen diluted with LD supplemented with 6, 8, 10 or 12 ml of green tea infusion / 100 ml of diluent and LD: Lake diluent.
* Each value represents the mean of 48 measurements.
** Values in a column with different letters differ significantly ($p < 0.05$).

This is the first study demonstrating the benefits of green tea in an avian model in respect of counteracting the lipid peroxidation that naturally occurred during *in vitro* storage of birds' semen. The positive results in semen quality obtained in the present study which accompanied with the inclusion of green tea in roosters' semen diluent may be attributed in part to that green tea contains a very high value of Catechin polyphenols that have antioxidant properties that are known to fight against oxidative stress (7). An accumulation number of population studies suggest that consumption of green tea beverage may bring positive health effects (20). One hypothesis explaining such effects is that the high levels of flavonoids in green tea can protect cells and tissues from oxidative damage by scavenging oxygen – free radicals. Chemically, the flavonoids found in green tea are very effective radical scavengers. A substantial number of human intervention studies with green tea demonstrate a significant increase in plasma antioxidant capacity 1h after consumption of moderate amounts of green tea (1 – 3 cups / d.)(21). There are initial indications that the enhanced blood antioxidant potential leads to reduced oxidative damage to macromolecules such as DNA, lipids and proteins. Green tea polyphenols may function indirectly as antioxidants through 1) inhibition of "pro – oxidant" enzymes, such as inducible nitric oxide synthase, lipoxygenases, cyclooxygenases and xanthine oxidase; and 2 induction of phase II and oxidant enzymes, such as glutathione S - transferases and superoxide dismutases (10). Mukhtar and Ahmad (18) reported that green tea Catechins have very potent antioxidative (radical scavenging) activity and the intake of them suppress or retard the process of life style related, life threatening diseases such as cancer, hyperlipidemia, hypertension, hyperglycemia. The mechanism for this role is unclear. One possible mechanism is that green tea consumption has a beneficial effect on lipids and lipoproteins. Sung et al. (24) showed that total antioxidant capacity of plasma after consuming 300 ml of green tea showed a significant increase of 7 % after 60 min and 6.2 % after 120 min, and after consuming 450 ml 12 % after 60 min and 12.7 % after 120 min over baseline value.

Although oxygen is necessary for organisms' life, it can be harmful agent in the form of active or free radical oxygen. Active oxygen can combine with anything in the body and oxidize it with consequent destruction of cell membranes, damage to DNA, and oxidation of lipids. The antioxidant properties of green tea have been shown to efficiently scavenge these toxins. Green tea's antioxidant activity is particularly important for preventing lipid peroxidation. Lipid peroxidation is a factor in the deterioration of lipids and fatty constituents of cells (12). One study concluded that at the typical UK daily consumption of 3 cups of green tea a day, tea has approximately the same antioxidant power as eating six apples, while another study found that one or two cups of green tea has the same ' radical scavenging capacity' as five portions of fruit and vegetables or 400 mg vitamin C equivalents (8).

The effects of green tea may not be limited to the scavenging of toxic oxidants. Green tea has many health benefits. It can help ward of fungal, bacteria and viral infections. It is beneficial for digestive disorders as it inhibits pathogenic bacteria associated with digestive infections; increases probiotic activity and helps regulate bowel function. It may help improve thinking and eyesight, strengthens arteries and reduces excess fats in the blood, clears phlegm from sore throats and neutralizes poisons, increases energy and potential oxygen uptake by cells, and clears heat and toxins from the body (19). The tannins in green tea can help stop diarrhea and inhibit flu viruses. It is recommended as an alternative to coffee, black tea and harsh stimulant (15).

Varilek et al. (30) reported that green tea exhibits inhibitory effects against *Salmonella typhi, E. coli. Campilobacter jejuni, Campilobacter coli, Heliobacter pylori, Vibrio cholera*e, Shigella, Clostridium, Psudomonas, Candida and others. Green tea Catechins possess antimicrobial properties that support immune system. They are strong antibacterial and antiviral agents which make them effective for treating all microorganisms. In studies green tea has even protected rats from cholera, and has been shown to inhibit the spread of disease (28). However, they can help restrain the growth of various bacteria that cause disease. Organisms found in freshly collected semen are probably derived from the cloaca because the spermatozoa from the vas deferens are usually free of bacterial organisms. Several investigators have reported that chicken semen collected by artificial ejaculates contains on the average 2.2 X 10^6 bacteria / ml (9). Certain of microorganisms identified in poultry semen include *Staphylococcus albus*, *Staphylococcus aureus*, *Esherichia coli*, *Proteus* spp., hemolytic *Streptococci* spp., *Alcaligenes* spp., diphtheroid *bacilli*, and *Bacillus* spp.

In conclusion, the present study provides good evidence that green tea has powerful antioxidant effect against lipid peroxidation that naturally occurred during *in vitro* storage of avian semen which clearly reflected on the improvement in semen quality of green tea treated groups as compared with fresh semen treatment (control group; T1) and semen extended with Lake diluent alone (T2).

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