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Original Article

Assessment of Bull Semen Cryopreservation Extended in Tris Extender Enriched with Green Tea Extract.

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Short title: Effect of green tea on cattle semen .

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Abstract

Background and Objective : Green tea has potential antioxidant capacity and free radical-scavenging properties. The present study aimed to clarify the effect of the watery green tea extract in tris semen extender on cryopreservability. **Materials and Methods:** 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2% of green tea extract were added to the Tris citrate egg-yolk fructose (TCYF) diluent. Semen was added to the different concentration of diluents to obtain 60 $\times 10^6$ motile sperm.ml^{-1}. Cooling and equilibration at 5°C proceeded and ended with freezing in liquid nitrogen. Thawed semen was evaluated for total sperm motility, viability, abnormality, membrane integrity (HOS) and normal intact acrosome%. **Results:** concerning chilled diluted semen, high motile and alive sperms % were found on addition of 0.2 and 0.4% green tea extract to the tris-basic extender, while all treatments with green tea extract exerted low abnormal sperm%. On the other hand, 0.6 and 0.8 % green tea extract addition showed significant increase in the membrane integrity and acrosome integrity% compared to the control. Concerning the thawed diluted semen, the addition of 0.4, 0.6, 0.8% green tea extract had maintained the motility % as that of the control, increased live sperm%, lowered the abnormal sperm% and had no significant effect on membrane or acrosome integrities% compared to the control ($P \leq 0.0001$). **Conclusion:** green tea extract supplementation in concentration of 0.2, 0.4, 0.6, 0.8% to cattle bull extender protected sperm from the hazard effect of cryodamage and offer better protection for semen quality parameters.

Key words: Cattle ; Semen; Cryopreservation; Tris; green tea.

INTRODUCTION

Sperm cryopreservation is the most widely applied tool for genetic resource conservation of farm animal, livestock production improvement, and spreading of genetic dissemination worldwide. However, exposing semen to cold shock and oxygen throughout collection, cooling and freezing/ thawing practices subjected spermatozoa to the deleterious effects of reactive oxygen species (ROS) overproduction resulting in alterations in sperm membrane, motility, morphology, viability, DNA and acrosome integrities[1,2] .

In general, the concentrations of ROS are well equilibrated by intracellular antioxidants; but, as ROS-scavenging capacity fail, sperm cells are subjected to oxidative stress resulting in the decrease of motility, mitochondrial activity, decline of plasma membrane integrity and initiation of sperm apoptosis, causing subsequently impaired fertilizing capacity[3,4,5] .

It has been known that the endogenous antioxidants failed to protect spermatozoa from oxidative stress induced by cryopreservation procedure[6,7] . The capacity of endogenous antioxidants was significantly diminished throughout cryopreservation process[8]. Incidentally, antioxidant addition is a realistic strategy to contract the damaging effects of freezing and crucial enhancement in semen characters and fertility[9,10] . Enrichment of semen extender with miscellaneous collection of antioxidant constituents extracted from plants has been of growing interest as a common strategy to counteract oxidative stress during semen cryopreservation[11,12] .

To date, various plant-derived remedies, particularly including carotenoids and flavonoids from apricots, strawberries and black grapes have been evaluated for their antioxidant capability to enhance the fertility as constituents of extenders and culture media or as dietary additions[13,2] .

Green tea derived from the *Camellia sinensis* plant has potential antioxidant capacity, anti-carcinogenic and free radical-scavenging properties principally owing to polyphenol components[14,15] .

The antioxidant potential of green tea is related to its high content of water-soluble polyphenols (epigallocatechin gallate, epicatechin gallate, epicatechin and epigallocatechin) which exert a direct antioxidants by scavenging ROS, or indirectly, by up-regulating antioxidant enzymes[16,17] Supplementation of Green tea infusion resulted in decreased lipid peroxidation, DNA damage and upgrade glutathione peroxidase, glutathione transferase capacity and increased sperm concentration in rat testes[18] .

Lately, green tea extract was successfully indicated as cryo-protective additive to semen extender of buck, ram cattle and buffalo semen[19,20 ,21,22] before cooling and cryopreservation. Enrichment of bull semen extender with 0.25% and 0.5% green tea extract improved the post-thawed sperm motility, sperm livability, and membrane integrity[19] . Inanc et al.[21] indicated that green tea extract supplementation to bovine semen extender proved to be beneficial in protection of sperm morphology and DNA integrity against sperm cryodamage and improving the total antioxidant potential of semen during cryopreservation.

Green tea may have anti-oxidative properties on bovine spermatozoa during cryopreservation, so, the aim of the current study was to explore the efficiency of supplementation of Green tea to bull semen extender on post-cooling and post-thawing semen quality.

MATERIALS AND METHODS

1.1. Preparation of green tea infusions:

Organic green tea leaves sourced from the Egyptian market. The leaves were ground in a coffee grinder for 60 s, and passed through a 0.037 mm sieve. 12 g of green tea powder was added to 60 mL of distilled water and heated at 80 °C for 30 min. Green tea extract was filtrated with Whatman number1 filter paper, then cooled to 25 °C and diluted in Tris-extender to obtain 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2% green tea extract.

Semen samples were collected from five cattle-bulls belonging to Ministry of Agriculture Egypt, using an artificial vagina each week for 5 weeks. The ejaculates initially evaluated for volume, concentration, sperm motility and % live sperm, then samples with > 70% motility and 80% normal sperm morphology were preceded to freezing procedure.

1.2. Semen processing :

Tris-citric acid-egg yolk-fructose (TCYF) diluents[23] was used as a control cryopreservation extender. Semen samples divided into similar seven aliquots, and extended to different concentrations of

green tea extract (0.0 (control), 0.2, 0.4, 0.6, 0.8, 1% and 1.2%), to obtain a final concentration of 60 million motile spermatozoa × mL-1. All samples were cooled slowly to 5 °C for 4 h. Semen was packed into 0.25 mL polyvinyl French straws (IMV, France). After an equilibration period, the straws were placed horizontally on a rack and frozen in a vapor of liquid nitrogen (LN_2) at 4 cm above the surface of LN_2 for 10 minutes and were then dipped in liquid LN_2.

1.3. Semen quality assessment :

Frozen straws were thawed at 37°C for 30 sec. Semen samples were assessed for sperm motility, sperm viability, sperm abnormality, sperm membrane integrity (HOS) and percent of normal intact acrosome after cooling and post-thawing.

1.3.1. *Sperm motility(%)* :

From each specimen 10 µl was laid down on a prewarmed clean slide and then covered with a prewarmed coverslip (25 × 25 mm). The slide was subjectively examined with a phase contrast microscope (20×) equipped with a heating plate at 37 °C and a closed TV circuit system[24]

1.3.2. *Live and abnormal spermatozoa (%)* :

The eosin (1% solution)/ Nigrosin (10% solution) stain was prepared according to Agarwal et al.[25]. One drop of well mixed semen sample was placed on a clean Boerner slide. Adding 2 drops of 1% eosin stain solution and mix well for 15 s. Then adding 2 drops of 10% nigrosine stain solution and mix well. Then make a thin smear film with 10-20 µl of the previous mixture. Let the slide to air dry and then nigrosine provides a dark background. While, live sperm has white or faint pink. On the other hand, the dead sperm head is red or dark pink. 200 sperms were counted per each slide.

Concerning the abnormal sperm percentage, head shapes revealing enlargement, small, tapered, pyriform, amorphous, vacuolated, double heads or all the previous combinations. The middle piece, or neck deformities were absence of tail, non-inserted or bent tails, irregular, bent middle piece, thin middle piece or a combination of the previous. The tail defects included, short, multiple, hairpin, broken, irregular width, coiled tail, proximal or terminal droplets.

1.3.3. *Sperm membrane integrity(%)* :

Sperm membrane integrity was assessed using the hypo osmotic swelling (HOS) test as described by Revell and Mrode[26] . Briefly, add 9.0 g of fructose and 4.9 g of trisodium citrate in a baker and continue to 1000 ml of deionized water with an osmolarity of 100 mOsmol kg-1. Dissolve well and then incubate 10 µl of diluted semen in 1 ml of the hypoosmotic solution for 40 – 60 min. at 35°C. The percentage of spermatozoa with curled tails (swollen/ intact plasma membrane) as figured by Revell and Mrode[26] was calculated after assessment of 200 spermatozoa.

1.3.4. *Acrosome integrity (%)* :

Acrosome integrity was assessed by fixing a film of semen with 10% neutral saline formaldehyde and then staining with 1:10 dilution of stock Giemsa with double distilled water overnight as described by Watson[27] . The slides were examined under oil immersion (100×). 200 spermatozoa were recorded for acrosomal cap integrity.

1.4. Statistical analysis:

Data were analyzed using the ANOVA test according to according to Snedecor and Cochran[28] (1989) to distinguish the significance between means of replicates in each treatment at ($P \leq 0.05$). The Duncan multiple range test was used to compare means.

RESULTS

The effect of different concentrations of green tea extract to the tris basic extender on cooled bull semen (5°C)

Table (1) Showed that the addition of 0.2% and 0.4 % green tea extract to the tris-basic-extender, had maintained sperm motility % in a high significant ($P<0.0001$) rank (93.33%), while the 1% and 1.2% green tea extract revealed the lowest sperm motility during the chilling phase.

Concerning the live sperm %, a parallel result was supporting the sperm motility in the 0.2 and 0.4% green tea extract. On the other hand, the high level of green tea extract (1.0% – 1.2%) had significantly decreased the live sperm %. In concern to the sperm abnormality, all concentrations of green tea extract showed a significant ($P<0.0001$) decrease compared to the control. The sperm membrane integrity (HOS) divulged prominent significant ($P<0.0001$) results concerning the addition of the 0.6% and 0.8% green tea extract as compared to control. Finally, the acrosomal integrity showed significant ($P<0.0001$) increase at the addition of 0.6%, 0.8% and 1.0% green tea extract as compared to control.

Table 1.
Semen parameters in cattle cooled semen diluted with green tea infusion extender.

Treatment	Motile sperm	Live sperm	Abnormal sperm	Sperm membrane integrity (HOS)	Acrosome integrity
Control	$88.33^{b} \pm 2.50$*	$91.56^{ab} \pm 2.19$	$18.33^{a} \pm 2.50$	$79.67^{a} \pm 0.50$	$78.33^{c} \pm 2.50$
0.2 % green tea extract	$93.33^{a} \pm 2.50$	$94.67^{a} \pm 2.00$	$10.00^{b} \pm 1.73$	$72.00^{bc} \pm 5.27$	$80.33^{c} \pm 1.32$
0.4 % green tea extract	$93.33^{a} \pm 2.50$	$93.89^{a} \pm 2.03$	$6.33^{c} \pm 2.65$	$75.33^{b} \pm 0.50$	$83.33^{b} \pm 2.50$
0.6 % green tea extract	$86.67^{bc} \pm 5.00$	$89.67^{bc} \pm 3.50$	$7.00^{c} \pm 0.87$	$83.11^{a} \pm 4.62$	$88.33^{a} \pm 2.50$
0.8 % green tea extract	$88.33^{b} \pm 2.50$	$92.33^{ab} \pm 2.45$	$10.00^{b} \pm 2.29$	$80.44^{a} \pm 5.92$	$88.33^{a} \pm 2.50$
1.0 % green tea extract	$83.33^{c} \pm 5.00$	$87.44^{c} \pm 5.32$	$7.33^{c} \pm 1.32$	$69.33^{c} \pm 6.38$	$88.67^{a} \pm 2.00$
1.2 % green tea extract	$83.33^{c} \pm 5.00$	$87.00^{c} \pm 4.66$	$7.33^{c} \pm 1.32$	$75.22^{b} \pm 3.23$	$85.00^{b} \pm 4.33$
F-cal	10.75	7.02	42.49	11.12	21.99
P<	0.0001	0.0001	0.0001	0.0001	0.0001

*Data are presented as Mean ± SD.
Different superscript (a, b,…) in the same column are significantly different using Duncan multiple range test at ($P<0.05$).

The effect of different concentrations of green tea extract to the tris basic extender on freezed thawed bull semen

The effect of green tea extract on semen parameters after thawing of cattle frozen semen was demonstrated in table (2). Concerning the motility percentage, the addition of 0.2, 0.4, 0.6 and 0.8 % green tea extract did not show a significant difference than the control, while the addition of 1.0 and 1.2% green tea extract were the worst. Concerning the live sperm percentage, the addition of 0.4, 0.6 and 0.8% green tea extract revealed the highest percentage ($P<0.0001$), while the addition of 1.0 and 1.2% green tea extract was the lowest. The addition of 0.2, 0.4, 0.6, 0.8 and 1.0% green tea extract showed the lowest abnormal morphology ($P<0.0001$), on the contrary, the addition of 1.2% revealed the highest abnormal morphology. Dealing with the sperm membrane integrity, the addition of 1.0% green tea extract showed the highest significant ($P<0.0001$) values, while the addition of 1.2% green tea extract was the lowest. Finally, the acrosome integrity percentage showed no significant effect after the addition of 0.6 and 0.4% green tea extract compared to control while the addition of 1.0 and 1.2% green tea extract showed the lowest percentages.

Table 2.

Semen parameters in cattle frozen-thawed semen diluted with green tea infusion extender.

Treatment	Motile sperm	Live sperm	Abnormal sperm	Sperm membrane integrity	Acrosome integrity
Control	$33.00^{a} \pm 2.58$*	$54.60^{c} \pm 5.83$	$13.40^{a} \pm 1.58$	$21.60^{f} \pm 0.52$	$65.60^{a} \pm 4.03$
0.2 % green tea extract	$31.00^{a} \pm 6.15$	$41.40^{e} \pm 2.67$	$08.20^{b} \pm 1.03$	$43.00^{c} \pm 0.00$	$57.30^{b} \pm 8.81$
0.4 % green tea extract	$35.00^{a} \pm 3.33$	$63.80^{a} \pm 2.86$	$09.40^{b} \pm 1.58$	$42.40^{d} \pm 0.52$	$62.40^{a} \pm 1.71$
0.6 % green tea extract	$33.00^{a} \pm 2.58$	$62.00^{ab} \pm 2.75$	$09.60^{b} \pm 1.96$	$43.20^{c} \pm 0.42$	$63.40^{a} \pm 1.71$
0.8 % green tea extract	$33.00^{a} \pm 4.22$	$60.40^{b} \pm 3.03$	$08.80^{b} \pm 2.15$	$48.60^{b} \pm 0.52$	$57.40^{b} \pm 1.71$
1% % green tea extract	$17.00^{b} \pm 10.33$	$46.20^{d} \pm 2.62$	$08.80^{b} \pm 1.55$	$57.40^{a} \pm 0.52$	$49.00^{c} \pm 6.99$
1.2% % green tea extract	$14.00^{b} \pm 5.16$	$32.40^{f} \pm 2.37$	$13.80^{a} \pm 2.62$	$26.00^{e} \pm 0.67$	$51.00^{c} \pm 5.16$
F-cal	24.61	128.11	15.76	6462.95	15.53
P<	0.0001	0.0001	0.0001	0.0001	0.0001

*Data in table presented as Mean ± SD

Different superscript (a, b,…) in the same column are significantly different using Duncan multiple range test at ($P<0.05$).

Discussion

Exposing semen to the cold shock and oxygen throughout collection, cooling and freezing/ thawing practices subjected spermatozoa to the deleterious effects of ROS overproduction resulting in alterations in sperm membrane, motility, morphology, viability, DNA and acrosome integrities[1,2,29] . Recently, a numerous scientific groups focused their researches to find strategies to counteract these damages via inclusion of various antioxidants in semen extender during cryopreservation process to improve the post-thawed semen quality.

In the current study, addition of low levels of green tea extract to the tris-basic-extender maintained high sperm motility % and improved live sperm%, sperm morphology%, membrane and acrosomal integrities% after cooling and post-thawing while higher doses of green tea extract revealed adverse effect in all sperm quality parameter studied . Earlier studies in different animal species yielded results in accordance with those found in the present study where the enrichment of dog and boar cooling extenders with green tea polyphenols indicated a significant protective influence on the sperm motility and viability[17,30] and the low concentrations of green tea extract showed significant improvement in human sperm motility[31] and viability[32] .

Khan et al.[33] indicated that supplementation of bull semen freezing extender with 0.75% green tea extract improved sperm motility and membrane integrity[19] . Inanc et al.[21] concluded that addition of 25 µg/mL catechin from green tea extract to bull freezing extender protected sperm morphology and DNA integrity from the damaging effect of cryopreservation and improved the total antioxidant capacity. Ahmed et al.[22] indicated that adding 1% green tea extract to buffalo semen extender improved structural and functional parameters,

antioxidant capacity, longevity and fertility. Furthermore, green tea extract addition to ram semen extender resulted in a significant enhancement in post-thawing semen quality[34,35].

In contrast to our results, Gale et al.[36] indicated that the enrichment of boar semen extender with green tea extract extender did not result in any favorable outcomes on sperm motility, livability, acrosome and membrane integrities. Additionally, in another study on boar sperm, Park and Yu[30] did not find any significant differences in semen quality criteria between the control and various concentrations groups of green tea extract addition. These contradictory results may be due species variations.

Green tea is considered as potential antioxidant[15,16] due to its high content of polyphenols (epigallocatechin gallate, epicatechin gallate, epicatechin and epigallocatechin) and also vitamins C, E, Selenium and Zinc which exert scavenging ROS, and up-regulating antioxidant enzymes[16,17,38] .

Polyphenols (Catechin) are considered to be the utmost vital active constituent in green tea extract with antioxidant capability 20 folds greater than vitamin C on scavenging ROS[39].Polyphenols in green tea extract might exert their antioxidant effects via interaction with constituents of the spermatozoa and prevent the lipid peroxidation prompted by ROS[40] .Adding catechin to semen extender was indicated to exert good protection of rat[41] , ram[20] and stallion[42] semen during cryo-preservation.

Conclusion

Green tea extract supplementation (in concentration of 0.2, 0.4, 0.6, 0.8%) to cattle bull extender protected sperm from the hazard effect of cryodamage and offer better protection for all semen quality parameters.

Ethics approval and consent to participate

The present study design was approved ethically by the Medical Research Ethics Committee at the National Research Centre, Dokki, Cairo, Egypt, with a registration number 19/043.

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