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Dose-Dependent Effect of Green Tea (*Camellia sinensis*) Extract in the Post-Thawed Fertility Indicators of frozen Bovine Semen Extender

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IA, HK and NZF perceived and designed the study. NBSK, MTT and SA performed the experiment. NA, SMHA and O analyzed the data.

Key words
Green tea extract, Cryopreservation, Buffalo bulls, Post-thaw quality indicators.

ABSTRACT

This study describes antioxidative activity of green tea (GT) extract on standard post-thawed quality indicators of frozen buffalo bull's spermatozoa. Semen samples were obtained from the bulls for three weeks through artificial vagina. Egg yolk was used as an extender, in which different concentrations of GT extract (0.0%, 0.50%, 1.0%, 1.5%, 2.0% and 2.5%) were added. Semen quality indicators studied were the percentage of motility, survivability, plasma membrane integrity and acrosome integrity of frozen buffalo bull sperms. The antioxidative effect of GT was observed at the comparatively lower concentration (0.1-1.5%) on the post-thawed quality indicators. On the other hand, the hight GT concentration (1.5%) significantly decreased the quality indicators of spermatozoa. Green tea extract enhanced the spermatozoa survivability, integrity of acrosome and plasma membrane of spermatozoa following freezing and thawing. It is suggested that the addition of 1.5% green tea extract (4 g in 200 ml methanol) can be safely used for enhanced effectiveness of fertility after thawing.

INTRODUCTION

Semen cryopreservation is an important modus operandi of assisted reproductive techniques (ART) commonly applied for enhancement of the reproductive efficiency in domestic animals including buffalos. It has been reported that Buffalo' bull spermatozoa are inclined to oxidative stress-induced damages because of having abundant polyunsaturated fatty acids that include arachidonic and docosahexaenoic acids in the plasma membrane and lower availability of scavenging enzymes in their cytoplasm (Janic *et al*., 2000). Freezing/thawing procedure of sperm is routinely performed for artificial insemination (Bucak *et al*., 2008). During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which in turn raises the susceptibility to lipid peroxidation (Bucak *et al*., 2011). Oxidative stress (OS) produced in these procedures due to high reactive oxygen species (ROS) are resulting in the reduction of intracellular ATP levels that instigate lipid peroxidation in the plasma membrane of sperm (Almeida and Ball, 2005). Thus, these ROS have a depressing effect on sperm motility and weaken its fertilizing capability (Zarghami and Khosrowbeygi, 2005; Fraczek*et al*., 2007). Furthermore, cryopreservation of bull semen has been associated with a reduction of sperm intracellular antioxidants such as GSH and superoxide dismutase (SOD). Despite intensive use of frozen semen in artificial insemination, there is still a need for improvement of the cryopreservation process, as about 40 to 50% of the viable sperm are damaged during freezing and thawing (Watson, 2000).

A number of studies have been carried out to discover the role of antioxidants in the fight against OS in the semen cryopreservation biology. In recent times, extensive research focus has been intended for the use of natural antioxidants derived from plants or herb derived antioxidant, since these antioxidants are well known for its

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lower cytotoxicity and are known to be better than synthetic antioxidants (Gupta and Sharma, 2006; Nagulendran *et al*., 2007; Sen *et al*., 2010). Green tea (*Camila sensis*) has been reported to provide enhanced antioxidant protection in comparison with the normal polyphenols in antioxidant vitamins such as C and E (Tedeschi *et al*., 2004). Additionally, its antioxidative activity has been reported stronger than butylated hydroxyanisole, butylated hydroxytoluene, and DL-alpha-tocopherol. Conversely, it has been reported that green tea polyphenols induced cytotoxicity which is lower than butylated hydroxyanisole, butylated hydroxytoluene and DL-alpha-tocopherol (Chen *et al*., 1994). Polyphenols that are the most imperative water-soluble constituent of green tea infusions have been associated with beneficial effects on health due to their strong antioxidative properties. They are also potent scavengers of ROS superoxide, hydrogen peroxide, hydroxyl radicals and nitric oxide. Epigallocatechingallate (EGCG), epicatechingallate (ECG), epicatechin (EC) and epigallocatechin (EGC) are fundamental green tea polyphenols (Wittayarat *et al*., 2013). Furthermore, in the last decade, it has been elucidated that EGCG has a role in the prevention of impulsive mutations and chromosomal damage stimulated by ROS in somatic cells (Roy *et al*., 2003).

The ability of spermatozoa to fertilize the ovum depends upon its capability to reach at the site of fertilization (oviduct) after insemination which is directly related to the morphological changes that occur during the cryopreservation process. Myometrial contractility is an essential component in the fertilization process because it is the mechanism by which spermatozoa are transported to the site of fertilization. In an effort to stimulate certain physiological events (uterine contractility) associated with breeding, several research groups examined the effect of green tea on the pig semen at the time of breeding (Cheng *et al*., 2001). Similarly, plasma membrane and acrosomal integrity have been positively correlated with fertility in bovine (Saack and White, 1972). Therefore, by ensuring the availability of morphologically normal, motile and healthy (with intact plasma membrane and acrosome) spermatozoa at the site of fertilization (in the oviduct), the efficiency of AI in buffalo husbandry management could be improved to increase its productivity.

Several studies have been demonstrated that the plant-derived antioxidant or herbs are associated with improvement of spermatozoa in freezing extender to reduce the oxidative stress induced by ROS and free radical during the process of cryopreservation in a different region of the world. In recent times beneficial and/ or antioxidative protection of green tea extract on the quality of canine, avian, mouse, Sahiwal and Achai bull semen has been reported (Wittayarat *et al*., 2013; Al-Daragi, 2011; Absehenas *et al*., 2011; Ali *et al*., 2014; Khan *et al*., 2017). On the other hand, no study has been carried out in buffalo bull semen extender biology to elucidate the response of the green tea extract on the post-thaw quality indicators that includes sperm motility, viability, plasma membrane integrity and acrosome integrity at different concentration despite the fact that buffalo play a significant role in Asian agriculture.

MATERIALS AND METHODS

Ethical approval

The current study was carried out after approval from the ethical committee of the Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture Peshawar.

Area of study

Execution of the current research trial was done to investigate the response of diverse levels of green tea extract on post-thaw quality of bull spermatozoa. Collection of experimental material was done from Nili Ravi buffaloes at Semen Production Unit, Harichand, district Charsadda. Following semen thawing, freezing and then preservation and after that different solution was prepared at National Agriculture and Research Centre, Islamabad to perform fertility parameters and then finally post-thaw parameters were performed at Semen Production Unit Harichand, District Charsadda.

Management practices

The experimental breeding buffalo bulls that were used for semen collection were kept in the clean and hygienic environment at Semen Production Unit, Harichand, district Charsadda. The bulls were housed individually and the pens were located from north to south direction so that to protect bulls from heat during summer. The experimental bulls were given good quality seasonal green fodder at 10% of body weight accompanied with 2-3kg concentrate every day. A supply of clean water was also ensured to the bulls. Twice a day shower was given to the breeding bulls along with physical exercise for 1 h two times a week. As per the schedule, the bulls were vaccinated against FMD, TB, HS and BQ. Necessary measures were taken for the handling of these bulls.

Semen collection

Semen was collected through artificial vagina (AV). Proper sterilization of all the equipment was made before using. Water temperature ranges from 45-50°C filled into AV to make up the desired temperature to 42°C which

is the ideal temperature for AV (Andrabi *et al*., 2008). The air was injected into AV to produce pressure so that it allows easy penetration of penis so that sufficient pressure was exerted to induce stimulus. The inner lining was lubricated through K-Y jelly (Sharma *et al*., 1957). The AV was covered with a jacket in order to prevent the spermatozoa from sunlight. A warm glass vial was also used for collection.

Two consecutive ejaculates were collected at weekly interval for Four weeks. Immediately the ejaculates were then transferred to the laboratory and were kept in a water bath at 33-34°C.The semen samples were subjected to gross (volume, color) and microscopic (percentage motility) evaluation. Semen ejaculates having motility more than 80% were considered for the study.

Procedure for extract collection from green tea

Green tea extract was prepared by using the standard protocol as previously explained by Chan *et al*. (2011). First, green tea leaves were powdered in a laboratory blender. Then 4 g of powdered tea leaves was taken and mixed it in 200 ml of methanol, then it was kept for 18 h at room temperature following centrifugation at 6200 rpm for 20 min. The supernatant was taken out and filtered through filter paper. The extract was then kept frozen for future use.

Preparation of semen extender (egg yolk-citrate extender)

Tris-citric acid (100 ml) extender was prepared as first added 3.03 g tris and 1.56 g citric acid in 50 ml distilled water in a beaker. Then properly mixed it for 10-15 min with the help of a stirrer and after that checked its pH which ranges from 7.0-7.2, then added 0.2 g fructose and mixed it for 5-10 min and then added 7% (7ml) glycerol and again mix it for 5-10 min and after that took an egg and collected egg yolk from it and added 20% (20ml) of egg yolk to the extender solution and mixed for 10-15 min. At the end added 0.01 g streptomycin sulfate to the solution. Then add distilled water to the beaker up to 95 ml and adjusted the pH upto 7.00 (Khan and Ijaz, 2007).

Inclusion level of green tea extracts in extended semen

In order to assess the potential impact of GTE, diverse concentration of green tea extract (0.00%, 0.50%, 1.0%, 1.50%, 2.00% and 2.50%) were added in the semen extender. Incubation of the green tea extract containing tubes was carried out for 30 min at 37°C to allow methanol to evaporate followed by the addition of semen to the tubes. Furthermore, in order to permit the uptake of the green tea extract by spermatozoa further incubation was done for the 5 min interval at 37°C. Equilibration of semen was done for 2 h via temperature changes from 37°C to 4°C. Extended semen were filled into 0.5 ml straws were used inside cold cabinet division and were frozen first at -120°C by holding in nitrogen vapors for 7 min, 4 cm high liquid nitrogen. Liquid nitrogen was used for keeping semen straws that were frozen at -196°C.

Post-thawed semen evaluation

At the time of analysis, one straw of semen from each treatment was thawed separately at 37°C for 30 sec to perform semen quality parameters.

For motility assessment, the semen straw was emptied in a test tube kept in a water bath. A small drop of 10µl of semen was placed on pre-warmed (37°C) glass slide and covered with a coverslip. The percentage motility of spermatozoa (Ijaz *et al*., 2009) was assessed at 40X under a phase-contrast microscope (Olympus BX51 TF, Japan).

Sperm viability was performed using the standard protocol as previously described by (Mughal *et al*., 2013). Eosine-Nigrosine staining method was used to perform this study (Bucak *et al*., 2007). The composition of stain was made by adding Eosine-Y (Merck, Germany) (1.67g), Nigrosin (Merck, Germany) (10g), and sodium citrate (2.9g), dissolved in 100 mL of distilled water. A suspension smear of spermatozoa was prepared by placing a small drop of semen on one side of a clean slide and a small drop of smear on the same slide just close to the semen drop and with the help of a stirrer gently mixed it for 3 min then prepare a smear with the help of another slide and then dry the smear. With the help of the brightfield, microscope assessed the sperm viability at 40X magnification. After that 200 spermatozoa were counted. Sperm that looks purple was declared as non-viable. Whereas sperm that looked white means stain did not pass through the cell membrane of that spermatozoa were declared as viable/live (Balestri *et al*., 2007). The percentage of viable/nonviable sperm was identified by counting 200 spermatozoa on the slide.

Plasma membrane integrity was determined through the hypo-osmotic test (host) (Gohar *et al*., 2014). Briefly, preparation of host solution (190 osm/L) was done via mixing 0.735 g of trisodium citrate dihydrate and 1.351g D (-) fructose in 100 mL of de-ionized distilled water. For the assessment of membrane integrity 500µL of the hypo-osmotic solution was incubated for 45 min at 37°C with 50 µL of each frozen-thawed semen sample and observed under a phase-contrast microscope (40X). Two hundred spermatozoa for each slide were counted, and the percentage of spermatozoa exhibiting tail curling (intact membrane) was assessed. The swelling of sperm cell tail shows integrity of sperm cell. Normal semen has 300mosm/L osmolarity.

The acrosome integrity test was performed with the help of formal citrate solution, prepared by adding 2.9 g trisodium dihydrate (Merck, Darmstadt, Germany) and 1ml of 37% formaldehyde (Scharlau, Barcelona, Spain) in 99 ml distilled water and then properly mixed it. Intact acrosome was identified by the formation of normal Apical Ridges. 200 spermatozoa were counted by their normal Apical Ridge observed with help of phase contrast microscope at 100X magnification (Khan and Ijaz, 2007).

Statistical analysis

The statistical analysis was done through analysis of variance using SPSS. Comparison of the treated groups with different concentration of the green tea extract was done using Duncan's multiple range test by ANOVA procedure, when the F-value was significant ($p>0.05$) for the percentages of spermatozoa showing different quality characteristics.

RESULTS AND DISCUSSION

Spermatozoa motility

Table I shows effect of different concentrations of GTE on sperm mobility, sperm viability, acrosome integrity (AI) and spermatozoa plasma membrane integrity (PMI) in bovine bulls.

Sperm motility is an indication of the energy status of spermatozoa, is an important factor in normal sperm function because poorly motile spermatozoa may not reach the fertilization site in oviduct due to mucus barrier (Mortimer, 1997). The current findings demonstrated the positive effect of the green tea extract on the motility of post-thawed spermatozoa, high motility rate 49.20±0.82 ($P<0.05$) was observed after addition of 1.5% of green tea extract in semen extender as compared with the control and other treated groups (Table I).

The green tea extraction (1.5%) significantly increased the spermatozoa motility, spermatozoa viability, acrosome integrity and spermatozoa plasma membrane integrity as compared to control.

The fertilization capability of sperm subsequent to cryopreservation is an imperative aspect of high-pregnancy rates in diverse mammalian species after insemination, and it is linked with the sperm excellence following freeze-thaw process. To our knowledge, we are the first to attempt the current experiment for the exploration of the positive impact of green tea in buffalo (bulls) spermatozoa that are associated with the fight against the lipid peroxidation that commonly happens throughout cryopreservation events. Furthermore, the affirmative response of the green tea in semen extender on the functional spermatozoa attributes that was obtained in the current finding might be ascribed to the availability of abundant polyphenols of the component in GT. These polyphenols are effective in lessening the production ROS during cryopreservation (Chyu *et al*., 2004).

Reproduction is a dynamic phenomenon of the mammalian species. The fertilization capability of sperm subsequent to cold storage is an imperative aspect of elevated fertility feature in diverse mammalian species after AI, and it is linked with the post-thaw sperm excellence. Semen distinctive characteristics are the gauge of flourishing aspect in the assisted reproductive technologies for the enhancement of reproductive management of the breeding buffalo bull used for artificial insemination diligence. Spermatozoa survivability, intact membrane integrity and that of acrosome are considered the essential factors in the assessment of semen excellence. Recently the application of green tea extract is the important research focus associated with the deterrence of a variety of mammalian malady and also its outcome on animal reproduction.

Table I.- Effect of different concentration of green tea (*Camellia sinesis*) extract (GTE) on post-thawed motility (P-value=0.011) and viability (P-value=0.000) characteristics, acrosomal integrity (AI; P-value 0.023) and plasma membrane integrity (PMI; P-value 0.013) (Mean±SD) in semen extender.

GTE / Conc (%)	Sperm motility (%)	Sperm viability (%)	AI (%)	PMI (%)
GTE1 (0.00)	28.06±1.10^{c}	64.85±2.21^{c}	60.10± 2.01^{d}	62.26±2.41^{d}
GTE2 (0.50)	38.26±3.25^{b}	73.68±2.66^{b}	65.13±2.20^{c}	65.20±1.70^{c}
GTE3 (1.00)	40.30±1.94^{b}	74.40±1.51^{b}	69.03±1.96^{b}	68.06±1.00^{b}
GTE4 (1.50)	49.20±0.82^{a}	83.13±3.00^{a}	78.19±2.05^{a}	75.83±1.45^{a}
GTE5 (2.00)	22.34±2.65^{d}	64.90±1.15^{c}	63.26±0.9c^{d}	62.17±0.75^{d}
GTE6 (2.50)	20.13±2.02^{d}	63.78±1.52^{c}	60.06±2.04^{d}	60.51±0.81^{d}

Different superscript *i.e.,* a, b, c and d in column indicated the level significance of green tea in semen extender. Level of significance was $P < 0.05$. GTE1, control extender without any addition of green tea extract; GTE2 indicate the addition of GTE at 0.5% in semen extender , GTE3, 1.00% in semen extender; GTE4, 1.5% in semen extender; GTE5, 2.00% of the green tea extract; GTE6, 2.50% of the green tea extract.

Expression of extract green tea has been elucidated in semen extender in diverse mammalian species from the different region of the world. Currently, it has been reported in rabbits, mouse, chicken and Achia cattle. On the other side, there is the dearth of literature on the effect of diverse green tea extract concentration in bovine semen extender. Consequently, here we attempted to clarify the impact of different level of extorting of the green tea in the semen extender for cry preserved buffalo bull spermatozoa excellence attributes.

Spermatozoa's having characteristic motility is the essential feature in attaining flourishing fertilization. Subfertility or infertility in the domestic animal has been associated with deficient motility of spermatozoa (Itach *et al*., 2011). The effect of green tea extract on progressive sperm motility has not been studied thus far in buffalo bull semen extender; although few reports in the semen of cattle bulls and other mammalian species have been recently reported. Ali *et al*. (2015) and Khan *et al*. (2017) have investigated observed better motility with a lower concentration of green tea extract bull semen. Increase tendency was observed in motility of semen samples containing green tea extract in the current investigation from 0.5 % and attained the significant beneficial level at 1.5% addition with control and higher concentration. The result obtained in the current finding was in corroborated withthe recent study carried out in Achai bull where the beneficial effect of the green tea extract was recorded at lower concentration (Khan *et al*., 2017). The optimal concentration of green tea extract in the extender was 1.5% (w/v) in the current study, which was in close vicinity of 0.75% described by Khan *et al*. (2017) in Achai bulls and Ali *et al*. (2014) for Sahiwal bulls. The slight difference in results obtained in semen extender in buffalo bull semen in the current study with aforementioned researchers might be due to the difference of species.

The functional mechanism of the green tea extract in the semen extender biology is not yet fully clarified. However, the improvement of spermatozoa motility that's established in the current research trail might be ascribed to the presence of antioxidants components in the green tea that exerted a positive impact on reactive oxygen species. These ROS have been associated with lipid peroxidation which has a depressing impact on spermatozoa functional attributes. Additionally,these functional mechanisms have been further elucidated by Duru *et al*. (2000) and Ozmen *et al*. (2007), whose research demonstrated that's lipid peroxidation of the spermatozoa membrane via Reactive oxygen species provoke the demolition of factors that manipulate functional aspects of spermatozoa including the fertilization capability and survivability. Furthermore, enhancement of motility characteristic of the spermatozoa observed in the current trails was corroborated with the finding of Aitken and Fisher (1994) and de Lamirande and Gagnon (1995). Consequently, the improvement of sperm motility subsequent to the addition of different concentration of green tea extract to semen extenders in the current study might be tempted via reduction of efficient reactive oxygen species.

The viability of buffalo semen is the essential feature in the reproductive management of buffalo. Sperm viability appraises an intact plasma membrane, which is vulnerable to commotion by ROS (Cocuzza *et al*., 2007). It can be influenced by many factors and additives. Among these factors, the survivability of spermatozoa in female oviduct is a very crucial feature. A dose-dependent increase in sperm viability through Inclusion of green tea extract in semen extender or sperm storage media has been reported in the recent past (Lombardo *et al*., 2012; Khan *et al*., 2017). In the current study also comparatively, lower concentrations of green tea extract enhanced semen excellence by increasing the motility, viability of the post-thaw buffalo bull whereas a higher concentration exerted the opposite effect.

Spermatozoa are not capable to contribute in the fertilization process of the egg subsequent to ejaculation in diverse mammalian species, and they must get hold of the competence to fertilize "capacitation" in the female reproductive tract (Yanagimachi, 1994). Binding to the zona pellucida arouse the spermatozoa to endure acrosome reaction (AR) where the fusion of the outer acrosomal membranes occur with the overlying plasma membrane. This functional exocytotic occurrence is associated with the release of hydrolytic enzymes which are fundamental for the fertilization process. Spermatozoa which acrosome have to stimulate loss impulsively subsequent to ejaculation or by physical damages during cryopreservation are not capable to participate in the fertilization of eggs. Accordingly, AI is a crucial gauge of potential sperm function associated with fertilization of the oocytes at Al. Hence, the appraisal of acrosome indemnity after the freeze-thaw process is an essential aspect to consider in the successful reproductive management of the buffalo production enterprise. In the current study, 78.19% spermatozoa were found acrosome-intact in semen via extender having 1.5% green tea extract, which diminishes considerably when the comparison was made with control. It has been demonstrated that acrosomal thrashing might be associated with a degenerative process following the death of the spermatozoa (Fazeli *et al*., 1997).

In the subcontinent region where buffalo are abundantly available, the most extensively applied excellence appraisal decisive factor *i.e.* post-thaw motility (PTM) of buffalo sperms single trait was not adequate as

a gauge for post-thaw sperm, excellence. As a substitute, the plasma membrane integrity of spermatozoa PMI, which is very much fundamental in sustainability of spermatozoa functional attributes that includes SM, sperm motility, capacitation, acrosome reaction and its integrity is essential for the reaction essential for connections between the spermatozoa and the female reproductive region for union of both gametes (Rodríguez-Martínez, 2003), is receiving significance as a gauge in the appraisal of fertilization potential of the buffalo bull spermatozoa. A substantial level of polyunsaturated fatty acids (PUFA) is found in the plasma membrane of buffalo sperm which a tendency to lipid peroxidation (Mandal *et al*., 2014; Niki *et al*., 2005). Consequently, effective antioxidant components are necessary to safeguard sperm cells against damage due to lipid peroxidation (Breque *et al*., 2003). The result obtained in the current study associated with plasma membrane integrity was close with the recent finding of Khan *et al*. (2017) who investigated the Plasma membrane integrity in Achai bull semen extender and demonstrated that the post-thawed percentage of spermatozoa with intact plasma membranes was higher ($P<0.05$) at 0.75% green tea extract inclusion level. In our study also, the beneficial effect of green tea extract has been exerted on PMI of buffalo bull semen at 1.5% concentration. This slight variation in the response of the green tea extract in the semen extender might be ascribed to the use of different experimental animal species. The current findings to a greater extent supported the preservative effect of Green tea extract due to the antioxidant capacity of green tea. The antioxidant property of green tea has recently been reported in the semen extenders of Achai bull and Sahiwal bull (Khan *et al*., 2017; Ali *et al*., 2014). Additionally, the potential reason for this functional mechanism might due to the presence of a substantial percentage of polyphenols in green tea which are associated with prevention of cell damage caused by lipid peroxidation (Perumalla and Hettiarachchy, 2011). In addition, in the recent past, it has been reported that polyphenols in green tea are associated with the fight against free radicals and may diminish or even assist to obstruct a number of the damage they cause (Thasleema, 2013). Furthermore, it has been demonstrated that epigallocatechin gallate (EGCG) is the most important catechin of green tea extract (48–55% of total polyphenols) (Ho *et al*., 1997; Kodama *et al*., 2010). These polyphenols in the green tea extract have a vibrant antioxidant feat (Dufresne and Farn worth, 2001) and are an efficient seeker of ROS superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide synthesized by diverse chemicals (Schroeder *et al*., 2003).

CONCLUSION

In conclusion, our study demonstrated that Green tea extract should be used in the semen extender biology to enhance the post-thaw buffalo bull spermatozoa fertility indicators. Hence the use of plant-derived natural antioxidant like green tea extract in assisted reproduction associated with sperm cryopreservation become a visible potential avenue for improving the buffalo reproductive characteristics as evident in the present study.

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Statement of conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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