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Effect of Green Tea Extract (*Camellia sinensis*) on Fertility Indicators of Post-Thawed Bull Spermatozoa

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Authors' Contribution
HK and MSQ designed the study. AG carried out experimental work and compiled the data. MK, SSAH, AS and AK helped in experimental work. SA and PK analyzed the data. HK wrote the article. All others helped in finalizing the manuscript.

Key words
Green tea extract, Post thaw, Achai bull semen.

ABSTRACT

Green tea extract is plant–dervied natural antioxidant. The objective of this study was to explore the antioxidative effect of green tea extract on the cryopreserved bull semen. Experiments were carried out on Achai breeding bulls of similar age at the Semen Production Unit, Harichand, District Charsadda, Khyber Pakhtunkhwa. Collection of semen was done using artificial vagina (42°C) for three weeks. Semen was extended in egg yolk extender at various inclusion levels (0.0%, 0.25%, 0.5%, 0.75 and 1.0%) of green tea extract. Testing was consummated for quality characteristics of post-thawed spermatozoa such as motility, viability and plasma membrane integrity. The data was statistically analyzed with one-way analysis of variance using SPSS. The highest significant ($P<0.05$) response was obtained in extenders containing 0.75% green tea extract for all studied parameters. Based on the findings of the current study, it can be concluded that supplementation of green tea extract in semen extender showed significant response on the post-thawed spermatozoa motility, viability and membrane integrity of Achai bull.

INTRODUCTION

Semen cryopreservation is an important technique, widely used in ruminate breeding progamme to increase animal yields for fullfilling the rising demand of meat, milk and work in developing countries. Freeze-thawing procedure of sperm is performed characteristically for artificial insemination (Bucak *et al*., 2008). During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, this raise up the level of lipid peroxidation (Bucak *et al*., 2011). Experiments have shown that oxidative stress produced in these procedures due to high reactive oxygen species (ROS) are associated with low quality of seminal material and death of sperm cells with abnormal morphology, thus resulting in a predominant impairment for profitable semen cryopreservation (Janice *et al*., 2000).

It is well known that natural antioxidants of bovine semen are not enough to safeguard the sperm integrity against oxidative stress during the process of cryopreservation (Sreejith *et al*., 2006; Nichi *et al*.,2006). Additionally, during freeze-thawing cycles, the intensity of naturally occurring antioxidants in bovine semen get diminished (Bilodeau *et al*., 2000). Lower fertility and conception rate have been reported in different animal studies with frozen-thawed semen (Maxwell *et al*., 1993; Bhosrekar *et al*., 2001). To cope up with these harmful effects, supplementation of antioxidant may be a feasible approach to improve cryopreservation techniques (Anghel

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et al., 2010) for successful artificial insemination.

Recently plant-derived antioxidants have been getting major focus due to their lower cytotoxicity and are considered to be better than synthetic antioxidants (Gupta and Sharma, 2006; Nagulendran *et al*., 2007; Sen *et al*., 2010; Ibrahim *et al*., 2014). Additionally, green tea (*Camila sensis*) holds greater antioxidant protection than the ordinary polyphenols in antioxidant vitamins such as C and E (Tedeschi *et al*., 2004), also its antioxidative activity is stronger than butylated hydroxyanisole, butylated hydroxytoluene and DL-alpha-tocopherol. Toxicity generated with tea polyphenols is lesser than butylated hydroxyanisole, butylated hydroxytoluene and dl-alpha-tocopherol (Chen and Wan, 1994). It is well documented that Green tea has valuable effects on health due to abundant amount of polyphenols that are the major water-soluble components of green tea infusions. Green tea polyphenols include epigallocatechin-gallate (EGCG), epicatechin-gallate (ECG), epicatechin (EC), and epigallocatechin (EGC) (Wittayarat *et al*., 2013). In addition to strong antioxidant and antioxidative characteristics, green tea polyphenols (GTP) are potent scavengers of ROS superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide produced. In the recent past, it has been confirmed that EGCG prevents spontaneous mutations and chromosomal damage induced by ROS in somatic cells (Roy *et al*., 2003) .

Plant-derived extracts are excellent sources of the natural antioxidant. Several studies documented that the plant-derived antioxidant or herbs are associated with improvement of spermatozoa in freezing extender. Antioxidative protection property of green tea extract on the quality of canine, avian and mouse semen have been confirmed (Wittayarat *et al*., 2013; Al-Daraji, 2011; Abshenas *et al*., 2011). On the other hand, comprehensive assessment of the green tea extract in the semen extenders for successful ruminant breeding plan is not well documented. Though recently one study has been reported in Sahiwal bull assessing green tea extract effect at low concentration (0.25% and 0.5%) in semen extender. Therefore, current study was an attempt to clarify the effect of different level of green tea extract (0.25, 0.5, 0.75 and 1.0) on post-thawed spermatozoa functional indicators in Achai breeding bulls; a dairy and light drought indigenous cattle breed of Khyber Pakhtunkhwa that have the potential for continued existence under a mountainous and sub-mountainous environment (Khan *et al*., 2008).

MATERIALS AND METHODS

Experimental design

Standard procedure as previously described was used for the semen collection through artificial vagina (42ºC) from four mature Achai bulls (Andrabi *et al*., 2008; Gohar *et al*., 2014). Experimental animals were nurtured in clean and hygienic environment maintained at Semen Production Unit Harichand, Distt Charsadda, Khyber Pakhtunkhwa, Pakistan. Ejaculates were collected twice a week for three weeks successively. Immediately after collection, the ejaculates were transferred to laboratory and kept in water bath at 37ºC. The semen samples were subjected to gross (volume, color) and microscopic (percentage motility) evaluation. Semen ejaculates having motility above 65% were included in this study.

Preparation of green tea extract

Standard protocol was utilized to prepare green tea extract as previously described (Chan *et al*., 2007; Ali *et al*., 2014). Briefly, green tea leaves were powdered in a laboratory blender. Four grams of powdered tea leaves were mixed with 200 ml of methanol, kept for 18 h at room temperature followed by centrifugation at 6200 rpm for 20 min. Supernatant was taken and filtered through filter paper. Extract was kept frozen until use.

Preparation of semen extender

Semen samples showing normal characteristics of motility above 65% were pooled to eliminate bull effect and to have adequate semen. Semen samples were pooled and extended in egg yolk citrate extender (Khan and Ijaz, 2007). Briefly, Tris HCl 24.20 g, citric acid 13.4 g, fructose 10g, glycerol 70ml, egg yolk 200 ml, streptomycin 1 g, benzyl penicillin 500,000 IU, distil water up to 1000ml, at 37°C within 10 min after collection. The concentration of spermatozoa was adjusted to 80 x 10^6 sperms per ml.

Table I.- Inclusion level of green teat extract in semen extender.

Sr. No.	Green tea extract (%age)	Extract volume (µl)	Total volume (ml) (extract+extender)
1	0.00	0.00	10
2	0.25	25	10
3	0.5	50	10
4	0.75	75	10
5	1.00	100	10

Inclusion level of green tea extracts in extended semen

The composition of the green tea extract in semen extender at different concentration has been indicated in Table I. To evaluate the effect of green tea extracts, different inclusion levels of green tea extract (0.0%, 0.25%, 0.5%, 0.75% and 1%) were added to individual test tubes. Tubes

containing green tea extract were incubated at 37°C for 30 min to allow methanol to evaporate followed by the addition of the extended semen to tubes. The tubes were incubated at 37°C for 5 min to allow uptake of green tea extracts by spermatozoa. Semen samples were transferred from 37°C to 4°C for 2 h for equilibration. Extended semen was filled into 0.5 ml straws in cold cabinet unit and was frozen first at -120°C by keeping in nitrogen vapors for 7 min, 4 cm above liquid nitrogen. Semen straws were then frozen at -196°C by dipping in liquid nitrogen.

Post-thawed semen evaluation

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

Spermatozoa motility

For the assessment of motility, one straw of semen was thawed in water bath at 37°C for 30 seconds and then semen straw was emptied in a test tube kept in water bath. A drop (10µl) of semen was placed on prewarmed (37°C) glass slide and covered with cover slip. Percentage motility was assessed at X40 under phase-contrast microscope (Olympus BX51 TF, Japan) attached with closed circuit camera following the standard procedure (Ijaz *et al*., 2009).

Spermatozoa viability

Eosin-nigrosin stain was used to evaluate the viability (live/dead %) of the frozen-thawed semen. Briefly described as a small drop of frozen-thawed semen was placed on a pre-warmed slide and mixed with a relatively larger drop of the supravital stain [1% (w/v) eosin B, 5% (w/v) nigrosin dissolved in 3% tri-sodium citrate dehydrate solution] to prepare a thin and uniform smear. After air-drying, the smear was observed under a phase-contrast microscope at 100x as previously described (Mahmood and Ijaz, 2006). Two hundred spermatozoa were counted for unstained heads of spermatozoa (live) and/or stained/ partial stained heads of spermatozoa (dead).

Plasma membrane integrity

Hypo-osmotic swelling test (HOS) as previously described was used to evaluate plasma membrane integrity (Gohar *et al*., 2014). Briefly, a hypo-osmotic solution (190 mOsm/L) was prepared by dissolving 0.735 g of tri-sodium citrate dihydrate and 1.351 g D (-) fructose in 100 mL of de-ionized distilled water. The test was performed by first mixing 500 µL of hypo-osmotic solution with 50 µL of each frozen-thawed semen sample and incubated for 45 minutes at 37°C. After incubation, the sample was mixed gently and examined under a phase-contrast microscope (40x). Two hundred spermatozoa per slide were counted, and the percentage of spermatozoa exhibiting tail curling (intact membrane) was determined.

Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Science (SPSS for Windows version 12, SPSS Inc., Chicago, IL, USA). Data was presented as mean ± S.E. Data was analyzed using one-way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

RESULTS

Post-thaw sperm functional assays of different level of green tea extract in semen egg yolk extender are expressed in Table II. Data were expressed as percentage of mean with standard error of the respective fertility indicator including sperm motility, viability and plasma membrane integrity of the post-thaw spermatozoa in Achai bull's semen with various concentration of green tea extract. The current findings demonstrated positive response of the green tea extract on post-thawed quality indicators. For motility of post-thawed spermatozoa, significant response of green tea extract in semen extender was attained at 0.75% ($P<0.05$) inclusion level in comparison with the

Table II.- Appraisal of green tea extracts inclusion in semen extender on post-thawed fertility indicators of Achai bull spermatozoa.

Fertility indicator of post-thaw spermatoza	Concentration of green tea extract in semen extender				
	0.00%	0.25%	0.50%	0.75%	1.00%
Molitiy ± SE	34.31±0.33[a]	36.21±2.5[a]	43.33±0.88[b]	52.64±0.66[c]	48.13±0.04[d]
Viability ± SE	49.50±1.2[a]	51.66±1.1[a]	57.52±1.4[b]	64.32±2.4[c]	59.21±1.4[d]
Plasma membrane integrity± SE	38.56±2.7[a]	40.32±2.0[a]	46.31±0.57[b]	53.34±0.88[c]	49.33±1.6[d]

Post thaw sperm functional assays of different level of green tea extract in semen egg yolk extender. Data are indicated as mean and S.E of the respective fertility indicator of the post-thaw spermatozoa. S.E indicated standard error; a,b,c within similar row indicate significant level at 0.05% among various treated groups for fertility indicator (molility,viability and plasma membrane integrity) of post-thaw spermatozoa of achai bull at different concentration of green tea extract in semen extender.

other treated groups at 0.5%and 0.25% including the control (0.0%). Likewise, the current result indicated that post-thawed viability of sperms were statistically maximum (P<0.05) at 0.75% inclusion level of green tea extract compared to the other treated groups at 0.25% and 0.50% and 0.0% (the control).

Furthermore, result obtained in the current study further 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 comparation with at 0.25% and 0.50% together with the control (0.0%). On the other hand, the significant decrease in the response of green tea extract toward the post thaw fertility indicator were also observed at 1% when compared with 0.75 % inclusion level. Thus current study elucidated the enhancement of the fertility indicators of the post-thaw spermatozoa at 0.75% inclusion level of green tea extract in semen extender.

DISCUSSION

Nowadays usage of frozen semen in ruminants has become a routine technique with widespread relevance in countries depending on artificial insemination to increase animal productivity. Semen proficiency is the gauge of successful insemination in ruminant breeding plans. Measurement of sperm functional indicators *i.e.* sperm motility, viability and plasma membrane integrity is imperative for the appraisal of potential fertilizing capacity of the cryopreserved processed semen (Huynh *et al*., 2000; Rodriguez-Martinez, 2003, 2006). Consequently, the high motility of post-thaw spermatozoa in conjunction with its entire structural integrity of diverse components is recognized as a decisive factor in assessment of semen characteristic for the fertility potential in A.I. Enterprise. On the other hand, production of reactive oxygen species in cryopreservation has been associated with deterioration of sperm motility, plasma membrane integrity, viability of bull semen (Bailey *et al*., 2000; Bilodeau *et al*., 2001; Chatterjee and Gagnon, 2001; Ansari *et al*., 2010, 2011, 2012). Hence, various studies attempted to explore the best method of semen preservation with different extenders for successful artificial insemination in ruminant breeding plan. In recent time, green tea has become a central focus of extensive research investigation in the field of reproductive biology for exploration of its potential effects on mammalian reproduction (Wittayarat *et al*., 2013; Al-Daraji, 2011; Abshenas *et al*., 2011). We believe to be pioneers in elucidating the beneficial effect of green tea extract on the post-thaw spermatozoa functional characteristics of Achai bull.

Current findings indicated that addition of green tea extract to the semen extender enhanced the sperm functional factors such motility, viability and plasma membrane integrity which also confirmed recently reported antioxidative protection of the green tea extract in Sahiwal bull spermatozoa (Ali *et al*., 2014). Additionally, some studies have reported positive effect of green tea extract for semen preservation in various mammalian species. Findings of the current study were in line with the observations as reported in several mammalian species including canine, rabbit and avian species (Wittayarat *et al*., 2013; Jirina and Anton, 2013; Al-Daraji, 2011). In the canine species, it was demonstrated that addition of green tea extract to tris-egg yolk extender enhanced the motility and viability of chilled canine sperm. In case of avian study, it was clarified that green tea had dominant antioxidant effect against lipid peroxidation that was naturally occurring during cryopreservation of avian semen, thus improved the avian semen quality. Based on these studies, together with current study, it was evident that low concentration of green tea exerted excellent effect in semen extender to enhance post thaw quality parameter. In our study, at 0.75 % concentration of green tea extract in semen extender, the optimal response toward spermatozoa motility, viability and plasma semen extender was observed. Likewise, in Sahiwal bull, the maximium response of green tea extract was evident at 0.5% concentration in semen extender. Recently enhancement of sperm motility has been elucidated in rabbit with low concentration of green tea extract (≤0.75 mg/L) (Jirina and Anton, 2013). Therefore, it is construed from these studies including the current finding that high fraction of motile and viable spermatozoa in processed semen samples might indicated that at low concentration, green tea extract have potentially prevented the damages usually occurring during the process of cryopreservation by ROS production. Thus, ascertained characteristically the potential antioxidative role of green tea extract against the oxidative stress produced by ROS during cryopreservation. On the other hand, various studies carried in field of reproductive biology has demonstrated that higher doses of antioxidants supplementation have been associated with detrimental effect whereas at physiological point the addition of such antioxidants are commonly not dangerous (Bouayed and Bohn, 2010). In recent study also, the response of green tea extract on fertility indicator was not more significantly improved at higher concentration (1.0%) that has been remarkably supported by the another recent study carried out in rabbit where interestingly decreased sperm motility was observed with increased concentration of green tea extract in semen extender (Jirina and Anton, 2013). Hence elucidation of dose dependent response of green tea extract in the semen extender has been evident from these studies.

However, further studies in more high concentration of green tea extract in the semen extender would clarify the dose dependent nature of the green tea in semen extender biology.

As an established fact, the green tea extract contain ample amount of catechin polyphenols having the antioxidant characteristics and are strongly associated with fight against oxidative stress (Chyu *et al*., 2004). Considering the current findings as well as earlier studies, it seems reasonable that the positive effects of green tea extract on semen quality may be ascribed to catechin polyphenols content on reduction of the oxidative stress that naturally occurred during cryopreservation of semen,despite the difference in experimental animal model. Though the functional mechanism through which green tea polyphenol is involved in the inhibition of oxidative stress during semen preservation is not clear and remains to be elucidated. However, it was demonstrated that polyphenols might bind to components of the sperm membrane and would have prevented the lipid membrane oxidation induced by free radicals (Hyon, 2004). Additionally, the other potential factors might be attributed to the association of green tea polyphenols with inhibition of egg yolk oxidation in semen extender (Ponglohapan *et al*., 2004). In most animal studies including ours, egg yolk extenders were widely used for the preservation of semen (Wittayarat *et al*., 2013; Quintero-Moreno *et al*., 2004).

CONCLUSION

The current study demonstrated the significant response of the green tea extract on the fertility factors such as viability, motility and plasma membrane integrity in cryopreserved Achai breeding bull semen. The findings generated from the current study could be used in the field of animal reproduction to further elucidate antioxidative role of green tea extract (*Camellia sinensis*) on the cryopreserved semen in the other breeds of cattle and buffalo in different region of the world under diverse environmental conditions.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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