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SHORT COMMUNICATION

Antioxidative Protection by Strawberry and Green Tea Extracts During Cryopreservation of Sahiwal Bull Semen

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ABSTRACT

The reactive oxygen species (ROS), produced during semen cryopreservation, have detrimental effects on sperm survivability. The negative impact of ROS may be countered by adding antioxidants. This study was designed to evaluate the effects of natural antioxidants (green tea and strawberry extracts) on post-thaw semen quality. Semen samples from three Sahiwal bulls were collected, pooled, extended in Tris-citric acid based extender and mixed with various dilutions of strawberry and green tea extracts. The post thaw parameters of semen motility, viability and plasma membrane integrity for 0.25% green tea supplementation (42.66±3.08, 54.13±3.36, 43.33±2.40) were significantly improved than control (29.66±1.85, 43.40±2.43, 33.33±2.06) ($P<0.05$). The strawberry supplements also improved the quality of semen but to alesser extent. So, it was concluded that addition of green tea extract in semen preservative can improve post-thaw quality of Sahiwal bull semen.

INTRODUCTION

Reactive oxygen species (ROS), found in the ejaculates, are produced by the sperms and leukocytes. ROS triggers the process of lipid peroxidation that results in irreversible arrest of sperm motility and sperm damage. Post-thaw sperm quality is adversely affected by ROS which include superoxide anion, hydrogen peroxide, nitric oxide and hydroxyl radicals (Aitken et al., 2010). Excessive ROS produced during the cryopreservation procedure have been associated with decreased sperm motility, increased morphological abnormalities and DNA fragmentation which ultimately impair the fertility (Fraser and Strzezek, 2007).

In mammals, the semen contains many antioxidants like superoxide dismutase, catalase, vitamin C, vitamin E hypotaurine, taurine, and albumin (Zini et al., 2002). During semen extension, their concentration decreases leading to oxidative stress. ROS must be continuously inactivated to maintain normal cell functions. For this purpose the restoration of threshold level of antioxidants in the semen is very important (Agarwal et al., 2003).

The chemical composition of strawberry fruit (*Arbutus unedo*) and green tea leaves (*camila sensis*) contains a wide range of antioxidants such as phenolic compounds (e.g. anthocyanins, gallic acid derivatives, tannins), vitamin C, vitamin E and carotenoids, tea polyphenols (catechines, flavanols, flavanones, phenolic acids, glycosides and the aglycons of plant pigments) tea caffeine, amino acids, saponins, epicatechin (Epigallo-catechin gallate, Epicatechin gallate, Epicatechin, Epigallocatechin (Gunduz and Ozdemir, 2014; Pawlowska et al., 2006). In recent studies it has been concluded that green tea has higher antioxidant activity even than the well-known polyphenols found in antioxidant vitamins like vitamin C and E (Tedeschi et al., 2004). Moreover, epigallocatechin gallate (EGCG) a substance found in green tea is 200 times more potent antioxidant than vitamins C and E, three times more

effective than Butylated hydroxyanisole (BHA), and twice as powerful as resveratrol which is found in red wine (Lin and Lin, 1997).

It has been clearly described that strawberry fruits and green tea leaves are the best sources of natural antioxidants and their effect as antioxidants have been studied in mouse (Abshenas et al., 2011), Rooster (Al-Daraji, 2011) and dog (Wittayarat et al., 2013) semen. Moreover, these are economical and easily available. Therefore, the present study was designed to evaluate whether the addition of green tea and strawberry extract to cryo-diluent can raise the level of resistance of spermatozoa against oxidative stress.

MATERIALS AND METHODS

Preparation of extracts

For the extraction, 50 grams of strawberry fruits were homogenized in blender and mixed with 150ml of 1% citric acid solution. After 18 hours, the mixture was centrifuged at 6200 rpm for 20 minutes. The supernatant was filtered and frozen till further use (Denev et al., 2010).

Powdered green tea leaves (4 grams) were mixed with 200ml of methanol. This mixture was kept for 18 hours at Mention temperature?? followed by centrifugation at 6200rpm for 20 minutes. The supernatant was filtered through filter paper and kept frozen until use (Chan et al., 2007).

Preparation of semen extender

Buffer for semen extender was prepared by mixing 1.34g citric acid (Merk, Germany), 2.42g N-Tris-(hydroxymethyl-aminomethane (MP Biomedical, France) in 73ml of distilled water. The pH of buffer was adjusted to 7.0 and supplemented with 1g Fructose (BDH, England), 20 ml Egg yolk, 7 ml Glycerol (BDH, England) and antibiotics (Penicillin, Streptomycin 50000 IU).

Semen collection and evaluation

Three mature normal Sahiwal bulls were selected for the present study and they were maintained in standard management conditions for the entire study period (December, 2013 to February, 2014). Semen was collected with the help of artificial vagina (42^{o}C) at weekly interval for three weeks (3 replicates). Frequency of semen collection was two ejaculates weekly. The semen samples were subjected to gross (volume, color) and microscopic (percentage motility) examinations. Ejaculates having motility above 60% were used for further processing.

Semen processing

Tris-citric acid extender with three levels of strawberry (0.5%, 1% and 2%) and two levels of green tea (0.25%, and 0.5%) were prepared in individual test tubes. Tubes containing green tee extracts were further incubated at 37°C for 30 minutes to allow methanol to evaporate. Semen samples from three bulls were pooled to eliminate bull effect and to have sufficient semen for replicates. The semen was given 10 minutes holding time at 37°C in water bath before dilution. During experiment 1; four aliquots of semen for strawberry (0.0%, 0.5%, 1%, 2%) and during experiment 2; three aliquots of semen for green tea (0.0%, 0.25%, 0.5%) extracts were diluted in single step at 37°C. For dilution, the concentration of spermatozoa was adjusted to 80 x 10^6 sperm per ml. The extended semen was further incubated for 5 minutes to permit uptake of strawberry and green tea extracts by spermatozoa.

Semen samples were cooled from 37°C to 4°C in 2 hours and equilibrated at this temperature for 4 hours. Extended semen was filled into 0.5ml straws in cold cabinet unit and was frozen first at -120°C by keeping in nitrogen vapors for 7 minutes, 4cm above liquid nitrogen. Semen straws were then frozen at -196°C by dipping in liquid nitrogen(Andrabi et al., 2008) .

Post-thaw sperm functional assays

One straw of semen from each treatment was thawed at 37°C for 30seconds to assess the semen quality parameters (Andrabi et al., 2008).

a. Spermatozoa motility

A drop (10µl) of semen sample was placed on a pre-warmed (37°C) glass slide and covered with a cover slip. Percentage of progressive motile sperm was assessed at three points under phase-contrast microscope (Olympus BX51 TF, Japan) at 40x attached with closed circuit camera. A spermatozoa moving due to its swimming was considered as motile. Mean of three observations was considered as a single data point (Andrabi et al., 2008).

b. Spermatozoa viability

A spermatozoan viability was evaluated by eosin-nigrosin supravital stain [1% (w/v) eosin B, 5% (w/v) nigrosin dissolved in 3% tri-sodium citrate dehydrate solution]. A small drop of semen sample was placed on a pre-warmed glass slide and mixed with a drop of the supravital stain to prepare a thin and uniform smear. After air-drying, two hundred spermatozoan heads were counted for staining in the smear under a phase-contrast microscope. An unstained spermatozoan head was considered as live sperm and stained spermatozoan head was considered as dead sperm (Mahmood and Ijaz, 2006).

c. Plasma membrane integrity

Sperm plasma membrane integrity was assessed using the hypo-osmotic swelling assay (HOS)(Andrabi et al., 2008). Briefly, tri-sodium citrate dihydrate (0.735 g) and D fructose (1.351 g) were dissolved in 100 mL of de-ionized distilled water to prepare HOS solution (190 mOsm/L). The test was performed by mixing 50 µL of each frozen-thawed semen sample with 500 µL of HOS solution and incubated for 45 minutes at 37°C. After incubation, a drop of the treated mixture was examined

under a phase-contrast microscope (40x). Swollen spermatozoa exhibiting tail curling were considered to have intact plasma membranes. Two hundred spermatozoa per slide were counted, and the percentage of intact sperms was counted.

Statistical analysis

Data was presented as mean ± standard error. The data was analyzed by one-way analysis of variance (ANOVA) using the statistical software, SPSS (SPSS Inc., Chicago, IL, USA). Differences were considered significant at P<0.05.

RESULTS

Data regarding the effect of natural antioxidants (Strawberry and Greentea extracts) on the sperm motility, viability and plasma membrane integrity is given in Tables1 and 2 respectively. Motility did not differ among groups following dilution and equilibration with supplemented extenders having green tea (0%, 0.25%, 0.5%) or strawberry (0, 0.5%, 1.0%, and 2%) extracts. Post-thawed motility of sperms was higher with 0.25% and 0.5% green tea extract supplementation, compared to the control (P<0.05; Table 2) and the difference between green tea treatments (0.25% vs 0.5%) was non-significant**.** In strawberry extract treated groups (Table 1), motility was improved (P<0.05) when treated with low concentrations (0.5%, 1%) as compared to the control group.

Post-thaw viability of strawberry treated samples was highest (P<0.05) at 1% (46.40±2.10%) followed by 0.5% (45.40±2.85%) as compared to control (38.46± 2.69%) and 2% (29.60±2.71%) treated groups (Table 1). In experiment 2,sperm livability did not differ between 0.25% and 0.5% green tea treated samples (54.13±3.36 and 52.60±3.62, respectively)but this was significantly higher (P<0.05) as compared to control.

Percentage of spermatozoa with intact plasma membranes was significantly higher (45.13±3.27 and 43.33±2.40) with 0.5% and 0.25% green tea extract inclusion levels, respectively than control (33.33±2.06). No significant difference was observed within the treatments (Table 2). In strawberry treated samples post thaw plasma membrane integrity did not differ between 0.5%, 1% and control treatments (38.66±2.59%, 40.66±2.16% and 33.86±2.53% respectively). However, at 2% inclusion level it was decreased significantly (24.73±2.96%). Overall, strawberry extract inclusion at more than 1% concentration has negative impact on post-thaw sperm parameters.

Table 1: Effect of Strawberry extract addition on post-thawed Sahiwal bull semen quality cryopreserved in Tris-citric acid, egg yolk, glycerol extender

Strawberry extract (%)	Characteristics of spermatozoa (%)		
	Motility	Viability	HOS Positive
0%	31.66±2.05[a]	38.46±2.69 [a]	33.86±2.53 [a]
0.5%	39.00±2.54 [b]	45.40±2.85 [a,b]	38.66±2.59 [a]
01%	38.66±2.36 [a,b]	46.40±2.10 [b]	40.66±2.16 [a]
02%	21.33±3.21 [c]	29.60±2.71 [c]	24.73±2.96 [b]

Data shown are mean ± SEM (n=15), [a,b,c] denote differences (P<0.05) in the columns

Table 2: Effect of Green tea extract addition on post-thawed Sahiwal bull semen quality cryopreserved in Tris-citric acid, egg yolk, glycerol extender

Green tea extract (%)	Characteristics of spermatozoa (%)		
	Motility	Viability	HOS Positive
0%	29.66±1.85 [a]	43.40±2.43 [a]	33.33±2.06 [a]
0.25%	42.66±3.08 [b]	54.13±3.36 [b]	43.33±2.40 [b]
0.5%	42.33±3.15 [b]	52.60±3.62 [b]	45.13±3.27 [b]

Data shown are mean ± SEM (n=15), [a,b] denote differences (P<0.05) in the columns

DISCUSSION

Following dilution up to equilibration time, the extenders having different concentrations of natural antioxidants did not differ with each other. This indicated that the strawberry or green tea extracts had no toxic impact for *in-vitro* cell survivability. Further, it is also evident that the availability of antioxidants is more crucial during cryopreservation rather than fresh semen handling. These results are in accordance with findings of El-Sisy et al. (2008) who found that addition of superoxide dismutase to extender did not have a significant effect on motility of buffalo bull spermatozoa after cooling to 5^{o}C. During cryo-preservation and post thawing semen evaluation, the green tea extract exhibited the improved post-thaw sperm motility, viability and plasma membrane integrity (HOS test) as compared to control. These findings are in line with reports about post thawed addition of different antioxidents in semen extenders during cryopreservation of Sahiwal bull (Ansari et al., 2011) and buffalo bull sperms (Andrabi et al., 2008).

The addition of strawberry extract improved the post thawed semen parameters only to limited extent. The plasma membrane integrity did not improve by its addition and all the parameters deteriorated aggressively by its addition at 2%.It indicated that strawberry had beneficial effects only at very low concentrations and further studies are required investigating its impact in lower concentrations. The results of experiment 1 also indicated the trends of strawberry for its toxic effects on sperm survival.

More pronounced effect on the post-thaw motility of green tea treated samples might be due to Green tea polyphenols that act as antioxidants by discarding reactive oxygen and nitrogen species and chelation (Frei and Higdon, 2003). Further, green tea is also rich

in Epigallocatechin 3-gallate (EGCG), that has been comprehensively studied in terms of *in vitro* and *in vivo* studies. In presence of ferrous (Fe), EGCG give hydroxyl radicals and interacts with proteins and phospholipids in plasma membrane and improves DNA fragmentation (Kim et al., 2014).

In conclusion, the present study demonstrated that green tea and strawberry extract improved post-thaw quality parameters of Sahiwal bull spermatozoa. This study provides information for improving semen quality by addition of natural antioxidant compounds (green tea and strawberry). Further studies required to investigate and purify the active ingredients present in these natural compounds.

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