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Response of Cryopreserved Nili Ravi Buffalo Bull Semen to Gallic Acid Inclusion in Semen Extender

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MT conducted an experiment. IR, MSY, HZ and SI design and supervised the experiment and provided the required facilities. IUK, AZK, S, IUK, AUR and MAK helped in data collection, data analysis, manuscript writing and review.

Key words
Gallic Acid, Cryogenic media, Buffalo, Semen, Cryopreservation

ABSTRACT

During cryopreservation, the spermatozoa faces osmo-chemical, mechanical and thermal stresses, which are predominant at dilution, cooling, equilibration, freezing and thawing stages. They damage functional and morphological characteristics of sperms. Beside these exogenous stresses there is an oxidative stress which damages the spermatozoa endogenously. These stresses can be controlled with the inclusion of antioxidants in semen extender at the time of cryopreservation. In the current study, semen from four (n=4) healthy Nili Ravi buffalo bulls was collected through artificial vagina and Gallic acid was added to the semen at 1 µM, 15 µM, 30 µM, 45 µM, 60 µM, and 100 µM. A total of six groups were prepared. One group was kept as control where no Gallic acid was added. After the addition of extender, semen was cooled to 4°C, filled in 0.5mL straws for 4 h and frozen in liquid nitrogen at -196 °C. The parameters evaluated were percentage motility, plasma membrane integrity (HOST assay), acrosomal integrity (NAR), viability (Live/Dead), DNA integrity (Acridine orange assay) and oxidative stress (TBARS assay). Five straws from each Gallic acid group were thawed individually in water bath at 37°C for 30 seconds. The addition of 15 µM GA to semen extender improved marginally the buffalo bull spermatozoa motility, viability and membrane integrity but still not sufficient to reach the statistical significance, while it has no protective effects on other parameters like Acrosomal integrity, DNA status and oxidative stress. However further studies are needed to assess the role of Gallic acid in different concentrations and in other animals.

INTRODUCTION

Cryopreservation of semen and artificial insemination are advantageous techniques in animal reproduction in which the cryopreserved semen is used to access proven genetics at far low cost than buying a bull. Beside the beneficial aspect of cryopreservation, spermatozoa face osmotic, thermal and oxidative shocks during the cryopreservation process (Bansal and Bilaspuri, 2011; Holt, 2000) which induce the production of reactive oxygen species (ROSs). ROSs are short lived free radicals which include hydrogen peroxide (H_2O_2), superoxide anion ($O^{\cdot}$), and hydroxyl radical ($OH^{\cdot}$) and lipid per oxidation products like peroxyl ($ROO^{\cdot}$), alkaoxyl ($RO^{\cdot}$) radicals (McCarthy *et al*., 2010).

Under physiological conditions, ROSs are produced in small amounts which are requisite for successful capacitation, hyper activation, acrosomal reaction and oocyte fusion (Agarwal *et al*., 2005). For a good fertilizing ability of spermatozoa, there should be a fine balance between ROS production and it's scavenging by antioxidants in semen (de Lamirande *et al*., 1997). When ROS production reaches beyond the physiological limits they cause lipid peroxidation of the cell membrane, and negatively affect the quality of post-thawed spermatozoa (Lucio *et al*., 2016).

Different exogenous antioxidants have been used to improve the quality of bovine cryopreserved semen with varying degrees of efficacy. Some of these exogenous antioxidants include alpha-tocopherol (Vit-E), ascorbate (Vit-C) (Akhter *et al*., 2011), cysteine (Sarıözkan *et al*., 2009) and butylated hydroxytoluine (BHT) (Memon *et al*., 2011). Similarly, different antioxidants such as alpha-tocopherol, ascorbic acid and n-propyl gallic acid were combined into cryo-medium to improve the post thawed sperm quality. Improved viability and motility of spermatozoa was noticed at a concentration of 15 µM n-propyl gallic acid (Shukla and Misra, 2005). The n-propyl

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gallic acid converts hydrogen peroxide and oxygen-free radicals catalytically to water and oxygen. Gallic acid (3,4,5, trihydroxybenzoic acid) is a polyphenolic food component commonly found in fruits and medicinal plants which has good antioxidant, anticancer, and anti-apoptotic properties (Kahkeshani *et al*., 2019). The literature showed that gallic acid (GA) scavenges hydrogen peroxide which consequently prevents lipid peroxidation of the spermatozoa membrane (Li *et al*., 2005, 2010). GA has more cryoprotective effects than α-tocopherol against hepatocyte toxicity at the concentration of 20mg/L. The protective effect of GA against liver hepatocytes has been attributed to the reduced formation of lactate dehydrogenase and malondialdehyde, and maintained level of glutathione (Li *et al*., 2010). Moreover, the GA supplementation also reduces the production of hypochlorus acid, nitrogen dioxide and tyrosyl radicals in human atherosclerotic lesions (Serrano *et al*., 2010).

Therefore, considering the antioxidant properties of GA, the current study was conducted to expound the effectiveness of GA supplementation in buffalo semen for the reduction of oxidative stress in the sperms during cryopreservation.

MATERIALS AND METHODS

Collection of semen and its evaluation

Four healthy fertile Nili-Ravi buffalo bulls of age between 5 and 10 years of a good record of semen quality were selected for semen collection. All the bulls received the same kind of ration. At the time of collection, the environmental temperature was 27°C. All the bulls were mounted and the semen ejaculate was obtained by artificial vagina. Two ejaculates per bull were obtained. The semen was kept in a water bath at 37°C and was assessed qualitatively. The average volume of semen obtained per ejaculate per bull was 4mL. Only the ejaculates having >80% motility were selected for experimental study. Extender was added to the semen samples and spermatozoa concentration was adjusted to 40 x 10^6 spermatozoa per mL. The extender used was Tris citrate egg yolk extender containing egg yolk [20% (v/v)] and glycerol [7% (v/v)] at 37C° (Ali *et al*., 2008).

Inclusion of gallic acid in extender containing semen

A stock solution of gallic acid (GA) was prepared by dissolving 85.06 mg of GA in 100mL distilled water to make total concentration of GA of 5000 µM. In our study the concentrations of GA used were; (0 µM, 15 µM, 30 µM, 45 µM, 60 µM, 100 µM). In order to add these concentrations to the extended semen, 25mL extended semen obtained from each buffalo bull was pooled in a beaker, so that the final volume of 100mL extended semen was obtained. The extended semen was divided into six equal aliquots of 16mL in each group and GA from the stock solution was added like 320µL to 16mL i.e. (100 µM), 192µL to 16mL i.e. (60 µM), 144µL to 16mL i.e. (45 µM), 96µL to 16mL (30 µM), 48µL to 16mL (15 µM). As a control, 320µL of 0.9% normal saline was added to the extended 16mL semen. In this way total of 6 concentrations were prepared. The GA added extended semen samples were incubated at 37°C for 5 min. to allow spermatozoa to absorb GA.

Semen cryopreservation

Prior to the freezing of semen in liquid nitrogen, it was passed through the process of equilibration. French Straws (0.5mL) were first labeled as GA-0, GA-15, GA-30, GA-45, GA-60 and GA-100 representing each concentration of GA, and then filled with semen and sealed. For equilibration straws of semen were transferred from 37°C to 4°C for 4 h. Then semen filled straws were first frozen at -120°C in freezing grill for 10 min. followed by freezing in liquid nitrogen at -196°C. A total of 30 straws were prepared per inclusion level. The container was then transported to the physiology laboratory at University of Veterinary and Animal sciences, Lahore for further evaluation of parameters such as spermatozoa motility, viability, membrane integrity, acrosomal integrity, DNA fragmentation and oxidative stress assessment. For analysis, ten (n= 10) replicates were used for each treatment.

Post-thawed semen evaluation

Spermatozoa motility

The straws were taken out from -196°C liquid nitrogen and thawed at 37°C. Semen drop from the straw was put on a pre warm clean glass slide and observed under a phase contrast microscope at 37°C for the assessment of Percentage motility of spermatozoa. All slides were examined at 400 times magnification. The mean taken from three observations was counted as single reading.

Spermatozoa viability

Spermatozoa viability was assessed through eosin-nigrosine stain method. For this purpose, 3% solution of sodium citrate was prepared by dissolution of 3g sodium citrate in 100mL distilled water. This solution was divided into two equal parts. In one part, 1g of eosin was added and in second part, 5g of nigrosine was added. Both solutions were incubated at 60°C for 30 min. in glass bottles. Then both solutions were mixed and incubated at 37°C for 12 h. A small drop from the post-thawed semen was put on a pre warm clean glass slide and mixed with a larger drop

of supravital stain [1% (w/v) eosin B, 5% (w/v) nigrosine in 3% tri-sodium dihydrate solution] to prepare a thin smear with the help of a spreader slide. The smear was air dried and examined under a phase contrast microscope at 100x. The viable spermatozoa were identified by their unstained heads (live sperm), however the heads of dead spermatozoa were stained either completely or partially. Two hundred spermatozoa were counted in each smear of the sample.

Spermatozoa plasma membrane integrity

For the assessment of plasma membrane integrity at each concentration of GA, Hypo osmotic swelling test (HOST) was used (Iqbal *et al*., 2010). For this purpose, a hypo osmotic solution (190 mOsm/kg) was prepared by dissolution of 0.735 g of tri-sodium citrate dihydrate (Merk Germany) with 1.351 g D (-) fructose to make the final volume up to 100mL. The initial osmotic pressure of the prepared solution was 120mOsm/kg but this was dropped down to final osmotic pressure of 75 mOsm/kg by drop wise addition of double distilled water. Osmolarity was read with the help of cryoscopic osmometer (Osmomat 030; Gonotech Gmbh, Germany). A 500µL of hypo-osmotic solution was mixed with 50µL of each frozen-thawed semen sample in sterilized glass tube and incubated at 37°C for 45 min.. Sample was gently mixed for uniform distribution of spermatozoa after incubation and then on a pre-warmed slide at 37°C, a drop (~ 5 µL) of treated mixture was put, covered with a cover slip and examined under phase contrast microscope (40x). Tail curling/swelling was counted as intact plasma membranes and was examined in 200 spermatozoa. To get a mean value, four observations were taken.

Spermatozoa acrosomal integrity

Acrosomal integrity was assessed by normal apical ridge (NAR) test. For this purpose, post-thawed semen from straws of each concentration of GA was collected in test tubes. A 20 µL formaldehyde citrate (1%) {tri-sodium citrate dihydrate 2.9% (99mL) mixed with 37% formaldehyde (1mL)} solution was mixed with 50 µL of semen sample. For normal apical ridge, two hundred spermatozoa were counted at (100x) under a phase contrast microscope. Acrosomal deformities like (absent, swollen and ruffled) were counted as damaged acrosome.

Spermatozoa DNA integrity

Acridine orange (AO) staining assay was used to test sperm DNA integrity. AO solution was prepared by mixing 20mL of 1% acridine orange in distilled water with 80mL of 0.1M citric acid and 5mL of 0.2M $Na_2PO_4.7H_2O$. The pH was adjusted to 2.5. Medium-thick smears of sperms were prepared on pre-cleaned slides, air dried, fixed for two h in freshly prepared Conroy's solution (3 parts methanol: 1 part glacial acetic acid), again air dried, and stained with 0.19 mg/mL acidic AO solution for 5 min. in dark. All slides were examined on the same day under a fluorescent microscope (Labomed, Lx 400, USA). AO reacts with double stranded DNA of Sperm heads and fluoresce green under a fluorescent microscope. Sperm heads exhibiting green fluorescence were considered as normal DNA i.e. (intact chromatin). But reaction of AO with single stranded DNA fluoresce red (sometimes orange-yellow) which were counted as damage chromatin (DNA). A total of 100 cells/slide were examined for each concentration of GA added semen groups and control group.

Spermatozoa lipid per-oxidation

Lipid peroxidation level was measured by a Thiobarbituric Acid (TBA) method as described by Ohkawa *et al*. (1979). A volume of 100µl of semen was mixed with 200µl of sodium dodecyl sulphate (8.1%), 1.5mL of 20% acetic acid (pH 3.5) and 1.5mL 0.8% thiobarbituric acid. Distill water was added to made final volume of 4mL. This solution was kept at 95°C for 1 h. The solution was cooled and more distill water was added to make 5mL of total volume. Afterwards, 5mL solution containing 15 parts of butanol and 1 part of pyridine was added to make 10mL solution. These tubes were properly shaken and centrifuged at 4000rpm for 10 min.. In order to measure the absorbance at 532 nm with spectrophotometer, the organic layer at the top of solution was carefully removed, and the results (lipid peroxidation) were expressed in nano moles (nm) of malondialdehyde (MDA).

Statistical analysis

Statistical analysis was conducted with the statistical package for social sciences (SPSS for window version 12, SPSS. Chicago, IL, USA). The data was analyzed using one-way analysis of variance (ANOVA). The group differences were compared with Duncan Multiple Range Test. Data was presented as mean ± S.E. Differences were considered significant at $P < 0.05$.

RESULTS

Table I shows effect of GA on sperm viability, spermatozoa plasma membrane integrity, acrosomal integrity, DNA Integrity, lipid per-oxidation of Spermatozoa. The observed motility was higher in control and 15 and 30 µM as compared to 45, 60 and 100 µM groups ($P < 0.05$). No statistical difference observed between control, 15 and 30 µM groups and similarly between 45, 60 and 100 µM groups ($P < 0.05$).

Table I. Effect of different concentrations of Gallic acid inclusion on various parameters.

Gallic acid Concentration	0µM	15µM	30µM	45µM	60µM	100µM
Spermatozoa motility (%)	46 ± 1.87[a]	50 ± 2.55[a]	46 ± 2.98[a]	42 ± 2.54[b]	47 ± 1.22[b]	41 ± 1.87[b]
Spermatozoa viability (%)	65.6 ± 1.80[a]	69.2 ± 2.31[a]	64 ± 1.14[a]	58.6 ± 2.13[b]	62.8 ± 0.8[b]	53.8 ± 1.35[b]
Spermatozoa PMI (%)	59.2 ± 1.49[b]	65.4 ± 2.01[a]	56.8 ± 1.06[b]	51.6 ± 1.63[b]	53.6 ± 2.69[b]	50.4 ± 1.5[b]
Spermatozoa acrosomal integrity (%)	76.2 ± 2.13[a]	75.4 ± 2.01[a]	72.4 ± 1.12[b]	69.8 ± 1.12[b]	72.4 ± 1.16[b]	67.3 ± 3.03[b]
Dna damage (%)	1.75 ± 0.25	1.5 ± 0.28	2.0 ± 0.4	2.0 ± 0.4	1.75 ± 0.25	2.25 ± 0.25
Spermatozoa TBARS assay	34.5 ± 19.64[c]	44.75 ± 21.40[a]	67.25 ± 35.8[a]	84.33 ± 81.8[b]	121.5 ± 81.96[b]	183.75 ± 165.6[b]

Superscript letters [a, b, c] indicate significance. Values with unlike superscript letters are different from each other while the values with alike superscript letters are not mutually different.

The viability ratio was higher in 15 µM group as compared to 45, 60 and 100 µM groups (P < 0.05). The lowest viability percentage was manifested in 100 µM treatment group. No difference was recorded among control, 15 and 30 µM groups (P < 0.05).

The plasma integrity score was higher with 15 µM concentration of GA compared to other groups (P < 0.05). No differences were observed for intact membrane integrity in control, 45, 60 and 100 µM groups (P < 0.05).

The spermatozoa acrosomal integrity of control and 15 µM was higher than the 30, 45, 60 and 100 µM groups. However, it was the same between control and 15 µM groups (P < 0.05).

Improvement in sperm DNA integrity in GA treated groups and control groups was not different from each other. The intact double stranded DNA was manifested by more than 97% sperm heads in each group (P < 0.05).

The results of thiobarbituric acid reactive substances revealed no difference between 15 and 30 µM groups but it was less than 45, 60 and 100 µM groups. The trend of malondialdehyde (MDA) production was increasing with increasing the dose of GA i.e. from 15-100 µM (P < 0.05).

DISCUSSION

Semen cryopreservation and artificial insemination is advantageous for the genetic improvement of livestock. Cryopreservation process damages spermatozoa due to the induction of ROS which consequently reduces the fertility rate (Ezzati *et al*., 2020). Various studies indicated that Gallic acid (GA) has antioxidant properties which scavenges reactive oxygen species (Giftson *et al*., 2010; Li *et al*., 2010). The present study uses GA as an antioxidant agent in semen extenders to reduce the freeze thawing shock and improve the fertility. GA interacts with ROS both in cytosol and membrane of the cell. Previously, GA has been used as a protectant in hepatocyte toxicity (Li *et al*., 2010), atherosclerotic (Serrano *et al*., 2010) and colon carcinogenesis in male Wister rats (Giftson *et al*., 2010). We found that the sperm motility was significantly higher in control and 15 µM groups compare to other groups. We found that higher concentrations of GA have toxic effects on spermatozoa motility. The same toxic effects were also manifested by n- Propyl gallate (an ester of GA) on sperm motility above 25 µM concentration in Murrah buffalo bull semen (Shukla and Misra, 2005). They also reported that at 15 µM n-propyl gallate improved sperm motility which is not in line with our study, where GA didn't improve sperm motility on same concentration as compared to the control group. But it should be kept in mind that they used the ester of GA instead of pure GA. As there is no reported literature regarding the use of GA in semen extender as antioxidant, but it has been used in other in-vitro studies where it showed varying beneficial effects (Lu *et al*., 2006; Panghal *et al*., 2020; Sobeh *et al*., 2017).

The spermatozoa viability was same between control and 15 µM but higher than in other groups. These results revealed that GA though has potent antioxidant potential in many types of cells, but in buffalo bull spermatozoa it was ineffective. The protective effects of GA as antioxidant regarding spermatozoa viability in our study are not correlated with findings on liposome cell viability, where GA and its salts showed good protective effects (Lu *et al*., 2006). Similarly, green tea phenols and GA were cytoprotective for hepatocytes against oxidative cell injury (Li *et al*., 2010).

The membrane integrity was better in 15 µM group than in other groups. The 100 µM showed lowest percentage of plasma membrane integrity. The basic mechanism seems to be the prevention of lipid peroxidation of spermatozoa membrane, which otherwise is more prone to lipid peroxidation. The protection of cell membrane by the use of GA against ROS attack has also been reported previously which is in line with our study (De-Bona *et al*., 2016). Regarding the acrosomal integrity the percentage was significantly higher in control and 15 µM groups than in other groups. These results are in line with Tokeshi *et al*. (2007) where the activity of GA against anti-hyaluronidase

activity to prevent polyspermy was weaker. GA prevent lead acetate induced gonadal degeneration in albino rats as depicted by low MDA level due to low lipid peroxidation and ROS load (Bello and Idris, 2018). Similarly, the GA prevented from reproductive toxicity of cyclophosphamide in rats by inhibiting superoxide dismutase and Glutathione-S-transferase (Oyagbemi *et al*., 2016) which indicates the specific combating action of GA against lipid peroxidation by ROS. We compared different concentrations of GA and found that GA at 15 and 30 µM has least toxic effects and good at preventing lipid peroxidation in post thawed semen which is in agreement with the previous studies.

In our results, DNA integrity data showed that there was no beneficial effect of GA inclusion in the buffalo bull semen extender. The reason seems to be the least effect of cryopreservation on DNA, as this damage was merely 1-3% in all the groups. Our findings are in line with the previously results where the spermatozoa DNA integrity was reported to be 1.5-2.2% in sexed bull sperms (Boe-Hansen *et al*., 2005).

CONCLUSION

Gallic acid (GA) has been used as an antioxidant to prevent oxidative stress in various cell lines during freeze thawing procedures. We found that GA, after using in semen extender, has no viable effects in preserving the physiological and biological characteristics of buffalo bull sperms after freeze thawing procedures. More research is needed to assess the cryo-protective effects of GA using the semen of different animal species or using more different GA concentrations or combination with other antioxidants.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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