Effect of supplementation of butylated hydroxytoluene on post-thaw sperm viability, motility and membrane integrity of Hariana bulls

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Aim: This study was aimed to see the beneficial effect of butylated hydroxytoluene (BHT) as a semen additive of Hariana bull semen.

Materials and Methods: The study was carried out in Hariana bulls. Twenty-four ejaculates from two bulls were used for this study. Each ejaculate was extended with standard glycerolated egg yolk tris extender and supplemented with BHT at two concentrations as 0.5 mM (T1) and 1.0 mM (T2). After dilution, equilibration and 24 h of cryopreservation, the samples were analyzed for progressive motility, sperm viability and membrane integrity.

Results: Progressive motility, sperm viability and sperm membrane integrity were significantly (p<0.05) increased in the samples fortified with BHT as compared to the control during the process of cryopreservation and thawing. The BHT concentration of 1 mM revealed better results as compared to 0.5 mM.

Conclusion: Addition of 1.0 mM BHT was found better in cryopreservation of Hariana bull semen compared to 0.5 mM BHT and control samples. The addition of BHT has improved the sperm quality by acting as an antioxidant thereby reducing the lipid peroxidation of the sperms.

Keywords: sperm, viability, butylated hydroxytoluene, membrane integrity, antioxidant, Hariana bull.

Introduction

Cryopreservation of sperm is the integral part of a successful artificial insemination program, which not only depends on the prefreeze sperm features, but also by the freezing protocols [1]. Cryopreservation ensures long-term storage of sperm and its subsequent use in the breeding of domestic as well as wild animals. In spite of lots of development in cryopreservation and cryopreservation protocols, the sperms suffer from irreversible damage both at structural and functional level leading to a reduction in viability, motility, and fertility [2].

Free radical generation is a major limitation during the process of sperm cryopreservation, which is generated from the non-viable sperms as well as from the extenders, which contain the molecular oxygen [3]. The free radicals target the sperm plasma membrane, and in specific the polyunsaturated fatty acids, membrane proteins, acrosome and sperm DNA causing lipid peroxidation of membrane lipids, chromatin cross-linking and fragmentation of sperm DNA [4]. These changes induce irreversible cell damage and sperms undergo induced apoptosis causing a consequent reduction in the sperm quality. Generation of free radicals during cryopreservation cannot be inhibited, but can be reduced by the introduction of suitable antioxidants in the extenders used for sperm cryopreservation [5,6]. In the recent past, many of the laboratories are focusing on various antioxidants and their dose dependent incorporation in the semen extenders to reduce the generation of free radicals and their deleterious effect on sperm [6-8].

Butylated hydroxytoluene (BHT) is a phenolic antioxidant being supplemented in the semen extender as a measure to prevent the membrane permeability changes in the sperms during cryopreservation [9]. BHT also exhibits antiviral activity and has been associated with the inactivation of lipid-containing viruses [10]. Chemically BHT is a synthetic analog of Vitamin E, and is involved in the auto-oxidation reaction, thereby converting the peroxy radicals to hydroperoxides [11]. These may be the best possible reasons for which, BHT has been used as a potential antioxidant additive in a number of species, but no literature is available in the Hariana bulls till date.

Antioxidant addition has been emerged as one of the most powerful way to overcome the excessive generation of free radicals during the time of semen
cryopreservation and hence most of the laboratories are involved extending the semen with antioxidants. We have already studied the beneficial effects of Vitamin E, Vitamin C and various proteins which provide cryo-protection to the sperms during freeze-thaw process (data unpublished), however, literature is scanty regarding the incorporation of BHT in semen extender, as an antioxidant to protect sperm cells during cryopreservation. This provided us an open area to design our study to observe the effect of BHT addition in the semen extenders for its antioxidant effect reflecting protection to sperm cells.

**Materials and Methods**

**Ethical approval**

No ethical permission was required to conduct the study as the method of semen collection was non-invasive. However semen collection was done as per standard collection method without harming animal.

**Experimental animals**

The present study was conducted on Hariana bulls of the age group between 5.5 and 6.5 years and weighing between 450 and 500 kg body weight, reared at the University Instructional Livestock Farm Complex, College of Veterinary Sciences, U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura which is situated in a semiarid zone of Northern part of India, in the state of Uttar Pradesh. The bulls were fed 21.15 kg of greens, 4.83 kg of concentrate including service ration and 4.95-5.77 kg of wheat straw per bull per day. Bulls were apparently free of infection and were undergone for regular vaccination as per the prescribed manual of certified semen laboratory.

**Semen collection**

A biweekly semen collection schedule was followed during the entire period of the study, which was carried out during January to March of year 2014. Semen was collected directly into a clean dry graduated centrifuge tube attached to the latex cone of the AV. Immediately after collection, tube containing semen were marked, transferred to the laboratory and placed in the water bath at 32-34°C for physico-morphological studies.

**Evaluation of seminal attributes**

The collected semen was evaluated (as per the standard guidelines framed in the semen biology laboratory, DUVASU, Mathura) and those which fulfill the criteria for cryopreservation were extended with glycerolated egg yolk tris (GEYT) extender with two different concentrations (0.5 and 1.0 mM) of BHT. Three stages of sperm evaluation were carried out viz. (i) after dilution with the extender, (ii) after equilibration(at 4-5°C for 4 h) and (iii) after 24 h of cryopreservation. Seminal attributes viz. progressive motility, sperm viability and membrane integrity (hypoosmotic swelling test [HOST]) were evaluated at all three stages.

**Preparation of BHT**

275.44 mg of BHT, Kosher grade (Sigma-Aldrich, St. Louis, USA) was dissolved in 25 ml of ethanol making a BHT concentration of 0.05 M. This solution was then added @ of 0.05 and 0.1 ml in the two glass test tubes. Ethanol containing BHT was then evaporated at 37°C in an incubator resulting in sticking of BHT in the inner wall of the test tubes. The semen was extended with GEYT extender up to 80 million spermatozoa/ml. To each of this test tube 5 ml of extended semen was added making a concentration of 0.5 mM BHT (T1) and 1.0 mM BHT (T2). The extended semen along with BHT was then kept at 37°C for 5 min to allow uptake of BHT by spermatozoa. Simultaneously, the same amount of extended semen was added to another test tube (without BHT), which was considered as control group. All the extended semen (with and without BHT) were then filled in 0.25 ml, French straw with the help of filling and sealing machine, equilibrated in a cooling cabinet (temperature 4-5°C) in a straw rack for 4 h.

**Processing of semen for freezing**

The extended semen with BHT concentration of 0 mM, 0.5 mM and 1.0 mM were considered as control, treatment group T1 and T2 respectively and were taken for cryopreservation using a biological freezer (liquid nitrogen vapor freezing). These samples containing the BHT were processed for cryopreservation as per the standard protocol (lowering of temperature from 4°C to −10°C @ 5°C/min, −10°C to −100°C @ 40°C/min, −100 to −140°C @ 20°C/min) developed in the semen biology laboratory, DUVASU, Mathura.

**Post-thaw evaluation of sperms**

After 24 h of cryopreservation, the straws containing the sperms were thawed in thawing unit (IMV, France) maintained at 37°C with holding time kept as 45 s. After thawing the straws were processed for retrieval of the sperms which was followed by physico-morphological evaluation viz. progressive motility, sperm viability and sperm membrane integrity (HOST) in the control and test samples (T1 and T2) [12]. Progressive motility was observed on a thermostatically regulated stage, whereas, the sperm membrane integrity was evaluated by exposing the sperms to hypo-osmotic solution (150 mOsmol/L). Sperm viability was enumerated by Eosin-Nigrosin staining [13].

**Statistical analysis**

Statistical analyses were performed using Statistical Package for Social Science (SPSS® Version 22.0 for Windows®, SPSS Inc., Chicago, USA). Data are presented as mean and their standard error (Mean±standard error of mean). Effect of different inclusion levels of BHT (antioxidant) were analyzed using one-way analysis of variance and significance was tested at 5% level (p<0.05). Duncan’s multiple range test was used to compare the treatment means for various sperm attributes.
Results

Sperm viability was evaluated in the control sample and in two treatment groups (T1 and T2) during three stages of sperm processing. Sperm viability exhibited significant (p<0.05) difference between control and two treatment groups. Group T2 exhibited the best results in terms of sperm viability (Table-1). A similar trend was seen for sperm motility and membrane integrity as shown in Tables-2 and 3. Progressive motility was significantly (p<0.05) increased in both T1 and T2 group as compared to the control and T2 exhibited the best results in terms of progressive motility in all the three stages of processing. Sperm membrane integrity was significantly (p<0.05) increased in T2 group as compared to both control and T1 group at all three stages of semen processing (Table-3).

Discussion

The present study was an attempt to evaluate the beneficial roles of BHT as an antioxidant additive to the semen samples collected from Hariana bulls during three stages of semen processing viz. extended semen, semen after equilibration and thawing after 24 h of cryopreservation. The results obtained from the study have been presented in Tables 1-3. Retention of sperm viability is the prerequisite of semen cryopreservation so as to use the sperms for artificial insemination. The cryo damaging effect during cryopreservation induces subtle changes in the sperms which not only causes a reduction in the sperm viability but also reduce motility of the sperms [1]. The major setback to the sperm is provided by the free radicals, which are generated during the time of semen cryopreservation. Free radicals are molecular oxygen exhibiting high reactivity, targeting the sperm plasma membrane, the lipids therein and the sperm DNA. The consequence to these events, there is the induction of cell death by the process of apoptosis leading to an ultimate reduction in the viability of the sperms [14].

Viability of the sperms after the process of freeze-thaw is always a challenging step as 30 to 40% of the reduction in the viability of the sperm is seen. The loss in the sperm viability has been associated with the changes in the temperature, and ultra-low temperature maintained during cryopreservation [1]. The viability of the sperm is also reduced due to the generation of free radicals during the time of cryopreservation leading to lipid peroxidation and ultimate reduction in the sperm viability [4]. To restore the viability, the semen extenders must have enough antioxidant defenses so as to fight against the generated free radicals to minimize the free radical mediated lipid peroxidation [6]. In the present study, the viability of the sperms has been significantly increased in the semen samples containing the BHT as additive. Both the semen samples exhibited an increase in the viability of the sperms as compared to the control samples at all the three stages of sperm evaluation. The addition of BHT has been shown to increase the antioxidant defense and as a consequence to this, there was a resultant increase in the viability % of the sperms [9,10,15,16].

Free radicals generated at homeostatic levels are significant in regulating the sperm physiological functions like sperm capacitation, acrosome reaction, hyperactivation and sperm-oocyte fusion [17]. However, not all reactive oxygen species (ROS) are beneficial to the sperms along with higher levels of free radicals, which induce oxidative stress to the sperms. As a consequence to this, there is substantial damage to the sperms involving the plasma membrane damage, leakage, and increased permeability leading
to reduced sperm motility, metabolic activity, longev-
ity and viability [18].

Semen processing during the time of cryopreser-
vation results in dilution of sperm antioxidant system
and along with this, the generated free radicals brings
damage to the polyunsaturated fatty acids present on
the sperm cell membrane [19]. The addition of BHT
has a protective role on the sperms as it is postulated
that BHT penetrates the sperm cell membrane and
thereby exerts its antioxidant defense [20]. BHT pen-
etration also increases the membrane fluidity and flex-
bility of the sperms along with conversion of peroxyl
radicals to hydroperoxides. By these mechanisms,
BHT acts as an antioxidant to the cryopreserved
sperms. Studies also have shown the role of BHT as
an anti-lipid peroxidation agent and hence offers pro-
tection to the sperms during cryopreservation [21].

Studies in many species of domestic animals
have also been shown that BHT exerts a protective
effect on the sperms during freeze-thaw process. The
beneficial effects of BHT are also dependent on the
concentration in the extender, which is also species
dependent and specific. Earlier studies have reported
the beneficial role of BHT is dependent on BHT con-
centration, sperm membrane composition, time for
incubation and the type of extender used for the pro-
cess of cryopreservation [19].

Extension of the semen with desired low concen-
tration of BHT is the prerequisite to get the best results
in terms of sperm protection. Bull semen extended
with 0.5-1 mM BHT has been shown better results as
compared to 3 mM or more. Supplementation with
higher concentrations of BHT has shown detrimen-
tal effects on the sperms opted for cryopreservation.
In our study, we reported the similar concentration of
BHT offering a protective effect on the sperm after
dilution, equilibration, and thawing. BHT concentration has also
exhibited a species specific variation [19-21].

Progressive motility serves as the index of fertili-
zation and serves as the most significant determinant
of the sperm capability for bringing out fertilization.
Motility is a complex factor, which is regulated strictly
by the flagellar assembly proteins along with the gen-
erated ATP by the mitochondria [5]. Sperm motility
has been shown to reduce by 50% after freezing and
thawing in most of the species of the animal, and this
may be a probable factor behind the reduction in the
sperm quality [1]. That is why it is imperative to keep
the sperm motility at a higher level after cryopreserva-
tion so as to achieve optimal fertility. The loss in the
motility of the sperms has also been associated with
the increase in free radicals during cryopreservation
and lipid peroxidation [6]. To restore the motility and
to reduce the free radical mediated oxidative stress, it
is essential to improve the antioxidant defense to the
sperms opted for cryopreservation. This optimization
can be achieved by supplementing the extender with
antioxidant additives [22]. In the present study, we
noted a significant increase in the post-thaw motility in
the samples containing the BHT as an additive. After
dilution along with equilibration, the sperms exhibited
a higher motility as compared to the control non-sup-
plemented groups. The study defined the antioxidant
role of BHT in the improvement of the sperm motility.

Sperm membrane integrity is the index of sperm
function as well as an indication of metabolic status.
Membrane integrity is associated with keeping the
sperms active in terms of regulation of the osmotic
balance of the cells. The loss in the membrane perme-
ability is a common limitation in the sperms opted for
freezing and thawing [7]. Membrane leakiness is due
to the peroxidative damage caused by the free radicals
as a result to the dilution of antioxidant defense. The
membrane intactness can be retained by increasing the
antioxidant defense and thereby reducing the free rad-
cal mediated oxidative damage to the cells [6]. BHT
supplementation has been shown the beneficial effect
to the sperms at all the three stages of handling and
processing of sperms.

For all the parameters consider under this study,
BHT used to a level of 1.0 mM BHT (T2) did not have
any toxic effect to the spermatozoa. Hence, our result
indicates that 1.0 mM BHT is suitable for freezing
bull spermatozoa in GEYT extender having 20% egg
yolk and 7% glycerol with 80 millions/ml sperm con-
centration of extended semen.

Extension of the semen with two concentrations
of BHT improved the post-thaw motility, viability
and membrane integrity of Hariana bull sperms. The
exact mechanism behind the process of improvement
of sperm quality after the addition of BHT has not
been understood from the study, but it was probably
due to the action of BHT as an antioxidant and a con-
sequence reduction in the lipid peroxidation [20,23].
Polyunsaturated fatty acid content of sperm plasma
membrane is higher and is highly prone to lipid per-
oxidation during the process of cold/heat shock in
freezing and thawing. BHT penetrates the plasma
membrane of the sperm cells and prevents the cell
membrane from free radicals [11]. That is why BHT
acts as an exogenous antioxidant. It is also possible
that BHT in the added samples may have boosted the
antioxidant defense to the sperms.

The present study investigated whether the addi-
tion of BHT has improved the quality of Hariana bull
sperm after dilution, equilibration, and thawing. The
results revealed a significant improvement in the qual-
ity of sperm in all the three stages with the addition of
1 mM BHT. The characters evaluated were progressive
motility, sperm viability and membrane integ-
rency. The findings of the study revealed a significant
improvement in the sperm quality with the addition of
both the concentrations of 0.5 mM and 1 mM BHT.

Conclusion

Extender containing the exogenous BHT revealed better post-thaw semen quality as compared
to the extender without BHT. Addition of BHT at a
concentration of 1 mM revealed better results as compared to 0.5 mM. BHT augmented post-thaw semen quality by the reduction in the generation of free radicals and thereby a consequent reduction in lipid peroxidation. Further studies are required to understand the role of BHT in improving the sperm quality and whether, this addition translates into the improved fertility of artificially inseminated sperms.

Authors’ Contributions

AP was the MVSc. Scholar of the department who carried out experimental research work and laboratory analysis of data. AS was guide of AP, under whose supervision the thesis was submitted. AS planned and designed the experiment. AK helped in design of experiment. DKS help in manuscript preparation. DY, SSY and AK (MVSc. Scholars) helped in sterilization of equipment used in semen experiment, preparation of semen extender/stains for semen evaluation and processing of semen during the research work. All authors read and approved the final manuscript.

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Competing interest

The authors declare that they have no competing interests.

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