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Improvement in post-thaw semen quality by minimizing the lipid peroxidation following herbal treatment in sub fertile buffalo bulls

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Abstract

The present experiment was conducted to study the impact of herbal treatment on post thaw semen quality and lipid peroxidation level in subfertile buffalo bulls. Three Murrah subfertile buffalo bulls maintained at bull station, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, India (Latitude/Longitude, 30.55°N, 75.54° E) with the history of poor semen quality (pre-freeze individual motility < 60%, post-thaw individual motility < 40%, Viability : < 70% and Abnormality: > 20%) were orally supplemented with herbal mixture (*Panax ginseng* roots, Shilajit, *Withania somnifera* roots, *Tribulus terrestris* fruits, *Turnera diffusa* leaves, *Ptychopetalum olacoides* bark of each 400 mg/100 kg body weight and 300 mg/100 kg body weight of *Pausinystalia yohimbe* bark) daily for 60 days of treatment phase. Two semen ejaculates per week per bull were collected during pre-treatment, treatment and post-treatment phases of 60 days each and extended with Tris egg yolk extender. The freezing of extended semen was carried out in a biofreezer (i.e. 4 °C to -15 °C @-30 °C/min, -15 °C to -140 °C @-50 °C/min). Further, the post thaw semen was assessed in terms of individual motility, viability, total sperm abnormalities, mitochondrial membrane activity (TMRM fluorescent dye), intact acrosome and plasma membrane integrity. Further, the lipid peroxidation level was assessed in terms of malondialdehyde (MDA). During the treatment and post-treatment phase, individual motility, viability, total sperm abnormalities, mitochondrial membrane activity and intact acrosome and plasma membrane integrity were significantly ($P<0.05$) higher. Moreover, lipid peroxidation was significantly ($P<0.05$) lower during treatment phase and it remained similar ($P>0.05$) during the post-treatment phase in contrast to pre-treatment phase.

Keywords: Herbal, lipid peroxidation, malondialdehyde, subfertile buffalo bulls and semen ejaculates

Introduction

Buffalo bull sperms are more fragile as compared to cattle bull sperm (Andrabi *et al.*, 2008; Kumaresan *et al.*, 2005) [2, 22]. Various factors such as temperature, humidity and nutrition have been ascribed to play a key role in freezability of buffalo bull sperm (Sharma *et al.*, 2017; Dahiya *et al.*, 2013) [36, 8]. Under stress due to mentioned factors, there is excessive production of reactive oxygen species (ROS) in somatic and spermatogenic cells (Shamsi *et al.*, 2010) [35], thereby creating an imbalance between production and utilization of ROS leading to oxidative stress (Nita *et al.*, 2016) [29]. Due to the higher contents of polyunsaturated fatty acids (PUFA) and low antioxidant enzymes in buffalo bull sperm, there is more lipid peroxidation leading to poor post thaw recovery in terms of motility, viability, morphology, plasma membrane integrity and acrosomal integrity (Ahmad *et al.*, 2014; Bucak *et al.*, 2010; Nair *et al.*, 2006) [1, 6, 27].

Several studies have been carried out in humans and animals to improve the fresh and post thaw sperm quality by *in-vitro* addition of vitamins, minerals, proteins, amino acids, herbs (Patel *et al.*, 2016; Dorostkar *et al.*, 2014; Partyka *et al.*, 2017; Liu *et al.*, 2004) [32, 11, 31, 26] with variable success. However, only few *in-vivo* studies have been carried out by using oral feeding of herbs in subfertile buffalo bulls. Studies have indicated that herbs and herbal substances containing wide range of bioactive constituents protect semen from ROS induced quality deterioration due to various stressors (Etuk and Muhammad. 2009; Parandin *et al.*, 2012) [12, 20]. In the present study, we studied the effects of oral supplementation of herbal mixture on various sperm parameters. The hypothesis of our study was that oral supplementation of herbal mixture containing *Panax ginseng* roots, Shilajit, *Withania somnifera* root, *Tribulus terrestris* Fruits, *Turnera diffusa* leaves, *Ptychopetalum olacoides*

bark and *Pausinystalia yohimbe* bark at the rate of 400 mg/100 kg body weight and 300 mg/100 kg body weight respectively, might improve the sperm freezability in subfertile buffalo bulls as indicated by Shivkumar *et al.* (2018) [38].

Material and Methods

Ethical approval

The present study was conducted after the approval of Institutional Animal Ethics Committee with reference number GADVASU/2016/IAEC/35/02 dated 17.07.2016.

Procurement of herb and chemicals

Herbs and chemical reagents were procured from the Indian Drugs and Botanical Herbs Company, New Delhi, India and Sisco Research Laboratories Pvt. Ltd., India, respectively.

Experimental animals

The study was conducted on three (n=3) subfertile buffalo bulls (aged around 5 years and 700-750 kilograms of body weight) with the history of poor semen quality (pre-freeze individual motility < 60%, post-thaw individual motility < 40%, Viability: < 70% and Abnormality: > 20%). Buffalo bulls were being maintained loosely in half walled concrete sheds in individual pens (covered area - 12 x 10 ft and uncovered area - 25 x 10 ft) at bull station, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, India (Latitude/Longitude, 30.55°N, 75.54° E). All the animals were being fed according to standard feeding schedule along with *ad libitum* green fodder. The bulls were being given an exercise for half an hour on alternate days.

Experimental design and semen collection

This experiment was having three treatment phases viz. pre-treatment, treatment and post-treatment phase of 60 days each. During the treatment phase, buffalo bulls were orally supplemented daily with herbal mixture containing *Panax ginseng* roots, Shilajit, *Withania somnifera* roots, *Tribulus terrestris* fruits, *Turnera diffusa* leaves, *Ptychopetalum olacoides* bark each of 400 mg/ 100 kg body weight and *Pausinystalia yohimbe* bark @ 300 mg/100 kg body weight of bulls. Two ejaculates per week per bull were collected with artificial vagina method.

Extension of Semen

The collected semen was extended with freshly prepared Tris egg yolk extender keeping sperm concentration of 80 million/ml (35°C). The extended semen having >70% progressive motility, >70% viability and < 20% abnormality were further passed for filling, sealing, and printing of straws.

Filling, sealing, and printing of straws

The extended semen was filled, sealed and printed in French mini straws (0.25 ml) using integrated filling, sealing and printing machine (IS4 System, IMV Technologies, France).

Equilibration of semen

The packaged straws were racked and kept for equilibration at 4 °C for 4 hours in a horizontal cold handling cabinet (Model E22301CP-2000, IMV Technologies).

Freezing of semen

Equilibrated semen straws were frozen in a programmable bio-freezer (Mini Digitcool, ZH 400, IMV Technologies,

France) according to Shivkumar *et al.* (2018) [38] and the protocol is as mentioned below.

- 4 °C to -15 °C - 30 °C/min.
- -15 °C to -140 °C - 50 °C/min.

Then, straws were picked up from freezing chamber and plunged and stored into liquid nitrogen (-196 °C) for further assessment of post-thaw semen quality parameters.

Post-thaw semen evaluation

Frozen semen straw was thawed at 37 °C for 30 seconds and immediately evaluated for semen quality parameters such as individual motility, viability, total sperm abnormalities, mitochondrial membrane activity, plasma membrane activity, acrosomal integrity and extent of lipid peroxidation in terms of MDA.

Individual motility

Post thaw individual motility was assessed according to Shivkumar *et al.* (2018) [38] with Computer Assisted Sperm Analysis software (Biovis CASA 2000, version 4.59, Expert Vision Labs Pvt. Limited, India). The sperm concentration of the sample was adjusted to 20 million sperms/ml using Tris buffer. A drop of semen (5 µl) was put on a pre-warmed Biovis Shukratara chamber (Expert Vision Labs Pvt. Limited, India) and individual motility was recorded under 10x of phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan) attached with the warm stage.

Sperm viability

The viability of post-thaw semen was assessed by Eosin-Nigrosin stain (Kumar *et al.*, 2004) [20]. Twenty µl of semen sample was mixed with 80 µl Eosin-Nigrosin stain (5% Eosin and 10% Nigrosin stain prepared in 2.9% sodium citrate solution). The suspension was incubated for 60 seconds at 37 °C by keeping on warm plate. Thereafter smear was prepared on grease free, pre-warmed slide from stain mixed semen. The percentage of sperm viability was calculated by examining 200 spermatozoa under the microscope with 40x magnification (Digital LCD Microscope). The sperm stained with pink colour was classified as dead and sperm with the clear bright head was classified as live.

Total sperm abnormalities

The total sperm abnormalities were assessed according to Kumar *et al.* (2004) [20]. In brief, 20 microliter of semen was mixed with 80 microliters of Rose Bengal stain at 37 °C (Rose Bengal 3g, Formalin 1 ml and distilled water 100 ml). A thin smear was prepared on the grease free, pre-warmed slide from semen stain mixture. The prepared smear was observed under Digital Microscope (40x) attached with LCD screen. Percentage of total sperm abnormalities were calculated by examining around 200 spermatozoa.

Mitochondrial membrane activity

Mitochondrial membrane activity was assessed according to Dalal *et al.* (2016) [9] using tetramethylrhodamine methyl ester fluorescent dye (Cat # T-668, Life Technologies). Semen sample (250 µl) was washed twice with PBS by centrifuging at 1000 rpm for 5 mins (37 °C). Then, 5 µl of working TMRM solution (50 µM) was added to each sample and incubated at 37 °C for 90 min. After incubation, the sample was washed with 1 ml of PBS at 1000 rpm for 5 min (37 °C) to remove all the unbound dye. The pellet was mixed well with 500 µl of PBS. 10 µl of washed sample and 8 µl of

ProLong Gold Antifade Mountant with DAPI (Life Technologies) were taken on a slide, covered with a coverslip. Slide was wrapped with aluminum foil and kept at 4 °C for 10 min. Then, the slide was examined under an upright fluorescent microscope (Nikon) with DAPI filter (420-480 nm) for high or low fluorescence in midpiece region as an indicator of mitochondrial membrane activity. The percentage of mitochondrial membrane activity was calculated by observing 100 sperms.

Plasma membrane integrity (HOST)

Functional integrity of the sperm plasma membrane was evaluated by hypo-osmotic swelling test (HOST) according to Jeyendran *et al.* (1984) [18]. The hypo-osmotic solution (100 mOsm/L) was prepared by dissolving 4.9 g of sodium citrate and 8.77 g of fructose in one liter of double distilled water. Frozen-thawed semen (10 µl) was mixed with 100 µl of hypo-osmotic solution and incubated at 37°C for half an hour. Simultaneously, 10 µl of semen was incubated in 100µl PBS under similar conditions. 10 µl of incubated semen both from hypo-osmotic solution as well as from PBS was placed on separate glass slides and covered with a cover slip. The slides were examined under 40x objective lens of phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan) for curled tail spermatozoa. About 200 spermatozoa were counted separately in PBS and hypo-osmotic solution in different fields. The number of curled tail spermatozoa in PBS was deducted from that in hypo-osmotic solution and the resultant figure was taken as the HOS-reactive sperm.

Acrosomal integrity

Acrosomal integrity of spermatozoa was assessed according to Zodinanga *et al.* (2015) [41] by Giemsa stain. Giemsa stain was prepared by dissolving 1 g of Giemsa powder in 66 ml of methanol and kept at 4°C overnight. Thereafter, 60 ml of glycerol was added and the solution was filtered and stored in air tight container. A smear (10 µl) of washed frozen-thawed semen was prepared on a clean glass slide, air dried and fixed in methanol for 30 minutes. After drying, the smear was stained in Giemsa working solution (stock Giemsa stain 3 ml, 0.1 M phosphate buffer 2 ml, pH 7.4 and double distilled water 35 ml) for 2 h. The slides with smears were then removed from the stain solution and rinsed quickly in double distilled water, air dried and then at least 200 spermatozoa were examined under the microscope with 40x magnification (Digital LCD Microscope). Acrosomal integrity was calculated by classifying the examined spermatozoa as intact acrosome (defined acrosomal cap) and damaged acrosome (partially and/or completely missing acrosomal cap).

Measurement of lipid peroxidation (Malondialdehyde)

Lipid peroxidation was determined by the method of Buege and Steven (1978) [7]. Briefly, 1.5 ml of extended semen (80 million/ml) was taken in an eppendorf tube. Two washings were given with 1.0 ml Phosphate buffered saline (pH 7.4) at 6000 rpm for 10 mins (37 °C). The sperm pellet was dissolved in 1 ml of 1% Triton solution (Cat # 30190, SRL Pvt. Ltd, India). Thereafter, agitation was carried out in a water bath at 25 °C for 30 min. Then, the supernatant was collected following centrifugation (10,000 rpm for 20 min at 4°C) and was stored at -80 °C for the assessment of malondialdehyde (MDA).

Reagents

1. 150mM Tris-HCl (pH 7.1) - 1.64 gm/90ml of DDW (Cat # 2049297, SRL Pvt. Ltd, India)
2. 10% Trichloroacetic acid (TCA) - 10gms/100ml of DDW (Cat # 204842, SRL Pvt. Ltd, India)
3. 0.375% Thiobarbituric acid (TBA) - 750mg/200ml of DDW (Cat # 659605, CDH Pvt. Ltd, India)

Procedure

Reaction mixtures were prepared as below:

- For semen: 100 µl extracted semen and 100 µl Tris-HCL buffer.

Reaction mixtures were incubated at 37 °C for 20 min. After incubation, 1 ml of 10% chilled TCA and 2 ml of 0.375% TBA were added and kept for 20 min in the boiling water bath (100 °C). Thereafter, the mixture was cooled and centrifuged at 10,000 rpm for 5 min (37 °C) and the supernatant was taken out in a cuvette. Optical density was recorded at 532 nm (UV-VIS spectrophotometer, systronics, India) against distilled water as a blank.

Calculations

$$\text{LPO (MDA, } \mu\text{mole}/10^9 \text{ spermatozoa)} = \frac{\text{OD} \times \text{volume of assay mixture}}{\text{Molar extinction coefficient} \times \text{volume of sample taken}}$$

Where

- The molar extinction coefficient for MDA is $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.
- OD: Optical density of the sample at 532 nm.

Statistical Analyses

All data are presented as the mean \pm standard error. Normality of data was checked by Shapiro-Wilk Test. Homogeneity of variance was analyzed by Levine's test. Data were analyzed by one way ANOVA followed by Turkey's HSD post hoc test for the comparison of supplementation phases (IBM SPSS Statistics version 22). Statistical significance was considered at $P < 0.05$.

Results

Semen quality parameters

Semen quality parameters obtained during all the phases of experiment were presented in Table 1. During pre-treatment phase semen quality parameters such as individual motility, viability, mitochondrial membrane activity, intact acrosome and plasma membrane integrity were lower as 32.85 ± 2.40 , 65.78 ± 2.13 , 34.29 ± 2.00 , 40.9 ± 0.72 and 39.8 ± 0.57 , respectively. Following herbal treatment individual motility, viability, mitochondrial membrane activity, intact acrosome and plasma membrane integrity were significantly improved to 41.72 ± 0.33 , 75.47 ± 1.59 , 45.00 ± 0.42 , 49.6 ± 1.51 and 62.0 ± 1.39 during treatment phase and 40.36 ± 2.40 , 70.90 ± 1.05 , 41.35 ± 2.20 , 48.9 ± 0.60 and 57.6 ± 0.99 during post-treatment phase, respectively. Total sperm abnormalities were very high in pre-treatment phase (37.22 ± 2.33), but following herbal treatment significantly reduced to 15.63 ± 1.40 and 20.03 ± 2.02 during treatment phase and post-treatment phase, respectively. However, there was no significance difference between treatment and post-treatment phase.

Table 1: Semen quality parameters (Mean \pm SE) following herbal treatment of subfertile buffalo bulls during all three different phases

Semen quality parameters	Post-thaw semen		
	Pre-treatment	Treatment	Post-treatment
Motility (%)	32.85 \pm 2.40 ^a	41.72 \pm 0.33 ^b	40.36 \pm 2.40 ^b
Viability (%)	65.78 \pm 2.13 ^a	75.47 \pm 1.59 ^b	70.90 \pm 1.05 ^b
Total sperm abnormalities (%)	37.22 \pm 2.33 ^a	15.63 \pm 1.40 ^b	20.03 \pm 2.02 ^b
Mitochondrial membrane activity (%)	34.29 \pm 2.00 ^a	45.00 \pm 0.42 ^b	41.35 \pm 2.20 ^b
Intact acrosome (%)	40.9 \pm 0.72 ^a	49.6 \pm 1.51 ^b	48.9 \pm 0.60 ^b
Plasma membrane integrity (HOST)	39.8 \pm 0.57 ^a	62.0 \pm 1.39 ^b	57.6 \pm 0.99 ^c

Values with different superscripts (^{a,b,c}) within a row differ significantly (Tukey's HSD, $P < 0.05$).

Lipid peroxidation (MDA)

The extent of lipid peroxidation was measured in terms of Malondialdehyde (MDA) and presented in Table 2. In our experiment MDA level was very high during pre-treatment phase (95.83 \pm 6.6 μ mole/10⁹ spermatozoa). However,

following herbal treatment MDA level significantly reduced to 75.39 \pm 1.5 μ mole/10⁹ spermatozoa. During post-treatment (87.47 \pm 2.99 μ mole/10⁹ spermatozoa) level of MDA was similar to pre-treatment phase (95.83 \pm 6.6 μ mole/10⁹ spermatozoa).

Table 2: Effect of herbal treatment on lipid peroxidation (MDA) in semen of subfertile buffalo bulls during all three different phases.

Lipid peroxidation (MDA, μ mole/10 ⁹ spermatozoa)	Pre-treatment	Treatment	Post-treatment
	95.83 \pm 6.67 ^a	75.39 \pm 1.5 ^b	87.47 \pm 2.99 ^a

Values with different superscripts (^{a,b,c}) within a row differ significantly (Tukey's HSD, $P < 0.05$).

Discussion

In the present study, herbal supplementation was given to subfertile Murrah buffalo bulls to improve semen quality. The enhancement of semen quality and reduction in lipid peroxidation (MDA) during treatment and post-treatment phases may be due to the antioxidant and adaptogenic nature of bioactive components of supplemented herbs. Ginsenosides (Saponins) of *Panax ginseng* (Lee *et al.*, 2005; Leung and Wong, 2010; Lee *et al.*, 2017) [23, 25, 24], Humic acid, fulvic acid and Dibenzo Alpha Pyrones of Shilajit (Rege *et al.*, 2015; Sharma *et al.*, 2003; Gallardo *et al.*, 2012) [33, 37, 13], sitoindosides VII-X and withaferin A of *Withania somnifera* (Bhattacharya *et al.*, 1997; Kulkarni *et al.*, 2008) [5, 19], *Ptychopetalum olacoides* (Antunes *et al.*, 2001) [3] and yohimbine of *Pausinystalia yohimbe* (Neha *et al.*, 2017) [28] reduced the lipid peroxidation by elevation of free radicals scavenging enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) levels as reported in the rat, mice and rabbits in chronic disease conditions (Bhattacharya *et al.*, 1997; Dhuley, 1998) [4, 10]. Improvement in semen quality may be due to the improved androgen level in the body due to *Tribulus terrestris*'s active component protodioscin as reported in the primates (Gauthaman *et al.*, 2002; Gauthaman *et al.*, 2003) [14, 15]. Administration of *Turnera diffusa* having Apigenin as an active component, pinocembrin, and acacetin in combination significantly suppressed aromatase activity (Zhao *et al.*, 2008; Kumar *et al.*, 2006) [40, 21]. Aromatase inhibition increases the levels of FSH (Tsjoen *et al.*, 2005) [39] and testosterone in adult men (Ronde and Jong, 2011) [34]. Increased testosterone level will potentially stimulate the sperm production and it is higher in adult buffalo bulls when compared to aged bulls (Javed *et al.*, 2000) [17].

Similar results related to sperm motility have also reported Shivkumar *et al.* (2018) [38]. However, very few scientific reports are available in humans and lab animals on semen quality and lipid peroxidation following supplementation of same herbals in combination. There is no adequate data is available on to compare our findings. So, further studies are needed to corroborate our results.

Conclusion

Based on our study it could be concluded that, the treatment of subfertile buffalo bulls for 60 days with *Panax ginseng* roots, Shilajit, *Withania somnifera* roots, *Tribulus terrestris* fruits, *Turnera diffusa* leaves; *Ptychopetalum olacoides* bark each @ 400 mg/100 kg body weight and *Pausinystalia yohimbe* bark @ 300 mg/100 kg body weight specially in combination improved the semen quality by reducing the lipid peroxidation without any adverse effects.

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