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Shivkumar
Department of Animal Husbandry & Veterinary Services, Government of Karnataka, Veterinary Dispensary Gonal, Yadgir, Karnataka, India

Ajeet Kumar
Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

AK Singh
Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

M Honparkhe
Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

Sumit Singhal
Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

Gurpreet Singh
Department of Veterinary Physiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

Prahlad Singh
Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India

Correspondence
Shivkumar
Department of Animal Husbandry & Veterinary Services, Government of Karnataka, Veterinary Dispensary Gonal, Yadgir, Karnataka, India

Minimization of oxidative stress by oral supplementation of herbs in subfertile buffalo bulls

Shivkumar, Ajeet Kumar, AK Singh, M Honparkhe, Sumit Singhal, Gurpreet Singh and Prahlad Singh

Abstract
The present study was conducted to minimize the oxidative stress in subfertile buffalo bulls by oral supplementation of herbal mixture. The study was conducted in three phases of 60 days each. Three subfertile buffalo bulls were supplemented with herbal mixture containing *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 of bulls. Blood samples were collected once in a week during all the three phases. Lipid peroxidation (MDA), superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) were estimated in blood samples. Herbal supplementation significantly ($P<0.05$) reduced the lipid peroxidation in supplementation (401.18 ± 20.06 µmole/gm Hb) and post-supplementation (259.69 ± 11.35 µmole/gm Hb) phases. The activity of SOD increased during supplementation (982.50 ± 111.63 U/gm Hb/min) and post-supplementation (1632.71 ± 140.57 U/gm Hb/min) phases. GPx activity was significantly ($P<0.05$) increased only during supplementation phase (35.17±6.06 U/gm Hb/min). In conclusion, the supplementation of herbs reduces the oxidative stress in subfertile buffalo bulls.

Keywords: Herbs, oxidative stress, superoxide dismutase, glutathione peroxidase, buffalo bulls

Introduction
Breeding bulls are considered as "half of the herd" and fertility of herd is dependent on the fertility of bull. It has been observed that considerable number of breeding buffalo bulls are being culled at various stages of breeding life due to oxidative stress mediated subfertility problems (Bansal *et al.*, 2011) [1]. Oxidative stress is an increased production of reactive oxygen species in the body (Rahal *et al.*, 2014) [11]. The normal physiological levels of reactive oxygen species are useful for the body growth, sperm maturation, capacitation, acrosome reaction, fertilization and transmembrane cell signaling (Bansal *et al.*, 2011; Rhee 2006) [1, 12]. On the other hand, a higher level of reactive oxygen species causes lipid peroxidation (LPO) of polyunsaturated fatty acid present in spermatogenic (Fujii *et al.*, 2003) [4] and somatic cells (Shamsi *et al.*, 2010) [13] resulting into the subfertility problems.
To counter this, body has an inherent enzymatic (catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase) and non-enzymatic (vitamin C, vitamin E, glutathione, hypotaurine, taurine, and albumin) antioxidant systems to maintain and balance the production and utilization of ROS (Bansal *et al.*, 2011) [1]. However, these enzymes are secreted in small amounts and are insufficient to overcome the problems caused by lipid peroxidation (Nair *et al.*, 2006) [10]. So, to minimize the oxidative stress mediated subfertility problems, *in vitro* supplementation of enzymatic antioxidant (SOD and GPx) and non-enzymatic antioxidants (Vit C, Vit E, zinc, manganese, taurine, hypotaurine and herbals) has been tried with variable results in semen. A very few studies have been carried out blood oxidative stress markers in human beings (Shamsi *et al.*, 2010) [13] but, there were no such studies being found on blood oxidative stress markers in the subfertile buffalo bulls.
Therefore, there is a lack of consolidated scientific information regarding the *in vivo* supplementation of herbs and their effects upon on blood oxidative stress markers. So, based on above facts, this pilot experiment was conducted with the objective to study the blood oxidative stress markers following herbal supplementation, as well as pre and post herbal supplementation in subfertile buffalo bulls.

Materials 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

This study includes three subfertile Murrah buffalo bulls (aged around 5 years and body weight around 700-750 kilograms each) maintained at bull station, GADVASU, Ludhiana, Punjab, India (Latitude/Longitude, 30.55°N, 75.54° E). Bulls were being kept loose in half walled concrete sheds in individual pens (covered area - 12 x 10 ft and uncovered area - 25 x 10 ft). All the animals were being fed according to standard feeding schedule along with ad libitum green fodder. The buffalo bulls were being given an exercise for half an hour on alternate days by manual exerciser. The selected subfertile buffalo bulls was having the history of poor semen quality in terms of pre-freeze individual motility < 70%, post-thaw individual motility < 40%, viability < 70%, total sperm abnormalities > 20%.

Experimental design

Herbs were mixed with concentrate feed as mentioned in the below Table 1 and fed to buffalo bulls for 60 days (supplementation phase). All the animals were examined for physiological parameters (mucous membrane, body temperature, respiration rate and pulse rate) and adverse clinical signs (salivation, lacrimation and sweating) during the experiment.

Table 1: Composition of Herbal supplementation

S. No.	Herbs	Quantity per 100 kg body weight
1	*Panax ginseng roots*	400 mg
2	Shilajit	400 mg
3	*Withania somnifera* root	400 mg
4	*Tribulus terrestris* Fruits	400 mg
5	*Turnera diffusa* leaves	400 mg
6	*Ptychopetalum olacoides* bark	400 mg
7	*Pausinystalia yohimbe* bark	300 mg

Blood collection

Blood was collected in heparinized vials (10 IU heparin/ml blood), once in a week from jugular vein using 18 G needle during pre-supplementation, supplementation and post-supplementation phases. Haemolysate was prepared from all the samples and stored at -80°C until estimation of oxidative stress markers.

Preparation of haemolysate

Preparation of haemolysate was carried out according to Shivkumar (2017) [16].

Procedure

The blood was centrifuged in a graduated tube at 3000 rpm for 15 minutes (37°C) and the level of plasma was marked on the tube. Then, the supernatant (plasma) and buffy coat were discarded. The sedimented erythrocytes were washed thrice with normal saline by adding up to the mark on the tube. The content was gently mixed (8-10 times) and centrifuged at 37°C (3000 rpm for 15 min) for thrice and the supernatant was discarded. To prepare haemolysate, distilled water was added to the sedimented erythrocyte slowly with constant stirring up to the marked level. Prepared haemolysate was stored at -80oC until the estimation of haemoglobin and oxidative stress parameters (MDA, SOD and GPx).

Estimation of hemoglobin

The hemoglobin was estimated by Cyanmethemoglobin method (Shivkumar, 2017) [16].

Preparation of Drabkin's Solution

- Potassium cyanide (Cat # 024638, CDH Pvt. Ltd, India) - 0.05 gm.
- Potassium ferricyanide (Cat # 1648314, SRL Pvt. Ltd, India)- 0.2 gm.
- Sodium bicarbonate (Cat # 1949157, SRL Pvt. Ltd, India) - 1 gm.
- Double distilled water - upto 1 ltr.

Procedure

Test tubes (10 ml) were set on a stand. To each tube, 5 ml of Drabkin's solution and 20 µl of haemolysate were added. Optical density was recorded immediately against distilled water at 540 nm using spectrophotometer (UV-VIS spectrophotometer, systronics, India).

Calculations

Hemoglobin (gm/dl) = Optical density of sample × 36.67

Estimation of oxidative stress markers

To assess the oxidative stress markers in haemolysate, estimations of Malondialdehyde (MDA), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) were carried out as below.

Lipid peroxidation (MDA)

Lipid peroxidation was determined as reported by Shivkumar (2017) [16].

Reagents

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

(Cat # 659605, CDH Pvt. Ltd, India)

Procedure

Reaction mixtures were prepare by adding 200 µl Haemolysate and 200 µl Tris-HCL buffer in a test tube and

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

$$LPO = \frac{OD \times \text{volume of assay mixture}}{\text{Molar extinction coefficient} \times \text{volume of sample taken}}$$

Where:

- The molar extinction coefficient for MDA is 1.56 × 105 M-1 cm-1.
- OD: Optical density of the sample at 532 nm.
- MDA was expressed in µmole/gm Hb.

Superoxide dismutase (SOD)

SOD activity was measured according Shivkumar (2017) [16].

Reagents

- 0.017 M Sodium phosphate buffer (pH 8.3)

Solution I: Dissolved 0.2652 gm of NaH2PO4 (Cat # 22249, SRL Pvt. Ltd. India) in 100 ml distilled water.
Solution II: Dissolved 0.2413 gm Na2HPO4 (Cat # 53046, SRL Pvt. Ltd. India) in 100 ml distilled water.
Solution I (7.36 ml) and solution II (92.64 ml) were mixed and further, diluted to 200 ml with DDW after adjusting pH to 8.3.

- 1.5 mM Nitro Blue Tetrazolium (NBT, Cat # 11207, SRL Pvt. Ltd, India): Dissolved 13.226 mg NBT in 10 ml of distilled water.
- 0.093 mM Phenazonium Methosulphate (PMS, Cat # 024014, CDH Pvt. Ltd, India): Dissolved 2.85 mg PMS in100 ml of distilled water.
- 2.34 mM Nicotinamide adenine dinucleotide (NADH, Cat # 77168, SRL Pvt. Ltd. India): Dissolved 16.6 mg NADH in10 ml of distilled water.

Procedure

The assay mixture of the test was prepared by adding 2.6 ml Sodium phosphate buffer, 100 µl NBT, 100 µl PMS and 100 µl diluted haemolysate (1:100 V/V) in a test tube. The sample was replaced by 100 µl of sodium phosphate buffer in the above assay mixture for control. The reaction was initiated by the addition of 100 µl of NADH (2.34 mM) to both test and control. The two readings of optical density (test and control) were immediately recorded at an interval of 60 sec after adding 0.1 ml of NADH at 560 nm by using spectrophotometer against distilled water as a blank.

$$SOD = \frac{\Delta T \times 2}{\Delta C}$$

Calculations

Where:

- ΔT: Change in OD Test at 60 second interval.
- ΔC: Change in OD Control at 60 second interval.
- The activity of SOD was expressed in U/g Hb/min.

Glutathione peroxidase (GPx)

Glutathione peroxidase activity was determined according to Shivkumar (2017) [16].

Reagents

- 2 mM reduced glutathione (GSH, Cat # 48938, SRL Pvt. Ltd. India): Dissolved 6.146 mg GSH in 10 ml of distilled water.
- 0.4 M Sodium phosphate buffer (pH 7) containing 4 x 10-4 M EDTA: Dissolved 2.835 gm Disodium hydrogen phosphate (Na2HPO4, Cat # 53046, SRL Pvt. Ltd. India), 2.395gm Sodium dihydrogen phosphate (NaH2PO4, Cat # 22249, SRL Pvt. Ltd. India), 5.5 mg EDTA in 30 ml DW and made volume to 50 ml after adjusting pH at 7.
- M Sodium azide (NaN3, Cat # 641335, CDH Pvt. Ltd. India): Dissolved 13.002 mg NaN3 in 20 ml of distilled water.
- 1.25 mM H2O2 (Cat # 845250, CDH Pvt. Ltd. India): Mixed 15 µl H2O2 + 100 ml distilled Water.
- 0.4 M Disodium hydrogen phosphate (Na2HPO4, Cat # 53046, SRL Pvt. Ltd. India): Dissolved 14.175 gm Na2HPO4 in 250 ml of distilled water.
- Metaphospharic acid precipitation solution (MPA): Dissolve 1.67 glacial Metaphospharic acid (Cat # 609605, CDH Pvt. Ltd. India), 200 mg EDTA (Cat # 40088, SRL Pvt. Ltd. India) and 30 gm sodium chloride (Cat # 41721, SRL Pvt. Ltd. India) to make volume up to 100 ml by distilled water.
- 5, 5 dithio bis (2 nitro) benzoic acid (DTNB) reagent: Dissolved 40 mg of DTNB (Cat # 32363, SRL Pvt. Ltd. India) in 100 ml of an aqueous 1% tri-sodium citrate.

Procedure

The reaction mixtures were prepared in separate tubes for the test and control by adding 0.1ml of 1:200 V/V diluted haemolysate, 0.2 ml sodium phosphate buffer (0.4 M, pH 7), 0.2 ml of reduced glutathione (2 mM), 0.1 ml of sodium azide (0.01 M) and 0.2 ml of distilled water. The tubes were incubated at 37oC for 5 mins. Hydrogen peroxide (0.2 ml, 1.25 mM) was added to 'test' and 0.2 ml of distilled water was added to 'control' tubes. Further, both tubes were incubated at 37oC for 3 mins. The reaction was stopped by adding 4 ml of MPA to both the tubes and centrifuged at 6000 rpm for 5 mins. Two ml of the supernatant from test and control tubes were dispensed into two tubes and 2 ml of Na2HPO4 (0.4 M) was added to both tubes. The two readings of optical density were recorded at an interval of 60 sec after adding 0.1 ml of 1 mM of DTNB at 420 nm by using spectrophotometer against distilled water as a blank.

Calculations

GPx activity= (10 (logT –logC)) / Hb × 200

Where:

- ΔT: change in OD Test at 60 second interval.
- ΔC: change in OD Control at 60 second interval.
- The GPx activity was expressed in U/gm Hb/min.

Statistical analyses

All data are presented as the mean ± standard error. Normality of data and Homogeneity of variance was analysed by the Shapiro-Wilk Test and Levene's test, respectively. Statistical analysis of data was analyzed by one way ANOVA using the IBM SPSS Statistics version 22. The comparison

between the groups was done with Tukey's HSD post hoc test at P<0.05.

Results

Estimation of oxidative stress markers such as MDA, SOD and GPx in blood gives the overall oxidative status of body cells. So, in our experiment we have supplemented the herbs and estimated the oxidative stress markers in blood and results obtained are discussed below.

Table 2: Effects of herbal supplementation on oxidative stress markers in blood (Mean ± SE) of subfertile buffalo bull during pre-supplementation, supplementation and post- supplementation phases.

Blood Oxidative markers	Herbal supplementation phases		
	Pre-supplementation	Supplementation	Post-supplementation
Lipid peroxidation (MDA, µmol / gm Hb)	612.12±50.15[a]	401.18±20.06[b]	259.69±11.35[c]
Superoxide dismutase (U/ gm Hb/min)	305.69±52.63[a]	982.50±111.63[b]	1632.71±140.57[c]
Glutathione peroxidase (U/gm Hb/min)	12.13±1.52[a]	35.17±6.06[b]	20.12±3.33[a,b]

Values with different superscripts ([a,b,c]) within a column differ significantly (Tukey' HSD,P<0.05).

Lipid peroxidation (MDA)

The average lipid peroxidation (MDA) in blood during different phases is presented in Table 2. Herbal treatment significantly (P<0.05) reduced the blood MDA level during supplementation (401.18 ± 20.06 µmole/gm Hb) and post-supplementation (259.69 ± 11.35 µmole/gm Hb) phases as compared to pre-supplementation phase (612.12 ± 50.15 µmole/gm Hb).

Superoxide dismutase (SOD)

The average activities of blood SOD during different phases are presented in Table 2. SOD activity in blood during pre-supplementation phase was very low (305.69 ± 52.63 U/gm Hb/min). Following herbal supplementation, the activity of blood SOD significantly (P<0.05) increased to 982.50 ± 111.63 U/gm Hb/min and 1632.71 ± 140.57 U/gm Hb/min in supplementation and post-supplementation phases, respectively.

Glutathione peroxidase (GPx)

The average activity of blood GPx during different phases is presented in Table 7 and Fig. 11. The activity of GPx in blood during pre-supplementation phase was low (12.13 ± 1.52 U/gm Hb/min), which increased significantly (P<0.05) following herbal supplementation to 35.17 ± 6.06 U/gm Hb/min. After the discontinuation of herbal supplementation, GPx activity of post-supplementation phase (20.12 ± 3.33 U/gm Hb/min) was similar to pre- supplementation phase.

Discussion

Results of our study indicated the strong antioxidant and residual effects of herbal supplementation. Similar results are published by Shivkumar (2017) [16] and Shivkumar *et al.* (2018) [15] in semen of subfertile buffalo bulls and also by Kim *et al.* (2011) [7] in serum of human being by supplementation of *Panax ginseng* in serum of humans.

Contrary to our findings, administrations of *Panax ginseng* in men and women did not significantly increase the serum SOD and GPx activity (Kim *et al.*, 2011) [7].

As extent of lipid peroxidation (MDA) and antioxidant activity (SOD and GPx) of blood plays a decisive role in the maintenance of fertility by protection of spermatozoa from oxidative damage (Shamsi *et al.,* 2010) [13]. It has been observed that blood superoxide dismutase has a positive correlation with the sperm count and negative correlation with the abnormal and dead spermatozoa in humans (Shamsi *et al.*, 2010) [13]. Glutathione peroxidase (GPx) is an antioxidant enzyme and it protects the sperm against peroxidative damage (Bilodeau *et al.,* 2001) [3].

In our study, improvement of blood antioxidants (SOD and GPx) and decreased MDA level may be due to the active metabolites of individual herbs such as *Withania somnifera* (Sitoindosides VII-X and Withaferin A), Shilajit (Dibenzo Alpha Pyrones, Humic acid and Fulvic acid), *Tribulus terrestris* (Protodioscin) (Ghosal 1990; Bhattacharya *et al.*, 1997; Gauthaman *et al.*, 2002; Sharma *et al.*, 2003) [6, 2, 5,] and *Turnera diffusa* (Apigenin) (Kumar *et al.*, 2006; Kumar *et al.*, 2008) [9, 8].

This was the first pilot study conducted on the blood oxidative stress markers to evaluate the effect of herbal supplementation on blood oxidative stress markers in subfertile buffalo bulls. However, recently a similar herbal supplementation study was published on semen of subfertile buffalo bulls by Shivkumar *et al.* (2018) [15] but, no reports are available on blood oxidative stress markers before, during and after supplementation of herbs in combination (*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) in humans and animals to compare our findings. This pilot experiment strongly recommends the further detailed study regarding herbal supplementation and its impacts on the blood oxidative markers and their relation with fertile and subfertile bulls.

Conclusion

Based on our study it could be concluded that, the herbal supplementation having mixture of *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 to the subfertile buffalo bulls for 60 days improved the blood superoxide dismutase and glutathione peroxidase activity. Thus increased activity of SOD and GPx reduced the extent of lipid peroxidation in blood of subfertile buffalo bulls without any adverse effects.

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