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; Pycnogenol to attenuate oxidative stress markers in semen. A very few studies have been carried out blood oxidative stress markers in buffalo 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) [19] and somatic cells (Shamsi et al., 2010) [15], 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) [11]. 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 herbs) 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) [15] 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>
<thead>
<tr>
<th>S. No.</th>
<th>Herbs</th>
<th>Quantity per 100 kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Panax ginseng roots</td>
<td>400 mg</td>
</tr>
<tr>
<td>2</td>
<td>Shilajit</td>
<td>400 mg</td>
</tr>
<tr>
<td>3</td>
<td>Withania somnifera root</td>
<td>400 mg</td>
</tr>
<tr>
<td>4</td>
<td>Tribulus terrestris Fruits</td>
<td>400 mg</td>
</tr>
<tr>
<td>5</td>
<td>Turnera diffusa leaves</td>
<td>400 mg</td>
</tr>
<tr>
<td>6</td>
<td>Ptychopetalum olacoides bark</td>
<td>400 mg</td>
</tr>
<tr>
<td>7</td>
<td>Pausinystalia yohimbe bark</td>
<td>300 mg</td>
</tr>
</tbody>
</table>

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)[10].

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 -80°C until the estimation of haemoglobin and oxidative stress parameters (MDA, SOD and GPx).

Estimation of haemoglobin

**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)[10].

**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 × 10^5 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 Na2HPO4 (Cat # 53046, SRL Pvt. Ltd, India) in 100 ml of distilled water.

Solution II: Dissolved 0.2413 gm Na2HPO4 (Cat # 53046, SRL Pvt. Ltd, India) in 100 ml of 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 in 100 ml of distilled water.
- 2.34 mM Nicotinamide adenine dinucleotide (NADH, Cat # 77168, SRL Pvt. Ltd, India): Dissolved 16.6 mg NADH in 10 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:
- \( \Delta T \): Change in OD Test at 60 second interval.
- \( \Delta C \): Change in OD Control at 60 second interval.
- The activity of SOD was expressed in U/gm Hb/min.

Glutathione peroxidase (GPx)
Glutathione peroxidase activity was determined according 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.
- 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.
- Metaphosphoric acid precipitation solution (MPA): Dissolve 1.67 glacial Metaphosphoric 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 37°C 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 37°C 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 = \((10 \times (\log T - \log C)) / Hb \times 200\)

Where:
- \( \Delta T \): change in OD Test at 60 second interval.
- \( \Delta C \): 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.

<table>
<thead>
<tr>
<th>Blood Oxidative markers</th>
<th>Herbal supplementation phases</th>
<th>Pre-supplementation</th>
<th>Supplementation</th>
<th>Post-supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxidation (MDA, μmol / gm Hb)</td>
<td>612.12±50.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>401.18±20.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>259.69±11.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase (U/gm Hb/min)</td>
<td>305.69±52.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>982.50±111.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1632.71±140.57&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (U/gm Hb/min)</td>
<td>12.13±1.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.17±6.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.12±3.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values with different superscripts (abc) 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 μmol/gm Hb) and post-supplementation (259.69 ± 11.35 μmol/gm Hb) phases as compared to pre-supplementation phase (612.12 ± 50.15 μmol/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 2. GPx activity of glycowithanolides from Withania somnifera (Sitostanesides 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) and Turnera diffusa (Apigenin) (Kumar et al., 2006; Kumar et al., 2008) [k, 5, 8]. 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 (Sitostanesides 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) and Turnera diffusa (Apigenin) (Kumar et al., 2006; Kumar et al., 2008) [k, 5, 8].

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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; Pychopetalum 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; Pychopetalum olacoides bark each @ 400 mg/100 kg body weight and Pausinystalia yohimbe bark @ 300 mg/100 kg body weight 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.

Acknowledgements

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References


