

*Original Research***Effects of Dietary Supplementation of Herbs on Semen Quality and Oxidative Stress in Subfertile Buffalo Bulls****S. Kumar<sup>1\*</sup>, A. Kumar<sup>2</sup>, M. Honparkhe<sup>2</sup>, A. K. Singh<sup>2</sup>, P. Malhotra<sup>2</sup>, V. K. Gandotra<sup>2</sup> and P. Singh<sup>3</sup>**<sup>1</sup>Department of Veterinary Clinical Complex, Veterinary College Gadag - 582101, Karnataka, INDIA<sup>2</sup>Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana - 141 004, Punjab, INDIA<sup>3</sup>Directorate of Livestock Farms, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana - 141 004, Punjab, INDIA**\*Corresponding author:** [kamthaneshivkumar@gmail.com](mailto:kamthaneshivkumar@gmail.com)

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**Abstract**

The study was conducted to assess the combined effects of herbs on semen quality and oxidative stress in subfertile buffalo bulls (*Bubalus bubalis*). A total of 144 semen ejaculates (16 ejaculates/bull) were collected from 3 subfertile buffalo bulls following oral supplementation 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 of bulls for 60 days. Semen was evaluated for quality and oxidative stress (lipid peroxidation, superoxide dismutase and glutathione peroxidase) during pre-supplementation, supplementation and post-supplementation phases (each phase of 60 days). Herbal supplementation significantly ( $P<0.05$ ) improved mass motility, individual motility, rapid progressive motility, curvilinear velocity, sperm concentration, viability and active mitochondria. Total sperm abnormalities and amplitude of lateral head displacement were significantly ( $P<0.05$ ) reduced during supplementation and post-supplementation phases. The level of malondialdehyde was significantly ( $P<0.05$ ) declined during supplementation and post-supplementation phases. The activity of superoxide dismutase significantly ( $P<0.05$ ) increased during supplementation and post-supplementation phases, whereas the activity of glutathione peroxidase was significantly ( $P<0.05$ ) increased only during the supplementation phase. It could be concluded that feeding of herbs in combination improved the semen quality and antioxidants activity in subfertile buffalo bulls.

**Key words:** Herbal Supplementation, Oxidative Stress, Semen Quality, Subfertile Buffalo Bulls**How to cite:** Kumar, S., Kumar, A., Honparkhe, M., Singh, A., Malhotra, P., Gandotra, V., & Singh, P. (2018). Effects of Dietary Supplementation of Herbs on Semen Quality and Oxidative Stress in Subfertile Buffalo Bulls. International Journal of Livestock Research, 8(7), 56-66. doi: 10.5455/ijlr.20171016013807

## Introduction

Poor semen quality is one of the major reasons for the culling of breeding bulls (Mukhopadhyay *et al.*, 2010). There are several factors (temperature, humidity, physical, physiological and nutritional stressors), which contribute to the poor semen quality (Agarwal *et al.*, 2005; Wellen *et al.*, 2010; Balic *et al.*, 2012). Under stressful conditions, there is an excessive production of reactive oxygen species (ROS) in semen. This leads to an imbalance between endogenous ROS production and its neutralization through scavenging activities in spermatozoa (Agarwal *et al.*, 2014). ROS is beneficial at normal physiological level by promoting sperm production, maturation, capacitation, acrosome reaction and fertilization processes (Bansal *et al.*, 2011). On the other hand, increased ROS level results into lipid peroxidation of polyunsaturated fatty acid present in sperm membrane (Fujii *et al.*, 2003).

The ROS production and scavenging activities in spermatozoa are carried out by inherent enzymatic and non-enzymatic antioxidant system (Bansal *et al.*, 2011). Moreover, buffalo bull sperm has higher polyunsaturated fatty acids and lower antioxidant enzymes as compared to cattle bull sperm (Nair *et al.*, 2006). Therefore, buffalo bull sperms are more prone to ROS-mediated lipid peroxidative damages (Nair *et al.*, 2006) leading to decreased fertility by increasing sperm abnormality and DNA fragmentation (Kadirvel *et al.*, 2009). It also lowers the sperm concentration, motility, viability, epididymal maturation, sperm mitochondrial membrane potential and even outcome of assisted reproductive techniques (Oral *et al.*, 2006; Shamsi *et al.*, 2010). ROS-mediated sperm damage occurs at two stages i.e. during spermatogenesis and freeze-thawing process (Agarwal *et al.*, 2014). The damages caused during freezing and thawing can be minimized by *in vitro* supplementation of enzymatic and non-enzymatic antioxidants (Walczak-Jedrzejowska *et al.*, 2013). One of the major limitations of *in vitro* supplementation of antioxidants is the inability of sperm to restore ROS-mediated damages during spermatogenesis as spermatozoa are terminally developed germ cells. Hence, there is need to supplement some antioxidants *in vivo*, which can minimize the ROS-mediated lipid peroxidative damages inflicted to spermatozoa during spermatogenesis and sperm maturation. In our study, oral supplementation of herbs viz. *Panax ginseng roots*, *Shilajit*, *Withania somnifera roots*, *Tribulus terrestris fruits*, *Turnera diffusa leaves*, *Ptychopetalum olacoides bark* and *Pausinystalia yohimbe bark* have been made to improve semen quality and antioxidant activity in subfertile buffalo bulls. The present work was planned with the objective to study the impact of *in vivo* herbal supplementation on semen quality and oxidative stress in subfertile buffalo bulls.

## Material and Methods

### Ethical Approval

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

## Procurement of Herb and Chemicals

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

## Experimental Animals

The present study was conducted on 3 subfertile buffalo bulls (aged around 5 years and 700-750 kilograms of body weight each) were having a history of poor semen quality (pre-freeze individual motility < 60%, post-thaw individual motility < 40%, viability < 70% and total sperm abnormalities > 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. The semen collection was being done twice a week with artificial vagina method.

## Experimental Design and Semen Collection

The experiment was comprised of three phases viz. pre-supplementation, supplementation and post-supplementation phases of 60 days each. During the supplementation phase, buffalo bulls were orally supplemented with herbal mixture daily (*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). 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. Semen was collected twice a week per bull from the three subfertile buffalo bulls during pre-supplementation, supplementation and post-supplementation phases of the experiment. Following semen collection, a total of 144 semen ejaculates (16 ejaculates/ bull/ supplementation phase) were subjected to the assessment of semen quality and oxidative stress parameters.

## Assessment of Semen Quality

### Semen Volume

Semen volume was noted directly from the graduated semen collection tube.

### Mass Motility

Mass motility was recorded (0-5 scale) according to Kumar *et al.* (2004) by placing a small drop of neat semen on pre-warmed glass slide without a coverslip under phase contrast microscope (10x) attached with warm stage (Nikon Eclipse E600, Tokyo, Japan).

### Sperm Concentration

The concentration of spermatozoa in the neat semen was determined by the pre-calibrated Bovine Photometer (Accucell Photometer, IMV Technologies, France). For Bovine photometry, semen sample (40  $\mu$ l) was diluted with normal saline 0.9% w/v (3960  $\mu$ l) using automatic semen dilutor (Hamilton Semen Dilutor, IMV Technologies, France). For dilution of a semen sample, foot paddle of auto-dilutor was pressed first to suck 20  $\mu$ l of air, then again pressed to suck semen (40 $\mu$ l) in the pipette. Finally, semen along with 3960  $\mu$ l of normal saline was dispensed in a disposable cuvette. This cuvette was placed into cuvette holder of the photometer. Information regarding ejaculate was entered into photometer to get the concentration in million/ml.

### Sperm Motion Traits

Sperm motion traits were analyzed by Computer Assisted Sperm Analysis (CASA) software (Biovis CASA 2000, Expert Vision Labs, India) according to Kumar *et al.* (2004). The sperm concentration of sample was adjusted to 20 million/ml using Tris egg yolk extender consisting of 20% egg yolk, 80% Tris buffer with 7% glycerol (Cat # 20118, SD Fine-chem. Limited, India) and 50  $\mu$ g/ml of Gentamicin (Cat # 15710064, Gibco, Life Technologies). Sperm motion traits were evaluated by placing a 5  $\mu$ l drop of diluted semen in pre-warmed Shukratara chamber (Biovis, Expert Vision Labs, India) under phase contrast microscope. The individual motility (%); rapid progress motility (%); slow progressive motility (%), curvilinear velocity (VCL,  $\mu$ m/sec); average path velocity (VAP,  $\mu$ m/sec); straight line velocity (VSL,  $\mu$ m/sec); linearity (LIN, %); Straightness (STR, %); wobble (WOB, %); beat cross frequency (BCF, Hz); amplitude of lateral head displacement (ALH,  $\mu$ m); Dance (DNC,  $\mu$ m<sup>2</sup>/sec), mean dance (DNM,  $\mu$ m<sup>2</sup>/sec) were analysed.

### Sperm Viability

The live sperm count was assessed by Eosin-Nigrosin stain (Kumar *et al* 2004). Twenty  $\mu$ l of semen sample was mixed with 80  $\mu$ l Eosin-nigrosin stain (5% Eosin and 10% Nigrosin stain prepared in 2.9% sodium citrate solution). Semen 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). 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) using tetramethylrhodamine methyl ester fluorescent dye (Cat # T-668, Life Technologies). Semen sample (250  $\mu$ l) was washed twice with PBS by centrifuging at 1000 rpm for 5 mins (37°C). Then, 5  $\mu$ l of working TMRM solution (50  $\mu$ M) 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  $\mu$ l of PBS. 10  $\mu$ l of washed sample and 8  $\mu$ l 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.

### **Assessment of Oxidative Stress**

#### **Extraction of Oxidative Stress Markers**

Sperm oxidative stress markers were extracted according to Castro *et al.* (2016) with some modifications. Briefly, 1.5 ml of extended semen (80 million/ml) was given two washing with 1.0 ml Phosphate-buffered saline (pH 7.4) at 37°C (6000 rpm for 10 mins). The sperm pellet was dissolved in 1 ml of 1% Tritone and agitated 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 (-80°C) for further assessment of oxidative stress markers such as MDA, SOD and GPx as mentioned below.

#### **Estimation of Malondialdehyde (MDA) Level**

The Lipid peroxidation in sperm was determined by the estimation of MDA (Buege *et al.*, 1978). Briefly, 100  $\mu$ l each of extracted semen sample and 150 mM Tris-HCl buffer (pH 7.1) were taken in a test tube and incubated at 37°C for 20 min. To this tube, 1 ml of 10% chilled Trichloroacetic acid and 2 ml of 0.375% Thiobarbituric acid were added and kept in boiling water for 20 mins. Thereafter, the mixture was cooled and centrifuged at 10,000 rpm at 37°C for 5 min. The supernatant was pipetted into a cuvette and optical density was recorded at 532 nm by using spectrophotometer (UV-VIS spectrophotometer, systronics, India) against distilled water as a blank.

## Calculations

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

Where, molar extinction coefficient for MDA is  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

## Assessment of Superoxide Dismutase (SOD) Activity

The SOD activity was measured by the method of Nishikimi *et al.* (1972). Following reaction mixtures were prepared (test and control): 2.6 ml of sodium phosphate buffer (0.017 M, pH 8.3), 100  $\mu\text{l}$  of nitro blue tetrazolium (1.5 mM) and 100  $\mu\text{l}$  of Phenazine methosulphate (0.093 mM) and mixed well. After that, 100  $\mu\text{l}$  each of extracted semen sample and phosphate buffer were added to test and control tubes, respectively. The reaction was initiated by the addition of 100  $\mu\text{l}$  of Nicotinamide adenine dinucleotide (2.34 mM) to both test and control tubes. Two readings of absorbance of the test and control mixtures were immediately recorded against distilled water (blank) at an interval of 60 seconds by using spectrophotometer at 560 nm.

## Calculations

$$SOD \text{ activity (IU}/10^9\text{spermatozoa}/\text{min}) = \frac{\Delta T \times 2}{\Delta C}$$

Where,  $\Delta T$ : change in  $OD_{\text{Test}}$  at 60 second interval;  $\Delta C$ : change in  $OD_{\text{Control}}$  at 60 second interval.

## Assessment of Glutathione Peroxidase (GPx) Activity

The glutathione peroxidase activity was determined by the method of Hafeman *et al.* (1974). The reaction mixtures for test and control consists of 0.1ml of extracted semen sample, 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 distilled water in two separate tubes. The tubes were incubated at  $37^{\circ}\text{C}$  for 5 mins. Hydrogen peroxide (0.2 ml, 1.25 mM) was added to 'test' and 0.2 ml distilled water was added to control tubes. Further, both tubes were incubated at  $37^{\circ}\text{C}$  for 3 mins. The reaction was stopped by adding 4 ml of Metaphosphoric acid precipitation solution to both the tubes and centrifuged at 6000 rpm for 5 mins. Two ml of the supernatant from test and control tubes were aspirated into two tubes and 2 ml Disodium phosphate (0.4 M) was added to both tubes. Two readings of optical density were recorded at 420 nm an interval of 60 sec after adding 0.1 ml of 5, 5-dithio-bis-2-nitrobenzoic acid (1 mM) against distilled water as a blank.

## Calculations

$$GPx \text{ activity (IU/10}^9 \text{ spermatozoa/min)} = \Delta T - \Delta C$$

Where,  $\Delta T$ : change in OD<sub>Test</sub> at 60 second interval;  $\Delta C$ : change in OD<sub>Control</sub> at 60 second interval.

## 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 Levene's test. Data were analyzed by General Linear Univariate Model with the bull as a random factor followed by Tukey'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

The effects of herbal supplementation on semen quality parameters are presented (mean  $\pm$  standard error) in Table 1.

**Table1:** Effects of herbal supplementation on semen quality parameters (Mean $\pm$ SE) of subfertile buffalo bulls during pre-supplementation, supplementation and post-supplementation phases

Semen Quality Parameters	Phases		
	Pre-supplementation	Supplementation	Post-supplementation
Volume (ml)	3.90 $\pm$ 0.19 <sup>a</sup>	4.13 $\pm$ 0.23 <sup>a</sup>	4.22 $\pm$ 0.20 <sup>a</sup>
Mass motility	3.27 $\pm$ 0.13 <sup>a</sup>	4.10 $\pm$ 0.16 <sup>b</sup>	3.47 $\pm$ 0.16 <sup>a</sup>
Sperm concentration (million/ml)	793.27 $\pm$ 68.25 <sup>a</sup>	1106.70 $\pm$ 68.69 <sup>b</sup>	981.53 $\pm$ 94.16 <sup>a,b</sup>
Viability (%)	70.30 $\pm$ 1.20 <sup>a</sup>	88.60 $\pm$ 0.95 <sup>b</sup>	85.30 $\pm$ 0.92 <sup>b</sup>
Total sperm abnormalities (%)	23.73 $\pm$ 1.06 <sup>a</sup>	10.03 $\pm$ 1.00 <sup>b</sup>	17.23 $\pm$ 2.54 <sup>c</sup>
Mitochondrial membrane activity (%)	56.54 $\pm$ 5.27 <sup>a</sup>	78.92 $\pm$ 2.42 <sup>b</sup>	62.07 $\pm$ 2.67 <sup>a</sup>

Values with different superscripts (<sup>a,b,c</sup>) within a row differ significantly (Tukey's HSD,  $P < 0.05$ ).

Semen volume during different phases of supplementation was similar ( $P > 0.05$ ). Mass motility, sperm concentration and percent active mitochondria were significantly ( $P < 0.05$ ) improved during the supplementation phase. The percentage of sperm viability significantly ( $P < 0.05$ ) increased during supplementation and post-supplementation phases. Further, total sperm abnormalities were significantly ( $P < 0.05$ ) reduced during supplementation and post-supplementation phases.

### Sperm Motion Traits

The results of sperm motion traits are presented (mean  $\pm$  standard error) in Table 2. The sperm motion traits such as individual motility, rapid progressive motility, and VCL were significantly ( $P < 0.05$ ) higher and ALH and DNC were significantly ( $P < 0.05$ ) reduced during supplementation phase as compared to pre-supplementation phase. Further, VCL, VSL, STR, BCF were significantly ( $P < 0.05$ ) increased and ALH, DNC and DNM were significantly ( $P < 0.05$ ) reduced during post-supplementation phase as

compared to pre-supplementation phase. The remaining sperm motion traits such as slow progressive motility, VAP, LIN, and WOB were similar in all the three phases.

**Table 2:** Effects of herbal supplementation on sperm motion traits (Mean ± SE) in semen of subfertile buffalo bulls during pre supplementation, supplementation and post-supplementation phases

Sperm Motion Traits	Phases		
	Pre-supplementation	Supplementation	Post-supplementation
Motile (%)	57.72 ± 5.53 <sup>a</sup>	73.55 ± 2.89 <sup>b</sup>	62.01 ± 3.05 <sup>a</sup>
Rapid Prog (%)	25.17 ± 5.48 <sup>a</sup>	34.36 ± 3.00 <sup>b</sup>	31.91 ± 3.67 <sup>a,b</sup>
Slow Prog (%)	22.82 ± 2.73 <sup>a</sup>	31.47 ± 3.53 <sup>a</sup>	26.05 ± 2.70 <sup>a</sup>
VCL (um/sec)	71.85 ± 5.20 <sup>a</sup>	90.85 ± 2.21 <sup>b</sup>	86.11 ± 19.16 <sup>b</sup>
VAP (um/sec)	47.76 ± 4.37 <sup>a</sup>	52.53 ± 3.64 <sup>a</sup>	54.58 ± 20.57 <sup>a</sup>
VSL (um/sec)	43.39 ± 4.55 <sup>a</sup>	47.44 ± 3.61 <sup>a,b</sup>	51.78 ± 3.81 <sup>b</sup>
LIN (%)	59.60 ± 3.71 <sup>a,b</sup>	65.72 ± 2.16 <sup>b</sup>	59.08 ± 1.50 <sup>a</sup>
STR (%)	84.63 ± 1.92 <sup>a</sup>	87.72 ± 1.57 <sup>a</sup>	92.49 ± 0.49 <sup>b</sup>
WOB (%)	68.28 ± 3.74 <sup>a,b</sup>	73.22 ± 2.07 <sup>a</sup>	62.58 ± 1.42 <sup>b</sup>
BCF (Hz)	10.35 ± 0.54 <sup>a</sup>	14.40 ± 1.28 <sup>a</sup>	19.28 ± 0.93 <sup>b</sup>
ALH (um)	6.18 ± 0.54 <sup>a</sup>	4.57 ± 0.36 <sup>b</sup>	3.26 ± 0.26 <sup>c</sup>
DNC (squm/sec)	390.77 ± 69.13 <sup>a</sup>	251.53 ± 20.67 <sup>b</sup>	231.38 ± 12.50 <sup>b</sup>
DNM (squm/sec)	10.85 ± 0.80 <sup>a</sup>	7.35 ± 0.76 <sup>a,b</sup>	6.54 ± 1.09 <sup>b</sup>

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

### Oxidative Stress

The results of oxidative stress during different phases of supplementation are presented (mean ± standard error) in Table 3.

**Table 3:** Effects of herbal supplementation on MDA level and antioxidant activity (SOD and GPx) in semen of subfertile buffalo bulls during pre- supplementation, supplementation and post-supplementation phases

Semen oxidative parameters	Phases		
	Pre-supplementation	Supplementation	Post-supplementation
Malondialdehyde (µmole/10 <sup>9</sup> spermatozoa)	51.52 ± 1.57 <sup>a</sup>	38.42 ± 1.67 <sup>b</sup>	40.22 ± 0.78 <sup>b</sup>
Superoxide dismutase (IU/10 <sup>9</sup> spermatozoa/min)	166.10 ± 3.15 <sup>a</sup>	193.28 ± 5.01 <sup>b</sup>	193.65 ± 4.09 <sup>b</sup>
Glutathione peroxidase (IU/10 <sup>9</sup> spermatozoa/min)	0.49 ± 0.02 <sup>a</sup>	0.93 ± 0.09 <sup>b</sup>	0.69 ± 0.12 <sup>a,b</sup>

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

The level of MDA was significantly (P<0.05) reduced in supplementation and post-supplementation phases. The activity of SOD was significantly (P<0.05) increased during supplementation and post-supplementation phases as compared to pre-supplementation phase. GPx activity was significantly (P<0.05) higher during the supplementation phase.

### Discussion

To the author's knowledge, this is the first study conducted to improve semen quality and antioxidant activity in subfertile buffalo bulls following oral supplementation of herbal mixture containing *Panax*

*ginseng* roots, Shilajit, *Withania somnifera* roots, *Tribulus terrestris* fruits, *Turnera diffusa* leaves, *Ptychopetalum olacoides* bark and *Pausinystalia yohimbe* bark daily for 60 days. In our study, herbal supplementation significantly improved the mass motility, sperm concentration, viability, normal sperm morphology, percent active mitochondria and sperm motion traits (individual motility, rapid progressive motility, VCL, VSL, STR, BCF, ALH, DNC and DNM). Moreover, we also found enhanced activity of antioxidants (SOD and GPx) and declined lipid peroxidation of sperm plasma membrane indicated by MDA level. However, no such study has been conducted to compare our results. Some discrete information are available regarding the individual use of herbs in small animals and humans (Kopalli *et al.*, 2015; Biswas *et al.*, 2009; Ambiye *et al.*, 2013; Adaay *et al.*, 2012; Ferrini *et al.*, 2015; Kumar *et al.*, 2008; Neha *et al.*, 2017). In our study, the improvement in semen quality and antioxidant activity might be due to the antioxidant function of active metabolites like Ginsenosides of *Panax ginseng* (Leung *et al.*, 2013), humic acid, fulvic acid and Dibenzo Alpha Pyrones of Shilajit (Sharma *et al.*, 2003; Ghosal, 1990), sitoindosides VII-X and withaferin A of *Withania somnifera* (Bhattacharya *et al.*, 1997), protodioscin of *Tribulus terrestris* (Gauthaman *et al.*, 2002), Apigenin of *Turnera diffusa* (Kumar *et al.*, 2006), *Ptychopetalum olacoides* (Antunes *et al.*, 2001) and yohimbine of *Pausinystalia yohimbe* (Neha *et al.*, 2017). In our study, there was higher activity of antioxidants such as SOD and GPx. SOD converts superoxide anion ( $O_2^-$ ) into  $O_2$  and  $H_2O_2$ , while glutathione peroxidase converts  $H_2O_2$  to  $O_2$  and  $H_2O$  (Birben *et al.*, 2012). Thus, ROS level in semen might have reduced to the normal physiological level during spermatogenesis and maturation thereby improved the semen quality and sperm motion traits.

## Conclusion

Oral supplementation of herbal mixture (*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 300 mg/100 kg body weight of *Pausinystalia yohimbe* bark) for 60 days to subfertile buffalo bulls, significantly improved semen quality and antioxidant activity without any adverse effects.

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## Conflict of Interest

The authors declare no conflict of interest.

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