Int.J.Curr.Microbiol.App.Sci (2018) *7*(3): 2543-2550

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 03 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.703.294

Effects of Dietary Herbal Supplementation on CASA Based Sperm Motion Traits in Subfertile Buffalo Bulls (*Bubalus bubalis*)

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Keywords

Subfertile buffalo bulls, Herbs, Pre-freeze, Post-thaw, CASA, Sperm motion traits

Article Info

Accepted:
20 February 2018
Available Online:
10 March 2018

A B S T R A C T

The study was conducted to evaluate the combined effect of herbs on pre-freeze and post-thaw sperm motion traits in subfertile buffalo bulls. Three subfertile buffalo bulls which were maintained on the basal ration along with this, they were orally supplemented 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 of bulls for 60 days of treatment phase. A total of 144 semen ejaculates (16 ejaculates/ bull/ treatment phase) were collected during pre-treatment, treatment and post-treatment phase (each phase of 60 days). Semen was extended with Tris egg yolk extender and freezing was carried out in a bio-freezer. Semen was evaluated for sperm motion traits in pre-freeze and post-thaw semen by Computer Assisted Sperm Analyser (CASA). Herbal supplementation significantly ($P<0.05$) increased the pre-freeze curvilinear velocity (VCL) during treatment phase and post-treatment phase, whereas VSL, STR and BCF were significantly ($P<0.05$) increased only during post-treatment phase. Moreover, herbal treatment significantly ($P<0.05$) improved the post-thaw individual motility during the treatment and post-treatment phase, whereas rapid progressive motility was significantly higher only during the treatment phase. It could be concluded that dietary supplementation of herbs in combination improved the sperm motion traits in subfertile buffalo bulls.

Introduction

Artificial insemination is expanding day by day to improve the production and genetic potential of indigenous cows. The success and efficiency of the artificial insemination primarily depends on semen quality of breeding bulls (Sharma *et al.*, 2017). The quality of semen depends on many factors such as genetic, climatic, physiological and nutritional status (Parisi *et al.*, 2014; Andrabi *et al.*, 2009; Koonjaenak *et al.*, 2007). Among these climatic factors, extreme temperature and humidity are producing excessive quantity of reactive oxygen species in the body cells and spermatozoa (Sharma *et al.*, 2017). Therefore, the poor semen quality and freezability are major problems in breeding

buffalo bulls (Khatun *et al.,* 2013). Reactive oxygen species are the oxygen-based highly reactive molecules (superoxide anion, hydrogen peroxide, and hydroxyl ion) (Krumova *et al.,* 2016), which have double pronged effects on sperm.

The normal physiological levels of ROS promote sperm motility, maturation, capacitation, acrosome reaction, fertilization and transmembrane signal transduction [Bansal and Bilaspuri, 2011; Rhee, 2006). On the other hand higher levels of ROS, causes lipid peroxidation (LPO) of polyunsaturated fatty acid present in sperm membrane (Shamsi *et al.,* 2010; Vaisberg *et al.,* 2005; Fujii *et al.,* 2003). Lipid peroxidation decreases the fertility by increasing the sperm abnormality and DNA fragmentation. It also lowers the sperm concentration, motility, viability, epididymal maturation, sperm mitochondrial membrane potential and even outcome of assisted reproductive techniques (Fujii *et al.,* Benedetti *et al.,* 2012; Shamsi *et al.,* 2010; Tunc *et al.,* 2009; Kadirvel *et al.,* 2009; Cheema *et al.,* 2009; Oral *et al.,* 2006; Greco *et al.,* 2005; Chatterjee *et al.,* 2001).

Therefore to alleviate subfertility problems, several therapeutic approaches such as allopathy, ayurveda and unani are being used. Moreover, evidences suggest that dietary supplementation of herbs improves semen quality in animals and human.

So, in this experiment the subfertile bulls were orally supplemented with *Panax ginseng* roots, Shilajit, *Withania somnifera* root, *Tribulus terrestris* Fruits, *Turnera diffusa* leaves, *Ptychopetalum olacoides* bark and *Pausinystalia yohimbe* bark. Our goal was to study the changes in the sperm motion traits of fresh and post-thaw semen of subfertile buffalo bull during the period of dietary supplementation herbs and as well as pre and post supplementation.

Materials and Methods

Ethical approval

The experiment was carried out 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%).

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

The trial was comprised of three phases viz. pre-treatment, treatment and post-treatment phase of 60 days each. During the treatment 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 during pre-treatment, treatment and post-treatment phases. A total of 144 semen ejaculates (16 ejaculates/bull/ phase) were subjected for the study.

Assessment of sperm motion traits

Sperm motion traits were studied according to Kumar *et al.*, (2004) by using Computer Assisted Sperm Analysis software (Biovis CASA 2000, version 4.59, Expert Vision Labs Pvt. Limited, India) with the set up as shown in table 2. The sperm concentration of the sample was adjusted to 20 million sperms/ml using Tris egg yolk extender and Tris buffer for pre-freez and post-thaw semen, respectively. A drop (5 µl) of diluted semen was placed in a pre-warmed Biovis Shukratara chamber (Expert Vision Labs Pvt. Limited, India). The motion traits were recorded under 10x of phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan) attached with the warm stage.

Statistical Analyses

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

Results and Discussion

Sperm motion traits

In present study, sperm motion traits of subfertile buffalo bull semen were assessed by Computer Assisted Sperm Analysis software (Table 1).

Pre-freeze semen

The sperm motion traits of pre-freeze semen such as, individual motility (57.72 ± 5.53 vs. 73.55 ± 2.89), rapid progressive motility (25.17 ± 5.48 vs. 34.36 ± 3.00) and VCL (71.85 ± 5.20 vs. 90.85 ± 2.2) were significantly ($P<0.05$) higher during treatment phase as compared to pre-treatment phase, respectively. However, ALH (6.18 ± 0.54 vs. 4.57 ± 0.36) and DNC (390.77 ± 69.13 vs. 251.53 ± 20.67) were significantly ($P<0.05$) reduced during treatment phase as compared to pre-treatment phase, respectively. Further, VCL (86.11 ± 19.16 vs. 71.85 ± 5.20), VSL (43.39 ± 4.55 vs. 51.78 ± 3.81), STR (84.63 ± 1.92 vs. 92.49 ± 0.49) and BCF (10.35 ± 0.54 vs. 19.28 ± 0.93) were significantly ($P<0.05$) increased during post-treatment phase in contrast to pre-treatment phase, respectively. Moreover, ALH (6.18 ± 0.54 vs. 3.26 ± 0.26), DNC (390.77 ± 69.13 vs. 231.38 ± 12.50) and DNM (10.85 ± 0.80 vs. 6.54 ± 1.09) were significantly ($P<0.05$) reduced during post-treatment phase as compared to pre-treatment phase. The remaining sperm motion traits such as slow progressive motility, VAP, LIN and WOB were similar ($P>0.05$) in all the three phases.

Post-thaw semen

Herbal treatment significantly ($P<0.05$) improved the post-thaw individual motility during the treatment phase (41.72 ± 0.33%) and post-treatment phase (40.36 ± 2.40%) in contrast to pre-treatment phase (32.85 ± 2.40%).

Table.1 Effects of herbal treatment on pre-freeze and post-thaw sperm motion traits (Mean ± SE) of subfertile buffalo bulls during different phases

Sperm motion traits	Pre-freeze semen			Post thaw semen		
	Pre-treatment	Treatment	Post-treatment	Pre-treatment	Treatment	Post-treatment
Motile (%)	57.72 ± 5.53^{a}	73.55 ± 2.89^{b}	62.01 ± 3.05^{a}	32.85 ± 2.40^{a}	41.72 ± 0.33^{b}	40.36 ± 2.40^{b}
Rapid Prog (%)	25.17 ± 5.48^{a}	34.36 ± 3.00^{b}	31.91 ± 3.67^{a,b}	12.30 ± 1.79^{a}	16.92 ± 0.62^{b}	15.63 ± 0.70^{a,b}
Slow Prog (%)	22.82 ± 2.73^{a}	31.47 ± 3.53^{a}	26.05 ± 2.70^{a}	16.64 ± 2.27	11.45 ± 1.00	20.49 ± 3.35
VCL (um/sec)	71.85 ± 5.20^{a}	90.85 ± 2.21^{b}	86.11 ± 19.16^{b}	73.82 ± 3.03	72.37 ± 2.37	72.99 ± 2.43
VAP (um/sec)	47.76 ± 4.37^{a}	52.53 ± 3.64^{a}	54.58 ± 20.57^{a}	43.16 ± 1.46	41.59 ± 1.07	41.73 ± 1.11
VSL (um/sec)	43.39 ± 4.55^{a}	47.44 ± 3.61^{a,b}	51.78 ± 3.81^{b}	38.51 ± 1.69	36.78 ± 1.31	35.90 ± 1.78
LIN (%)	59.60 ± 3.71^{a,b}	65.72 ± 2.16^{b}	59.08 ± 1.50^{a}	47.36 ± 1.28	46.23 ± 1.73	49.73 ± 2.48
STR (%)	84.63 ± 1.92^{a}	87.72 ± 1.57^{a}	92.49 ± 0.49^{b}	81.78 ± 2.02	82.73 ± 2.06	82.62 ± 2.05
WOB %()	68.28 ± 3.74^{a,b}	73.22 ± 2.07^{a}	62.58 ± 1.42^{b}	54.34 ± 0.80	52.65 ± 1.22	53.15 ± 2.23
BCF (hz)	10.35 ± 0.54^{a}	14.40 ± 1.28^{a}	19.28 ± 0.93^{b}	16.17 ± 0.65	15.64 ± 0.45	14.90 ± 10.82
ALH (um)	6.18 ± 0.54^{a}	4.57 ± 0.36^{b}	3.26 ± 0.26^{c}	2.72 ± 0.07	2.64 ± 0.11	7.19 ± 4.56
DNC (squm/sec)	390.77 ± 69.13^{a}	251.53 ± 20.67^{b}	231.38 ± 12.50^{b}	178.21 ± 12.11	179.46 ± 8.72	161.65 ± 11.47
DNM (squm/sec)	10.85 ± 0.80^{a}	7.35 ± 0.76^{a,b}	6.54 ± 1.09^{b}	6.85 ± 0.39	6.50 ± 0.48	7.36 ± 0.49

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

Table.2 Analysis set-up for CASA (Biovis CASA 2000, version 4.59)

Optic calibration	
Parameters	**Settings**
Magnification	Objective 10 X Phase Image Pixels - 1.48 pixels/unit
Camera frequency (FPS)	160
Frame rate (FPS)	60
Frames acquired (FPS)	60
Detection of motility parameters	
Non progressive limit (µm/sec)	0-10
Slow progressive limit - (µm/sec)	10-25
Rapid progressive limit - (µm/sec)	>=25
Detection of velocity parameters	
Maximum velocity for tracking (µm/sec)	150
Minimum VCL (µm/sec)	> 25
Minimum VAP (µm/sec)	> 10
Minimum VSL (µm/sec)	> 1
Minimum track Length (% of frames)	51
Shape and size	
Area (µm)	1-9999
Axis major (µm)	5-16
Axis minor (µm)	3-10
Stage configuration – BiovisShukratara	
Chamber depth (micron)	10
Chamber area (mm^2)	100 × 0.01
Volume (vL)	5

Further, rapid progressive motility was significantly higher during the treatment phase (16.92 ± 0.62%) as compared to pre-treatment phase (12.30 ± 1.79%). However, other post-thaw sperm motion traits such as slow progressive motility, VCL, VAP, VSL, LIN, STR, WOB, BCF, ALH, DNC and DNM were similar (P>0.05) across the three phases.

Improvement of sperm motion traits may be due the complex interactions or synergetic or adaptogenic or antioxidant effects of bioactive components 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 with a ferin 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).

Improvement of sperm motion traits without any adverse effects following herbal treatment might be due to *Panax ginseng*, which enhances the level of cAMP-responsive element modulator messenger (Hwang *et al.,* 2010; Park *et al.,* 2007) and ginsenoside Rg1 (Dae and You 2013). Similar results have also been reported in fresh semen of bulls, men,

mice, rats and cocks following individual supplementation of Shilajit, *Withania somnifera* and *Tribulus terrestris* (Salgado *et al.*, 2017; Khaleghi *et al.*, 2016; Eskandari *et al.*, 2016; Sharaway *et al.*, 2015; Mostafa and El-Khalik 2014; Ambiye *et al.*, 2013; Mishra *et al.*, 2012; Adaay *et al.*, 2012; Biswas *et al.*, 2010; Ahmad *et al.*, 2009; Grigorova*et al.*, 2008; Hadi, 1970).Contrary to our data in vitro addition of *Panax ginseng* extract to boar semen has shown no significant effects on sperm motion traits (Gray *et al.*, 2016).

However, no scientific extensive data are available in bulls for the comparison of our results. So, further, studies are needed for the detailed understanding of the effects of the herbs on semen quality of subfertile buffalo bulls.

Present study clearly indicated that, the dietary supplementation of 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 for 60 days improved the sperm motion traits of pre-freeze and post-thaw semen samples in subfertile buffalo bulls.

Acknowledgements

The authors are thankful to the Director of Livestock Farms, GADVASU, Ludhiana, Punjab (India) for providing animals for the experiment.

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How to cite this article:

Shivkumar, Ajeet Kumar, M. Honparkhe, A.K. Singh and Brar, P.S. 2018. Effects of Dietary Herbal Supplementation on CASA Based Sperm Motion Traits in Subfertile Buffalo Bulls (Bubalus bubalis). *Int.J.Curr.Microbiol.App.Sci.* 7(03): 2543-2550.
doi: https://doi.org/10.20546/ijcmas.2018.703.294