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Biophysical studies of administered *Shilajit* on rat bone tissue

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Abstract

The purpose of the current study was to examine the effect of Shilajit; a herbomineral, supplementation on the mechanical strength of bone tissue.

Thirty six animals in two batches of eighteen animals each were assigned to three groups: Group A (Control), Group B (PS 100) and Group C (PS 200). The treatment period was continued for ten weeks. Bone tissue mechanical strength along with antioxidative enzymes, alkaline phosphatase (ALP) and morphometric parameters were assessed.

Mechanical strength of the bone tissue (both femur as well as tibia) was found to be significantly enhanced upon shilajit supplementation. Also, the activities of anti oxidant enzymes in liver of both *Shilajit* administered groups were found to be significantly higher compared to control group.

These findings suggest that *Shilajit* is very efficacious and competent in the maintenance of bone health.

Bone loss can be cured using large number of medications comprising Hormone replacement therapy (HRT), bisphosphonates, selective estrogen-receptor modulators (SERMs), testosterone, parathyroid hormone etc. these anti-resorptive medications slow or stop the bone resorbing portion of the bone remodeling cycle but do not affect the bone forming portion of the cycle[9]. However, they are not devoid of side effects. They are known to be associated with an increased risk of breast cancer, endometrial cancer, thrombosis, stroke and other heart diseases, abdominal pain, nausea, headache, severe digestive disorders, heart burn, difficulty in swallowing, musculoskeletal pain, hot flashes, leg cramps and pulmonary embolism. There is an increasing need to explore complementary and alternate medicines having least side effects with desired potency and activity.

Shilajit is used as a panacea in the traditional systems of medicine[11]. Native/raw *Shilajit* is almost always found to be

contaminated with toxic polymeric quinines, microbial debris and heavy metal ions which are accumulated during the process of *Shilajit* formation and also due to adulteration. Because of considerable difficulty in the collection of *Shilajit* from steep rocks, its scant availability in anyone locale, and the absence of any set standard for its quality control, unscrupulous traders often mix shilajit with clay particles, gums, resins and cows' urine loc. cit. *et al., loc. cit.* Several natural phenomenons also contribute to the complex composition of *Shilajit*. These are associated with both biotic (pedological) and abiotic (geochemical) processes. *Shilajit* humus appreciably changes its chemical character in different natural habitats. The mean residence time of *Shilajit*, in its natural habitats, ranges from a few decades to several thousand years. It is helpful in number of metabolic disorders like diabetes, Alzheimers, arthiritis. Also, it is very powerfull rejuvenator and has a great ovogenic and spermatogenic potential. Inspite of such a huge activity spectrum, *Shilajit's* complete potential is still unexplored especially the potential to modulate bone resorption and formation cascades.

Skeletal homeostasis is dependent upon the fine balance between bone formation and bone resorption mechanism. Bone loss often occurs as a result of an imbalance between the two. Osteoporosis is a pathological condition characterized by a reduction of bone mass and microarchitectural integrity, making patients more susceptible to fractures. Osteoporosis generally occurs with age and in post-menopausal women. The pathogenesis of osteoporosis is associated with many factors like age, environmental, genetic, biomechanical, chronic disease and various hormones[2,5].

The mechanical properties of the organic component of bone are required for composite modeling of bone tissue. The mechanical function provides the structural framework for the organism that permits support, locomotion and protection of bones. Knowledge of mechanical properties of collagenous component of bone is essential required for understanding ductility and strength of the bone[4,10].

In the current study, the extent of antioxidant defense system and alteration in alkaline phosphatase by processed *Shilajit* has been assessed.

To carry out the present study, twelve week old healthy male wistar strain rats weighing 180-200gms were procured from the Central animal house of Panjab University, Chandigarh. The animals were acclimatized in the department animal house for two weeks in plastic cages under hygienic conditions and were provided feed and water *ad-libitum*. They were monitored for their health and body weight every week. *Shilajit* (Dabur) given during the treatment period was obtained from the market.

The animals were divided into three major groups: Group A(control): control rats were given water orally for a period of ten weeks; group B (treated): Animals were given processed *Shilajit* (PS; 100mg/kg/day) orally for a period of ten weeks; group C (treated): Animals were given processed *Shilajit* (PS; 200mg/kg/day) orally for a period of ten weeks.

Blood from the animals of each

control and experimental groups was taken from the ocular vein of the eye. The serum obtained is used for the analysis of alkaline phosphatase, blood calcium and phosphorous. Then they were sacrificed and their liver and long bones *i.e.* femur and tibia were removed. The liver was dissected out and cleaned with ice-cold saline, blotted dry and immediately transferred to the ice chamber. Various oxidative stress related parameters were estimated. Soft tissues were removed from the intact surfaces of the bone. Length (mm), diameter (mm) and weights (mg) of the bone samples were noted using a centimeter scale, Vernier caliper and digital weighing balance respectively. Then the cross-section area (mm^2) was calculated from the diameter obtained. Then the bones were stored at 20°C until use.

Biochemical analysis

Lipid peroxidation :

Lipid peroxidation in erythrocyte lysate was determined by the measurement of malondialdehydes (MDA) levels in plasma on the basis of MDA reacted with thiobarbituric acid at 532nm, according to the method of Wills *et al.*,[13]. Calculated values were expressed as nmol mg^{-1} protein for MDA.

Glutathione reduced :

Estimation of GSH was performed in the lysate of erythrocytes by the method of Moron *et al.*,[8]. The GSH contents were expressed in terms of µmol g^{-1} tissue.

Superoxide dismutase :

Superoxide dismutase assay was performed according to the method of Kono[6]. Enzyme activity was expressed as units/mg protein.

Catalase :

The activity of the Catalase enzyme was estimated by the method of Luck[7]. The enzyme activity was expressed as mM of H_2O_2 decomposed min^{-1} mg^{-1} protein.

Alkaline phosphatase :

Alkaline phosphatase was measured by using p-nitrophenol phosphate as the substarte[1], where p-nitrophenyl phosphate was used as a substrate, which is hydrolyzed by alkaline phosphatase to yield p-nitrophenol at an alkaline pH. Results are expressed as µmoles of p-nitrophenol liberated/mg protein.

Mechanical studies :

Breaking strength of all the bones was measured using texture analyzer. Texture analyzer (Model TA-XT2i from stable micro systems UK) is used with two probes for the measurement of mechanical strength of bones. For measuring breaking strength a three point bending rig was used; 75mm diameter aluminium plateau probe is used. The bone sample is placed on the probes. Another probe is used to break the bone sample in two pieces. The following settings were made for sample measurements. Pre-test speed 2.0 mm/s, test speed 1.0mm/s, post test speed 10mm/s, rupture test distance 6.0mm, force 1.0kg/s, time 0.10s and count 5. The force required to break the bone sample from the fixed distance is called the rupture force and signifies the firmness of the bone. This force is noted.

Statistical analysis :

Data for other estimations were expressed as the mean± the standard deviation (S.D.). One way analysis of variance (one-way ANOVA) was used to evaluate the differences among the groups. Newman–Keuls post hoc test was then used for the multiple comparisons between different groups. Differences were considered statistical significant at $p<0.05$. Statistical analysis software, SPSS (14 version) was adopted for processing the data of the present study.

Body weight of rats of each group was taken every week. Changes in the body weight of animals seen every week during the experiment are presented in table 1. Animals in group C (*Shilajit* 200) showed 17.7% increase in body weight, group B (*Shilajit* 100) showed 21% and group A (control) showed 14% increase in the body weight. In the groups B and C, the body weight gain was found to be higher than that of the group A.

Table-1. Results of change in body weights with time

Treatment	Week	Week	Week	Week	Week	Week	Week	Week	Week	Week	Weight gain	Weight gain %
Group A (control)	202	204	206	204	214	210	216	262	242	230	28	13.86
Group B PS(100)	190	190	194	190	220	212	230	236	252	230	40	21.05
Group C PS (200)	197	196	174	170	196	196	206	232	234	242	35	17.77

Biochemical analysis :

In *Shilajit* administered groups (Gp B and Gp C), the superoxide dismutase activity was found to be higher in comparison to control (Gp A). the activity of catalase also increased in *Shilajit* administered groups (GpB and Gp C) when compared to the control animals. *Shilajit* administration caused significant increases in the reduced glutathione activities of liver. Its activities increased gradually with increasing dose of *Shilajit*. Activities of anti oxidant enzymes in liver of both *Shilajit* administered were found to be significantly higher compared to control group (table 2).

Table-2. Results of concentration of antioxidative enzymes in control and treated groups

Treatment	LPO (activity/mg protein)	GSH (activity/mg protein)	SOD (activity/mg protein)	CAT (activity/mg protein)
Group A (control)	5.04±1.29	73.39±4.09	0.33±0.001	0.42±0.031
Group B PS(100)	7.14±1.04*	122.79± 17.58	0.36±0.009	0.50±0.028
Group C PS (200)	5.02±1.08	187.59±32.00	0.38±0.016	0.55±0.038

± standard deviation from the mean; significance *p<0.05; **p<0.01; ***p<0.001 w.r.t control

Table 3. shows the results of the variation in concentration of alkaline phosphatase in liver and in serum as seen in the present study. It was observed that the treated group showed modulation in the ALP activity in comparison to control. It was observed that the alkaline phosphatase concentration increased in Gp C but not much change was observed in Gp B.

Table-3. results of ALP of control and treated in liver and serum

Treatment	Liver (µmoles/mg protein)	Serum (µmoles/mg protein)
Group A (control)	0.370±0.050	4.180±0.311
Group B PS(100)	0.378±0.023*	3.124±0.736
Group C PS (200)	0.566±0.042**	4.9995±0.744

± standard deviation from the mean; significance *p<0.05; **p<0.01; ***p<0.001 w.r.t control

Mechanical studies :

The length (mm), diameter (mm) and weight (mg) of both femur and tibia were estimated using a centimeter scale, Vernier calipers and digital weighing balance, respectively. Then the cross sectional area (mm^2) was calculated from diameter. Table-4 shows the results of changes in the weight of bones. It was observed that there was an increase in weight of bones in rats of treated groups in comparison to rats of control.

Table-4. Weight of femur and tibia in control and treated groups

Treatment	Weight (mg)	
	Femur	Tibia
Group A (control)	784 ± 17	588 ± 39
Group B PS(100)	790 ± 67	596 ± 29
Group C PS (200)	869 ± 50**	880 ± 26**

± standard deviation from the mean; significance *p<0.05; **p<0.01; ***p<0.001 w.r.t control

Weight of tibia and femur are found to increase with shilajit dose. One way ANOVA statistics is applied further to confirm our observation between femurs and tbias of subgroups within a group. The differences in the mean value among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p<0.01$). From the table it is evident that the increase in weights seen in tibia of treated animals is much more than seen in corresponding femur. In fact, the increase in weight seen in Gp C is very high group Gp B then seen for the case of Gp B from Gp A. both the groups show increase as compared to the control and a significant difference is observed with increasing concentration of *Shilajit.*

Table 5 shows the results of changes introduced in length, diameter and cross sectional area in both the femur and tibia. It was observed that there were no changes in the bone length of treated groups in comparison to the control animals.

Table-5. Length, diameter and area of cross section of femur and tibia in control and treated groups.

Treatment	Length (mm)		Diameter (mm)		Area (mm^2)	
	Femur	Tibia	Femur	Tibia	Femur	Tibia
Group A (control)	35.567±0.686	39.317±0.864	3.50±0.158	2.73±0.869	9.62±0.870	6.36±0.410
Group B PS(100)	35.90±1.305	39.580±1.498	3.54±0.214	2.83±0.934	9.90±1.19	6.90±0.448
Group C PS (200)	35.678±0.967	39.033±0.837	3.62±0.273	3.20±0.273	9.22±1.48	8.53±0.344

± standard deviation from the mean; significance *p<0.05; **p<0.01; ***p<0.001 w.r.t control

The analysis of the table of the breaking test allows conclusions regarding the physical and mechanical properties of the bone. Shilajit intake results in change of mechanical properties of the bone. Table 6 shows the results of changes in toughness in both femur and tibia. It was observed that there was an increase in breaking strength and toughness of bones in rats of treated groups in comparison to the rats of the control group.

Table-6. Breaking strength and toughness of femur and tibia in control and treated groups

Treatment	Breaking strength (N)		Toughness (N/m^2)	
	Femur	Tibia	Femur	Tibia
Group A (control)	219±13	168±6	150.867±6	73± 4.382
Group B PS(100)	283±31***	200±13**	200.767±6**	122±2.656*
Group C PS (200)	246±11*	246±4**	176.800±13*	96±11.087

± standard deviation from the mean; significance *p<0.05; **p<0.01; ***p<0.001 w.r.t control

During the breaking test, the actual strength of the bones was recorded every 0.1mm during the lowering of stamp. After the total failure of the bone, the software programme indicated the maximum load, area below the curve and the breaking strength. Because of the fluctuations of the graphs during the test probably because of the consolidation-the energy to failure was sometimes visibly inaccurately, because it corresponds to the area below the graph. This is a phenomenon of the bone and not of the breaking test. The breaking strength is the last measured point of the running graph.

The present result provides evidence that *Shilajit* treatment over a period of two months causes a slight increase in the body weight.

The results of animal experiments given in tables above (table-2) clearly show that *Shilajit* induced a dose related increase in the activity of all anti-oxidative enzymes in the liver. *Shilajit* in its natural habitat exists as a redox mixture of the iron containing hydroquinone-semiquinone-quinone complex. One electron reduction of superoxide by *Shilajit* would produce a compound which, in turn, would catalyze the cleavage of the generated hydrogen peroxides and equivalents into water and oxygen and regenerate the original compounds. The regenerative cycle of the antiradical-antioxidant activity of *Shilajit* is apparent[3]. The above findings are consistent with the therapeutic uses of shilajit as an Ayurvedic rasayan against oxidative stress-induced diseases and geriatric complaints. Serum alkaline phosphatase (ALP) is a bone marker. ALP is produced primarily in the liver and in the bone. Normally, the liver produces more ALP than the other organs or the bones. Some physiological conditions may produce large amounts of this enzyme into the blood stream. These conditions can be rapid bone growth (during puberty), bone disease, or damaged liver cells. With shilajit administration, no variation in the ALP levels were seen in the case of *Shilajit* (100) group, in contrast, ALP levels were found to be increased in case of *Shilajit* (200) treatment. In serum, it was observed that *Shilajit* (100) treatment has reduced the ALP levels. But *Shilajit* (200) was found to be successful in increasing the serum levels of alkaline phosphatase. Increase in the concentration of ALP on shilajit treatment could be attributed to the enhanced bone growth.

Three months old Wistar rats were used and shilajit was supplemented orally to the rats for ten weeks in order to determine the degree of bone strengthening. The length of the femur was not influenced by the shilajit treatment. But diameter and cross-section area of femur were found to have altered. It was observed that there were no changes in the length of bones of treated groups in comparison to the control. All, the groups comprise fully mature rats. Hence bone length is not affected. *Shilajit* has been used in traditional medicine as a bone strengthening agent. In this study, we showed that administration of *Shilajit* had profound effects on the rat bones. The success of osteoprotective therapy is often examined by immunohistochemical studies of osteoblastic growth factors and gene expression and measurement of bone mineral density, by histomorphometric studies or mechanical tesing[12]. Usually mechanical testing is done at the diaphyseal femur or tibia region.

The most important biophysical parameter to assess the mechanical strength of bone is the breaking strength of the bone. Breaking strength was found to be altered in all the treated groups in comparison to the control groups. For *Shilajit* (PS 100) treated group, breaking strength has been found to be significantly higher both in femur ($p<0.001$) and in tibia ($p<0.01$). In contrast, for *Shilajit* (200) treated group, the increase observed in breaking strength was much less than observed in shilajit (100) treated group in comparison to the control. In case of femur of *Shilajit* (100), increase observed was significant ($p<0.05$) as compared to *Shilajit* (200). Likewise, the toughness (energy absorption) in treated

groups of both femur and tibia was much higher than the control. For both the bones, increase observed in toughness *Shilajit* (100) treated group was much higher than *Shilajit* (200). The above mentioned studies have provided evidence that shilajit treatment over an extended period of two months induces a profound change in the bone mechanical properties.

Thus we conclude that shilajit is very efficacious and competent in maintaining the bone health. As herbomineral, shilajit can be quite effective in the treatment of many path physiological conditions. This herbomineral preparation can offer a promising approach for the long term use in the treatment of number of diseases. But this exudate still needs to be explored more. Further studies into mechanisms underlying the relationships between shilajit and bone may improve our understanding.

References :

1. Bergmeyer, H.U. (1963). Phosphatase (phosophomonoesterase) determination in serum with p-nitrophenyl phosphate. In: Methods of enzymatic analysis. Academic press. New York. pp. 783.
2. Bhardwaj Payal, Durg Vijay Rai and Mohan Lal Garg (2013) Zinc as a nutritional approach to bone loss prevention in an ovariectomized rat model. *Menopause*, *20*(11): 1184–1193.
3. Bhattacharya, A.P. Sen and S. Ghosal (1995). *Phytotherapy Res. 9:* 56-59.
4. Hawkins D. (2001) Tissue mechanics. Human performance laboratory, University of California, Davis. Lecture available at: http://dahweb.engr.ucdavis.edu/dahweb/126site/126site. htm
5. Khan A. (2005) *J. Obstet Gynaecol Can*, *27:* 345–9.
6. Kono, Y. (1978). *Arch Biochem Biophys*, *186:* 189-195.
7. Luck H. Catalase, methods of enzymatic analysis E.D Bergmeyer HO, (1971) Academic Press, New York, London, P. 855.
8. Moron, M.J., J. W. Depierre and B. Mannlrvik (1979). *Biochem Biophys Acta 5:* 62-67.
9. Prentice, R.L., M.B. Pettinger, R.D. Jackson, J. Wactawski-Wende, A.Z. Lacroix and G.L. Anderson, *et al*., (2013) *Osteoporos Int. 24:*567–80. Epub 2012 Dec 4.
10. Rai D.V., G. Kumar, P. Tiwari and D.C. Sexena (2008) *J. Biomech*, *41*(1): 20-24.
11. Shibnath Ghosal, Jaws Lal and K Sushil Singh (1991) *Soil Biol. Biochem. 23* (7): 673480.
12. Sturmer, E. K., Seidlova-Wuttke, D., Sehmisch, S., Rack, T., Wille, J., Frosch, K. H., W. Wuttke and K. M. Sturmer (2006). *J. Bone Miner Res 21*(1)*:* 89-96.
13. Wills, E. D. (1966). *Biochem. J. 99:* 667-676.