CODEN (USA): IJPB07 ISSN: 2320-9267

Indian Journal of Pharmaceutical and Biological Research (IJPBR)

Journal homepage: www.ijpbr.in

Original Research Article

Effects of shilajit on the bone tissue of alcohol administered rats

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ARTICLE INFO:

Article history:

Received: 30 December 2015
Received in revised form:
13 March 2016
Accepted: 19 March 2016
Available online: 30 March 2016

Keywords:

Shilajit, bone, femur, tibia, breaking strength, alcohol

ABSTRACT

Purpose: The purpose of the current study was to examine the effect of Shilajit; a herbomineral, supplementation on the mechanical strength of alcohol treated rat bone. **Methods:** Experimental animals each were assigned to six 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); group C (treated): Animals were given processed shilajit (PS; 200mg/kg/day); group D: animals were given 30% alcohol; group E: animals were given 30% alcohol and shilajit (100mg/kg/day) orally; group F: animals were given 30% alcohol and shilajit (200mg/kg/day) orally for ten weeks. Bone tissue mechanical strength along with bone weight, liver antioxidative enzymes and alkaline phosphatase (ALP) were assessed for all the treatment groups. **Results:** Mechanical strength of the bone tissue (both femur as well as tibia) was found to be significantly enhanced upon shilajit supplementation to alcohol treated group. Also, the activities of anti oxidant enzymes and alkaline phosphatase in the liver of alcohol administered groups were restored upon shilajit administration. **Conclusion:** These findings suggest that shilajit is very efficacious and competent in the maintenance of bone health

Introduction

Abuse of alcohol is known to disturb the bone metabolism and causes osteoporosis. Chronic ethanol abuse is correlated with osteoporosis condition, decreased bone mass, and increased risk of fractures[1]. This is assumed to be the direct effect of ethanol on the bone tissue or indirect effects through altering vitamin D3 and calcium regulating hormones. Chronic alcohol consumption can interfere with bone growth and remodeling process, resulting in decreased bone mineral density and increased risk of fractures. Bone strength is affected by number of factors like hormonal, nutritional, environmental and lifestyle factors, including tobacco and alcohol consumption[2].

Bone is a major storage depot for number of minerals. Variety of nutrients also plays a major role in the bone formation and resorption mechanism. The absorption of calcium takes place in the small intestine. An adequate amount of calcium in the bloodstream is essential for the proper functioning of muscles and nervous tissue. The biological system has the mechanism to monitor the calcium concentration and also respond through the action of vitamins, hormones and growth factors, for the regulation of distribution of calcium. Alcohol consumption may disrupt this fine balance by modulating the hormones that regulate calcium metabolism as well as the hormones that influence calcium metabolism indirectly [3]

Numerous studies have shown relationship between the alcohol consumption and bone deterioration[4]. However, a few studies indicate that moderate alcohol consumption may help reduce osteoporosis and decrease fracture risk in postmenopausal women [5]. For example, in study of more than 14,000 subjects, reported that women age 65 and older who consume alcohol on more than 5 days a week had a greater risk of skeletal deformity in comparison to those who consumed alcohol only once in a week. Studies also investigated the effect of moderate alcohol consumption on rats following surgical ovariectomy to mimic the menopausal condition. Chronic alcohol consumption exerts harmful effects on the bone growth metabolism and its maintenance at all the age groups. The action of alcohol on the growing bone tissue is especially deleterious, because at this time point, bone tissue is in the growing stage and any interferences in the cellular mechanism may hampers the overall growth of the bone.

Shilajit, an ancient traditional medicine has been recognized of having a number of pharmacological activities and has been used as a rejuvenator and for treating a number of pathological conditions [6]. Modern scientific research has systematically

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validated a number of properties of shilajit and has proven that shilajit is truly a panacea in oriental medicine [7]. This traditional medication is an integral part of the health care system in a number of developing countries including India. However, these medications can only find a place for themselves in the medical field if their authentication will be evaluated scientifically and documented systematically. So the aim of the current study was to evaluate the effects of shilajit administration on the bone tissue of rats having supplemented ethanol for a period of ten weeks.

Material and methods

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. Ethanol given during the treatment period was provided by Bengal chemicals and Pharmaceuticals Ltd. Mumbai, India.

The animals were divided into six 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; group D: animals were given 30% alcohol orally for a period of ten weeks; group E: animals were given 30% alcohol and shilajit (100mg/kg/day) orally for ten weeks; group F: animals were given 30% alcohol and shilajit (200mg/kg/day) orally for 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) and weights (mg) of the bones samples were noted using a Vernier caliper and digital weighing balance respectively. Then the bones were stored at $20^{o}C$ until use.[8,9]

Biochemical analysis

Protein was estimated in all the samples extracted from all the groups by the method of Lowry et al (1951)[10].

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 (1966). Calculated values were expressed as nmol mg^{-1} protein for MDA.[11]

Glutathione reduced

Estimation of GSH was performed in the lysate of erythrocytes by the method of Moron et al (1979). The GSH contents were expressed in terms of µmol g^{-1} tissue.[12]

Superoxide dismutase

Superoxide dismutase assay was performed according to the method of Kono (1978). Enzyme activity was expressed as units/mg protein.[13]

Catalase

The activity of the Catalase enzyme was estimated by the method of Luck (1971). The enzyme activity was expressed as mM of H_2O_2 decomposed min^{-1} mg^{-1} protein.

Alkaline phosphatase

Alkaline phosphatase is measured by using p-nitrophenol phosphate as the substrate ,where p-nitrophenyl phosphate was used as a substrate, which is hydrolyzed by alkaline phosphatase to yield p-nitrophenol at an alkaline pH. Results were expressed as µmoles of p-nitrophenol liberated/mg protein.[14]

Mechanical studies

Breaking strength of all the bones is 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 is used; 75mm diameter aluminum 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. [15]

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.[16]

Results

Body weight of rats of each group was observed every week. Table 1 shows the weight of rats of each group over a period of ten weeks.

Table 1: Body weights of all the treatment groups recorded over a total treatment period

Treatment	1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week	7th Week	8th Week	9th Week	10th Week	Wt. Gain %
Gp AControl	202	204	206	204	214	210	216	262	242	230	13.86
Gp B PS (100)	190	190	194	190	220	212	230	236	252	230	21.05
Gp C PS (200)	197	196	174	170	196	196	206	232	234	242	17.77
Gp D ALC (30%)	198	200	212	212	230	256	236	264	286	266	34.34
Gp E ALC (30%)+PS (100)	190	188	188	197	207	215	201	230	237	243	27.89
Gp F ALC (30%)+PS(200)	204	200	200	206	230	228	236	262	249	245	20.10

Changes in the body weight of animals seen every week during the experiment are presented in table 1. Animals in Gp C showed 17.7% increase in body weight, group b showed 21% and Gp A shows 14% increase in body weight. In groups B and c, the body weight gain was found to almost same as that of group A. Animals in Gp D showed 34.34% increase, Gp E showed 27.89% and Gp F showed 20.10% increase in body weight.

Biochemical analysis

Protein estimation

Table.2 indicates the levels of protein concentration in the liver of control and treated animals. The protein concentration was found to enhance in shilajit supplemented group and also in the alcohol and shilajit combined group (Gp F).

Table 2: Results of change in protein concentration in control and treated groups

Treatment	Protein concentration
Gp AControl	49.78 ± 2.83
Gp B PS (100)	57.10 ±2.23
Gp C PS (200)	68.40 ±5.81**
Gp D ALC (30%)	58.44 ±4.53
Gp E ALC (30%)+PS (100)	59.96 ±4.72
Gp F ALC (30%)+PS(200)	77.02 ±10.11**##
N=6, ±standard deviation from mean, Significance *p<0.05, **p<0.01, ***p<0.001 w.r.t control; # p<0.05, ## p<0.01, ### p<0.001 w.r.t alcohol treated group (Gp D)	

Liver antioxidant enzymes

Malondialdehyde levels were found to increase significantly in the alcohol administered group in comparison to the normal control group. Superoxide activity also increased in alcohol treated group. However, catalase activity was found to decrease in alcohol group. Furthermore, shilajit administration restored the levels of theses antioxidant enzymes.

]Table 3: Activities of antioxidative enzymes in control and treated groups

Treatment	(LPO) µ moles of MDA min^{-1} mg^{-1} protein	(GSH) µ moles GSH mg^{-1} protein	(SOD) Units/µl	(CAT) µ moles H_2O_2 decomposed min^{-1} mg^{-1} protein
Gp AControl	5.04±1.29	73.39±4.09	0.33±0.001	0.42± 0.031
Gp B PS (100)	7.14±1.04*	122.79±17.58*	0.36±0.009	0.50±0.028
Gp C PS (200)	5.02±1.08	187.59±32.00**	0.38±0.016	0.55±0.038
Gp D ALC (30%)	10.97±1.61***	201.64±22.87	1.33±0.252**	0.34±0.012*
Gp E ALC (30%)+PS (100)	8.30±0.89**#	200.86±25.54**	0.92±0.010**##	0.39±0.096
Gp F ALC (30%)+PS(200)	7.88±0.33*##	155.87±15.58*	0.40±0.138**##	0.45±0.278##
N=6, ±standard deviation from mean, Significance *p<0.05, **p<0.01, ***p<0.001 w.r.t control; # p<0.05, ## p<0.01, ### p<0.001 w.r.t alcohol treated group (Gp D)				

Bone markers

The table 4 shows the results of the variation in concentration of alkaline phosphatase enzyme in liver and serum. It was observed that the treated groups showed modulation in the ALP activity in comparison to the control. From the table, it can be seen that the ALP concentration increased in Gp C, but the increase was not up to significant levels. ALP activity significantly increased in Gp D in comparison to the control and Shilajit administered groups.

Table 4: Concentration of ALP in control and treated groups

Treatment	Liver (10^{-3} µmoles/mg protein)	Serum (10^{-3} µmoles/mg protein)
Gp AControl	0.370±0.050	4.180±0.311
Gp B PS (100)	0.378±0.023	3.124±0.736
Gp C PS (200)	0.56±0.042	4.995±0.744
Gp D ALC (30%)	1.049±0.113**	2.710±0.774
Gp E ALC (30%)+PS (100)	0.573±0.075##	2.453±0.162
Gp F ALC (30%)+PS(200)	0.717±0.098#	5.320±0.141
N=6, ±standard deviation from mean, Significance *p<0.05, **p<0.01, ***p<0.001 w.r.t control; # p<0.05, ## p<0.01, ### p<0.001 w.r.t alcohol treated group (Gp D)		

Mechanical studies

In this study, we have shown that administration of shilajit had profound effects on the bones of alcohol treated rats. The weight and lengths of femur and tibia are noted. The weight of both the bones i.e. femur as well as tibia was found to decrease with the alcohol administration. One way analysis of variance statistics was applied to further confirm our observations between femur and tibia. A considerable difference is seen in the weights of alcohol treated rat bones in case of shilajit (100) and shilajit (200). Shilajit supplementation is more affected at a dose level of 200mg/kg/day.

Table 5: Bone weight recorded for control and treated group

Treatment	Weight (mg)	
	Femur	Tibia
Gp AControl	784 ±17	588±38
Gp B PS (100)	790±67	588±26
Gp C PS (200)	869±50**	596±29
Gp D ALC (30%)	663±48**	488±32***
Gp E ALC (30%)+PS (100)	652±43**	509±23**
Gp F ALC (30%)+PS(200)	790±87###	596±40##

N=6, ±standard deviation from mean, Significance *p<0.05, **p<0.01, ***p<0.001 w.r.t control; # p<0.05, ## p<0.01, ### p<0.001 w.r.t alcohol treated group (Gp D)

The analysis of the table of the breaking sterngth allows conclusions regarding the physical and mechanical properties of the bone tissue. Shilajit intake results in change in the 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 shilajit treated groups in comparison to the rats of the alcohol treated group. However, no significant change in the toughness was observed in the case of tibial bone.

Table 6 Breaking strength and toughness of control and treated groups

Treatment	Breaking strength (N)		Toughness (N/m^2)	
	Femur	Tibia	Femur	Tibia
Gp A Control	219±13	168±0.6	150±6	73±2
Gp B PS (100)	283±31***	200±33**	200±6***	122±11
Gp C PS (200)	246±11*	186±4*	176±23***	96±4
Gp D ALC (30%)	199±8	143±3*	127±17	66±7
Gp E ALC (30%)+PS (100)	229±32#	125±17**	146±39***	52±13*
Gp F ALC (30%)+PS(200)	221±20	189±6##	174±9***	105±4

N=6, ±standard deviation from mean, Significance *p<0.05, **p<0.01, ***p<0.001 w.r.t control; # p<0.05, ## p<0.01, ### p<0.001 w.r.t alcohol treated group (Gp D)

Discussion

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. The above findings are consistent with the study which shows that shilajit can be used as a therapeutic agent against oxidative stress-induced diseases and geriatric complaints [16]

Serum alkaline phosphatase (ALP) is a bone marker. ALP is produced primarily in the liver and in the bone. The Increased levels of ALP found in the liver in the alcohol intoxicated rats could be attributed to the dysfunction of liver as a result of chronic alcohol exposure. Also serum ALP levels were found to be less in all the treatment groups. This could be due to the damaged liver because of alcohol exposure which fails to excrete alkaline phosphatase enzyme into the circulation.

The results of mechanical strength provide evidence that chronic alcohol intake in rats induces profound changes in bones and mechanical properties. Alcohol impairs mainly an osteoblastic activity that results in reduced bone formation and mineralization .In our study significant decrease in weights of both the femur and tibia along with a considerable decrease in

breaking strength of long bones is seen with chronic alcohol intake. [17,18]This result is also validated in our previous study which showed that acute and chronic dose of alcohol affect the load carrying capacity of long bone in rats Chronic alcohol consumption leads to osteoporotic condition leading to decrease in bone mineral content This leads to lowering bone mass thereby reducing the bone weight. Our experimental results show lowering of bone strength with increasing alcohol dose. This is in tune with the studies of Kanis et al (2004) who concludes that high intake of alcohol confers a substantial risk for fractures in human beings. Also there is no significant decrease in bone lengths of treated animals in any group. Our study comprises fully mature rats, therefore, the length of both the bone tissue in all the treatment groups remain unaffected. Chronic ethanol administration may cause changes in bone matrix synthesis and mineralization process thereby changing the bone microarchitecture. [19,20]This change in bone microarchitecture may have resulted in decreased bone strength. Also, Alcohol is shown to have direct effects on the osteoblast cells and increases the endogenous secretion of calcitonin Furthermore, chronic alcoholism leads to poor nutrition (Rico 1990). Furthermore, the cellular mechanisms by which chronic alcohol intake enhances the risk of fracture as a result of decreased mineralization remain unclear.[21,22] Results of administration of alcohol-treated rats with different doses of shilajit have provided evidence that chronic treatment with shilajit has proved to be very useful in restoring the physical characteristics of bone. Dietary chronic ethanol causes negative effects on the rat skeleton and the study shows the tendency of shilajit to restore the negative effects caused by alcohol. The results of the study show that chronic ethanol exposure to the rats drastically reduces the strength of the mature bone. The technique herein tests only extrinsic properties, thus it cannot be determined from these data whether these effects were due to differences in tissue quality or cross-sectional geometry. Therefore, a more detailed study of bone mechanical properties is needed to determine the difference in whole bone strength seen herein is due to the changes in the bone quantity or bone quality.

Conclusion

The results also provide evidence that chronic alcoholism in rats induces profound changes in bones and its mechanical properties. Bone strength of alcohol treated groups was much lower than that of control and shilajit treated groups. This may suggest that in alcohol treated groups, alcohol leads to mineral loss. But when treated with shilajit, increase in breaking strength suggests that the minerals were either replaced during bone formation or minerals did not leach out during alcohol intake. So this further suggest that if alcohol intake causes increase in the probability of risk of fractures, then shilajit intake is quite effective in negating the negative effects caused by chronic alcohol intake. These effects of alcohol and then of shilajit may be exerted directly or indirectly through many cell types or the changes could also have been induced at gene level. Thus, 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.

Conflict of interest statement

We declare that we have no conflict of interest.

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Cite this article as: **Payal Bhardwaj,Mehak Goel, Durg Vijay Rai.** Effects of shilajit on the bone tissue of alcohol administered rats. **Indian J. Pharm. Biol. Res.2016; 4(1):74-80.**