Effect of alcohol on biochemical properties and thermal stability of weight bearing bones in male Wistar rats

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Alcohol consumption poses significant risk for osteoporosis development. The present study deals with changes in biochemical properties and thermal stability of weight bearing bones in alcohol noshed rats. About 48 male Wistar rats were equally divided into control (Gr. I) and treatment groups Gr. II-IV) subjected to 10, 20 and 30% ethanol administration, respectively. At the end of study, rats were sacrificed by decapitation under deep anesthesia and tibiae and fibulae bones were resected and used for biochemical (DNA, RNA, proteins and ash/inorganic minerals concentration) and thermogravimetric analyses (TGA). Administration of ethanol at higher doses (Gr. III & IV) had depressing effects on the nucleic acids and protein concentrations. The inorganic mineral content was also found to be lesser than that of control. The TGA revealed an increasing %weight loss in Gr. III and IV. This increase in %weight loss is due to decrease in mineral content causing calcification of bones and resulting in osteoporosis. The present study provides an insight that chronic consumption of alcohol negatively affects the biochemical properties and stability of bones, augmenting risks of osteoporotic fractures. However, at lower doses, alcohol administration may be helpful in bestowing thermal strength to bones.

Keywords: Ash content, Osteoporosis, Thermogravimetric analysis (TGA)

Corrosion is a fundamental process playing an insignificant role in economics and safety of metals. Among metals, Mild steel is a widely used commercial metal for fabrication, transportation and storage. The acid solutions are widely used in industries for acid pickling, industrial acid cleaning, acid descaling and oil well acidizing processes for mild steel and other alloys. Different concentrations of hydrochloric acid are most commonly used in these industries for this purpose. The most practical method for protection against corrosion of mild steel is the use of inhibitors, especially in acidic media and hence it leads the corrosion researchers to study the effect of various corrosion inhibitors on mild steel in hydrochloric acid environment.

Bone is the collagenous matrix impregnated with mineral salts, especially calcium hydroxyapatite. Its cellular, vascularised structure serves as storehouse of calcium and phosphorous and contributes in the regulation of the mineral metabolism. Trace elements such as iron, fluorine, zinc, magnesium, copper, lead, etc. play an important role in bone remodeling and maintenance of structural and functional integrity of bones. Mechanical strength of the bone tissue depends mainly on the nano-size apatite crystals. It is generally considered that the mineral resists compression and the collagen fibres withstand torsion and tension.

Bone undergoes lifelong remodeling which involves complex physiological mechanism that is governed by several biophysical and biochemical processes. The bone growth and remodeling are modulated by genetic and systemic factors. Although peak bone mass appears to be largely under genetic control, it can be influenced by hormonal, nutritional, environmental and lifestyle factors, including tobacco and alcohol consumption.

Long-term alcohol consumption can interfere with bone growth and remodeling, resulting in decreased bone density and increased risk of fracture. The cause of osteoporosis is a multifactorial entity in which alcohol consumption is known to play a vital part. The direct and indirect effects resulting in osteoporosis through the many cell types, hormones,
and growth factors that regulate bone metabolism and mechanisms are still under investigation. Also, the degree to which alcohol contributes to the declining skeletal status of the entire population is not yet known. Alcohol exposure is known to affect gene expression at both the nucleic acids and protein levels\textsuperscript{10}. It is likely that alcohol and its metabolites modulate the activity, stability, structure, or localization of some proteins or their interaction with other molecules. This alteration in protein structure and function may play a critical role in mediating the physiological and behavioral responses to the pathological consequences of ethanol.

Alcohol administration to young and rapidly growing rats significantly reduced bone growth, volume\textsuperscript{12,13}, density\textsuperscript{14}, rigidity\textsuperscript{15} and strength\textsuperscript{12,13,16}. Alcohol associated osteopenia appears to involve direct effect on bone cells that leads to suppression of new bone formation, and indirect effects by modulating the role of mineral regulating hormones\textsuperscript{13}. In addition, a dose dependent reduction in bone cell DNA synthesis was also observed\textsuperscript{17-19}. However, moderate consumption of alcohol is not related to bone loss, rather reported beneficial\textsuperscript{20,21}. Although, the relationship between alcohol ingestion and bone disease is evident, the mechanism by which alcohol induces osteoporosis remains unclear. Many dietary factors affecting bone strength have been evaluated in health and diseased condition but alcoholism has been implicated to have adverse affects on bone mineral density, and hence bone strength\textsuperscript{4,16}.

In the present study we investigated the effect of alcohol consumption on the changes in the biochemical properties, in which protein and nucleic acid content have been estimated. Besides, the difference between the thermal stability of bones of control and treatment groups of rats were evaluated for understanding the risk of osteoporosis.

**Materials and Methods**

**Animals**

Around 48 adult male Wistar rats, weighing 170-210 g were procured from the central animal house of Panjab University, Chandigarh and were acclimatized in the department animal house for 2 wk in plastic cages under hygienic conditions. The animals were divided into control and experimental groups. Control rats were fed on standard animal chow and water *ad libitum*. The treatment group animals were further divided into 3 subgroups viz., Gr. II-IV and supplied with the oral administration of 4.6, 9.2 and 13.8 g dehydrated ethanol (Bengal Chemicals and Pharmaceuticals Ltd., Bombay, India) (10, 20 and 30% v/v), respectively for 8 wk. At the end of the study, the rats were sacrificed by decapitation under deep anesthesia. The study was approved by Institutional Animal Ethical Committee, Panjab University, Chandigarh, India.

**Sample Preparation**

Rats of each control and experimental group were sacrificed to resect tibiae bones. The bone marrow was flushed out with normal saline after giving a cut on diaphyseal ends of the bone and the dry weight of bones of each groups were taken and powdered in pestle and mortar. The samples were defatted with chloroform/ methanol (1:1 in volume). Then the residue was demineralized in 0.5 M EDTA/50 mM Tris-HCl (pH 7.4) containing 1M NaCl. Demineralization was continued for 2 wk after which the demineralized bone powder was dissolved in 0.1 mL of 0.1N NaOH. 20 µL of sample was diluted to 100 µL by addition of 80 µL of distilled water. Proteins and nucleic acids were estimated in the demineralized bone powder solution\textsuperscript{22,23}.

**Biochemical estimations**

The biochemical estimations included the determination of nucleic acid (DNA and RNA), protein concentration and ash content in control and treated rat bone powder.

**DNA estimation**

DNA was estimated by diphenylamine (DPA) method. To 2 mL of sample, 4 mL of DPA reagent was added and the samples were kept in boiling water bath at 90°C for 10 min. After cooling, the optical densities (OD) of the samples were read at 595 nm with double beam spectrophotometer. DNA content was estimated using the linear standard curve of standard solution of DNA.

**RNA estimation**

RNA content was measured by Orcinol method. To 2 mL of sample, 3 mL of Orcinol reagent was added and the samples were kept in boiling water bath at 90°C for 15 min. After completion of incubation period the samples were cooled and the colour formed was read at 665 nm. RNA content was estimated using the linear standard curve.
Protein estimation

Proteins were estimated in the bone powder by the method of Lowry et al.\textsuperscript{22}. To 20 µL sample, 680 µL of 
\( \text{dH}_2\text{O} \) and 3 mL of Lowry’s reagent were added. After mixing thoroughly, the samples were incubated at 
room temperature (22-27°C) for 10 min. Then 300 µL of 1N Folin’s reagent was added and further incubated for 30 min at 37°C. The blue colour so 
formed was read for its absorbance at 620 nm with 
double beam spectrophotometer. Protein concentration in the sample was determined from a 
linear BSA standard curve.

Ash content analysis

The ash content of the bone specimens of both control and treated groups was measured to get the 
percentage of organic and inorganic matrix in bone. Six bone samples of control and treated groups were 
placed in crucible and heated to 800°C for 24 h in a muffle furnace (Laboratory Model, manufactured by 
Narang Scientific Works Pvt. Ltd., New Delhi). Taking the initial and final weight of the samples the 
percentage weight loss for each group gave the ash content.

Thermal gravimetry analysis (TGA)

The powdered form of control and treated bone 
samples Gr.II-IV were placed in the crucibles and 
their initial weight was recorded. The technique was 
carried on TG-assembly (851 E Metallur Toledo) 
which utilizes a microbalance to measure the weight 
loss continuously as a function of temperature. The 
temperature can be adjusted as it is regulated by the 
flow of helium gas. The temperature was increased at 
a constant rate of 10°C/min and was increased up to 
1000°C for all the samples. A moving light point 
indicating an increase of temperature and 
corresponding weight loss in the material is recorded 
on the paper to develop TG analysis of the sample.

Statistical analysis

Statistical analysis was performed by Students ‘t’ 
Test to estimate the significant difference between the 
mean values of two groups.

Results

Biochemical estimations

Nucleic acid concentration

Nucleic acid (DNA and RNA) concentration in 
the tibial bone powder of control and treated rats 
were estimated. The results showed insignificant

increase in the DNA content of Gr. II compared to 
control but a significant (\( P < 0.05 \)) decrease in DNA 
concentration was observed at higher alcohol doses 
i.e. Gr. III and IV. Similar trend was observed for 
RNA content (Fig. 1).

Protein estimation

The protein content of tibia of control and 
treatment groups of rats was analyzed. The results 
showed an insignificant increase in total protein 
content in Gr. II and a significant (\( P < 0.01 \)) decrease 
was observed in Gr. III and IV as compared to the 
control (Fig. 2).

Ash content analysis

The ash content was measured as shown in the 
Table 1. The bones of control group contained

<table>
<thead>
<tr>
<th>Sample</th>
<th>( % ) Weight loss</th>
<th>Mineral content (%)</th>
<th>Organic content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(70-270°C)</td>
<td>(270-550°C)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.15</td>
<td>18.28</td>
<td>72.5</td>
</tr>
<tr>
<td>Gr. II</td>
<td>9.04</td>
<td>16.66</td>
<td>74.3</td>
</tr>
<tr>
<td>Gr. III</td>
<td>9.76</td>
<td>21.6</td>
<td>68.94</td>
</tr>
<tr>
<td>Gr. IV</td>
<td>11.79</td>
<td>21.88</td>
<td>66.33</td>
</tr>
</tbody>
</table>

Fig. 1 — Variations in nucleic acid content of control and treatment 
groups. [The star marks signify a significant decrease in nucleic 
acid concentration in Gr. III and IV. *\( (P < 0.05) \) wrt Control]

Fig. 2 — Variations in protein content of control and treatment 
groups. [The star marks signify a significant decrease in protein 
content in Gr. III and IV. ** \( (P < 0.01) \) wrt Control]
approximately 30% organic matrix and 70% inorganic matrix. A relative increase in the values (74.3%) of mineral content of Gr. II was observed in comparison to control (72.5%). However, in case of Gr. III and IV the loss of inorganic matrix increased with the increasing doses of ethanol. The mineral content was found to be 68.9 and 66.33%, respectively. A relative increase in the organic matrix from 25.7% of Gr. III to 33.67 % of Gr. IV was observed.

Thermogravimetric (TGA) analysis
Thermal analysis of bone powder of control and treated groups were carried out. Tables 1 and 2 summarize the results of % weight loss by dry heating of all samples from the control and treated groups at temperatures 70-270, 270-550 and 650-850°C. At all stages, the %weight loss in Gr. III and IV were higher than that of control, while Gr. II exhibited a lesser loss of weight. At 270-550°C, the %weight loss in Gr. III and IV was 21.6 and 21.88%, respectively (Table 1), while in Gr. II bone sample it was 16.66% as compared to control (18.28%) (Table 2). Similarly, decrease in % weight loss in Gr. II (3.22%) as in contrast to control (3.6%) was observed which corresponded to an insignificant increase in carbonate fraction of bone in Gr. II. Similarly, a steep increase in % weight loss of 5.29% in Gr. III to 5.97% in Gr. IV was observed (Table 2).

The mineral content of bone was also found to be 72.5% in control that reduced to 68.94 and 66.33% in Gr. III and IV, respectively. In Gr. II, however, the mineral content was found to be greater (74.3%) than the control (Table 1). The % weight loss in Gr. III and IV followed an ascending trend but there was lesser loss in weight in Gr. II as summarized in Table 1. Conversely, the organic content of treatment Gr. III and IV increased to 31.36 and 33.67%, respectively from 27.5% in control (Table 1).

Discussion
The present study provides substantial evidence of the dose dependant effects of alcohol consumption on bone remodelling. The results have demonstrated that lower alcohol doses are actually beneficial for imparting thermal strength and durability to the bones. However, higher alcohol doses and long-term alcohol consumption disrupt the ongoing balance between the bones desorption and remodelling of bone tissue, contributing to alcoholic bone that results in decreased bone density and increased risk of fracture. Various studies suggest that these effects are exerted directly or indirectly by affecting physiology of bone cells, hormones metabolism including parathyroid hormone (PTH), calcitonin and vitamin D, and growth factors that regulate bone metabolism24-26. The imbalance may result in part from alcohol induced inhibition of osteoblast and other specialized cells that are responsible for the production of new bone cells. Small doses of alcohol cause calcium to leave body fluids such as blood, entering into the cells and increase the production of PTH, a hormone responsible for the transport of calcium from bone into the body fluid (blood). Higher alcohol doses are associated with hypocalcemia and impaired ability to produce PTH24. Moreover, level of calcitonin has been found to increase briefly during short term alcohol consumption. Calcitonin is the hormone that, contrary to PTH, helps in deposition of calcium on bones, and thus enhanced levels of calcitonin helps in strengthening of bones as observed in our study25,26. The otherwise negative effects of alcohol consumption are also related to low levels of activated vitamin D and proteins that protect the vitamin D during transport27. These effects have been observed equally in male as well as female subjects, however, there are significant evidences of the development of osteoporosis in post-menopausal women as well as men with gonadal dysfunction28. It is well known that estrogen plays a vital role in suppressing osteoclastogenic cytokine production in T-cells and osteoblasts29. In addition to suppressing these cytokines, estrogen has been shown to induce the apoptotic death of osteoclasts30. During peri-menopause and post-menopause, the levels of
Estrogen reduce and the bone desorption outpaces bone resorption laying the foundations of osteoporosis and bone fracture. Alcoholic men frequently have decreased levels of the male steroid hormone testosterone (produced mainly in the testes), and female alcoholics experience increased metabolic conversion of testosterone (produced in the ovaries and adrenal glands) to the female steroid hormone estradiol. Because estrogen deficiency is a major contributing factor for the development of osteoporosis, alcohol might indirectly affect bone through estrogen.

Further in the study, the effects of alcohol doses on nucleic acids of bone cells have been investigated. The results of nucleic acid estimation in bone powders of control and treated bones showed a decreasing concentration of DNA and RNA in the treatment groups III and IV subjected to 20 and 30% ethanol intake, respectively. These results are in agreement with the studies of Preedy and Peters who demonstrated that nucleic acids and protein content were reduced in bones of ethanol fed dietary restricted rats. Studies have also suggested that ethanol is capable of having deleterious effects at cellular level in bone. Two separate groups have observed a dose-dependent reduction in bone cell DNA synthesis, as assessed by incorporation of [3H] thymidine. In similar studies on chick calvarial cells, observed an inhibition of osteoblast proliferation by ethanol.

Similar to nucleic acids, the protein concentration was also found to be significantly (P < 0.01) deteriorating in the treatment groups at higher ethanol doses compared to the control group. There was, however, no change in total protein content in Gr. II. The decrease in the total protein content was significant at higher doses of ethanol indicating alcohol affects the osteoblastic activity and their number. Chronic consumption of ethanol causes anti-proliferative effect on osteoblast cells.

Surprisingly, lower doses of alcohol (10%) treatment in Gr. II resulted in an increase in inorganic mineral content of bone as compared to that of control. This may be due to the fact that bone mineral density is augmented under the effects of moderate doses of ethanol (Table 1). However, at higher doses the trend reversed and it was observed that the inorganic mineral content of bones of rats belonging to Gr. III and IV degraded further. The bone tissue became more flexible and soft with increasing demineralization making the bone mechanically incompetent. These observations support the earlier studies that mineral content is an important parameter, which determines the bone strength.

Thermal analysis of bone powder of control and treated groups were carried out in which a relative increase in the % weight loss in by the samples of Gr. III and IV compared to control has been observed. However, Gr. II showed a slight decrease in % weight loss as compared to the control. This shows that intake of higher doses of ethanol causes decrease in mineral content and also that the mineral content affects the stability of bone collagen i.e. it stabilizes bone collagen thermally.

Weight loss has been computed in three distinct phases. Initially, a gradual weight loss was observed up to 270°C; the second phase is that of rapid weight loss between 270-550°C. Third phase shows a gradual weight loss above 650°C. Dry heating from 70-270°C corresponds to the loss of water (adsorbed moisture and tissue bound water) whereas dry heating from 270-550°C corresponds to the combustion of the organic fraction in bone (fat, collagen, bone marrow) which leads to evolution of combustion gases (water and carbon dioxide) and pyrolysed material (giving methyl ions as detected by mass spectrometer).

At 550°C, the % percentage weight loss is due to water and organic content of bone. Thus, values of mineral content of control and treated bone samples were computed and were found to be close to the values obtained from ash analysis (Table 1). Percentage weight lost between 270 and 550°C amounts to the weight loss due to organic phase. Results of thermal analysis of control and treated groups show the Gr. II exhibiting a decrease in %weight lost compared to control while a relative and a gradual increase in %weight loss in Gr. III and IV compared to control, indicating that increasing doses of ethanol intake may cause decrease in the mineral content (Table 1 and 2). Interestingly, there was an increase in organic content of bone at higher alcohol doses in Gr. III and IV as compared to control. Our results support the earlier studies by Kronick and Cooke in which they found increase in the thermal stability of collagen fibers with increased degree of calcification (as seen in doses of 10% ethanol intake). The mineral phase of bone is carbonated apatite i.e., calcium phosphate with some incorporated carbonate ions. Qualitative analysis of bone to determine the
thermal decomposition behaviour of bone\textsuperscript{41,44} show that water content of bone increases with increasing degree of demineralization\textsuperscript{45}. This is because the space occupied by mineral in bone is now being occupied by water, which supports the earlier studies that increase in mineralization is at the cost of available water volume\textsuperscript{46}. Percentage weight loss between temperature range of 650 and 850°C corresponds to the weight loss due to loss of carbonate fraction of bone\textsuperscript{42} and similar trend was obtained as mentioned above for the temperature range of 270-550°C. Decrease in % weight loss in Gr. II (3.22%) as compared to control (3.6%) was observed which corresponded to an insignificant increase in carbonate fraction of bone in Gr. II. Similarly, a steep increase in % weight loss of 5.29% in Gr. III to 5.97% in Gr. IV was observed, indicating a subsequent loss in the carbonate fraction of bone. Thus, decrements in the carbonate content are found in doses of higher concentration of 20 and 30% ethanol. However, increment is found in dose of 10% ethanol compared to control suggesting thereby that at lower doses ethanol may have beneficial effects on bone density. However, at higher doses, depletion of bone mineral starts. The results are in accordance with the previous findings\textsuperscript{45-48}. All these results confirm that chronic consumption of ethanol not only leads to a lower mineral (inorganic) content but also results in a sharp increase in water and organic content of bone which consequently, interferes with the bone metabolism resulting in augmented risk of osteoporotic damages\textsuperscript{49}.

Conclusion

The present study evidently shows that administration of alcohol at higher doses impairs the osteoblasts number and activity by reducing the nucleic acid, protein and inorganic minerals content of bones. Moreover, the stability of bones is also severely affected due to high alcoholic intake, resulting in bone demineralization. All these result in calcification of bones, thereby augmenting the risk of osteoporosis in susceptible individuals. The lower doses (10%) of alcohol, however, have been shown to result in increased bone density and stability. The present work, thus, can be considered as a foundation for further investigation on detailed dose dependent effects of alcohol on bone structure and the risk of osteoporosis.

References


