Vitamin E improved bone strength and bone minerals in male rats given alcohol

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A R T I C L E I N F O	A B S T R A C T
	Objective(s): Alcohol consumption induces oxidative stress on bone, which in turn increases the risk of osteoporosis. This study determined the effects of vitamin E on bone strength and bone mineral content in alcohol-induced osteoporotic rats. ***Materials and Methods:*** Three months old Sprague Dawley male rats were randomly divided into 5 groups: (I) control group; (II) alcohol (3 g/kg) + normal saline; (III) alcohol (3 g/kg) + olive oil; (IV) alcohol (3 g/kg) + alpha-tocopherol (60 mg/kg) and (V) alcohol (3 g/kg) + palm vitamin E (60 mg/kg). The treatment lasted for three months. Following sacrifice, the right tibia was subjected to bone biomechanical test while the lumbar (fourth and fifth lumbar) and left tibia bones were harvested for bone mineral measurement. ***Results:*** Alcohol caused reduction in bone biomechanical parameters (maximum force, ultimate stress, yield stress and Young's modulus) and bone minerals (bone calcium and magnesium) compared to control group (*P*<0.05). Palm vitamin E was able to improve bone biomechanical parameters by increasing the maximum force, ultimate stress and Young's modulus (*P*<0.05) while alpha-tocopherol was not able to. Both alpha-tocopherol and palm vitamin E were able to significantly increase tibia calcium and magnesium content while only alpha-tocopherol caused significant increase in lumbar calcium content (*P*<0.05). ***Conclusion:*** Both palm vitamin E and alpha-tocopherol improved bone mineral content which was reduced by alcohol. However, only palm vitamin E was able to improve bone strength in alcohol treated rats.

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Introduction

Alcohol consumption is a global problem. According to a report by WHO, about 16.0% of drinkers aged 15 years or older engage in heavy episodic drinking worldwide (1). Based on National Health and Morbidity Survey 2011, it was reported that the average onset of drinking alcohol was 21 years old (2). Alcohol consumption has been associated with several deleterious effects such as liver injury (3), cognitive impairment (4) and impairment in embryo development (5).

Osteoporosis, a very common metabolic disorder of the skeleton, has been correlated with several risk factors. Among them is the strong association between low bone mass and chronic alcohol abuse (6). Alcohol abuse is also a risk factor for impaired fracture healing and its chronic abuse will lead to prolonged fracture repair (7). As a consequence, alcoholics who had fractures are associated with longer hospitalization and increased morbidity (8).

One factor that plays a central role in alcohol-induced osteoporosis is the excessive generation of free radicals, which in turn causes oxidative stress. The reactions in alcohol metabolism eventually lead to reactive oxygen species formation (9). Proinflammatory condition created by alcohol also lead to increase in cytokine production which may induce damage in many organs including bone (10).

Vitamin E, one of the lipid-soluble vitamins, was shown to have beneficial effects on bone in various animal models such as nicotine-induced rat model (11), ovariectomized rat model (12, 13) and orchidectomized rat model (14). Vitamin E (alpha-tocopherol and gamma-tocotrienol) has also been shown to exert anabolic effects on bone in normal male rats (15). Dose-response studies of alpha-tocopherol on bone have been carried out in normal female rats (16) and in mature male rats (17). Both studies found that high dose of alpha-tocopherol did not lead to bone loss, but in fact may exert beneficial effects on bone health. One of the mechanisms of vitamin E is its ability to scavenge free radicals and prevent oxidative damage (18).

Alcohol-induced osteoporosis is mediated via free radicals and lipid peroxidation as described above. The occurrence of osteoporosis can be debilitating and affect quality of life. In search for an alternative remedy to prevent alcohol-induced osteoporosis, vitamin E was postulated as a good choice due to its

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antioxidant properties. In order to prove the hypothesis, this study was carried out to determine the effects of vitamin E on bone strength and bone minerals in alcohol-treated rats.

Materials and Methods

Animals and treatments

Forty Sprague-Dawley male rats aging 3 months old weighing between 250-300 g were randomly allocated into 5 groups, 8 rats in each group. The selection of the gender and age of rats in this study was made based on previous study which has developed the binge alcohol model (19). These rats are considered young adults (20). The rats were kept in cages (with 12 hr light/dark cycle) and given rat chow and deionised water ad libitum. The groupings and the treatment regime are listed in Table 1.

Absolute alcohol 100% (Sigma, USA) was used in the study which was then prepared as 20% (vol/vol) ethanol/saline solution. For the dosage of 3 g/kg, 0.4 ml per 100 g body weight was administered intraperitoneally in the mornings of three alternate days a week and no injection for the remaining four days. This exposure model mimics binge drinking in human and the dose used was proven to exert effects on bone (21). The dosage of alcohol used in this study yielded blood alcohol concentration of nearing 300 mg/dl (19) which is equivalent to blood alcohol concentration commonly seen in chronic alcohol drinkers (22).

Three grams of palm vitamin E (Sime Darby, Malaysia) or alpha-tocopherol (Sigma, USA) was dissolved in 50 ml olive oil (Bertolli, Lucca, Italy) to obtain a solution of 60 mg/kg rat weight. An amount of 0.1 ml per 100 g of body weight was given to the rats via oral gavage. Palm vitamin E used in this study contained 162.2 mg/g alpha-tocopherol, 167.1 mg/g alpha-tocotrienol, 35.4 mg/g beta-tocotrienol, 266.2 mg/g gamma-tocotrienol and 90.7 mg/g delta-tocotrienol. The dosage of vitamin E used in this study has been shown to be effective in preserving bone in other osteoporotic animal models (11, 23). Based on calculations described by Nair & Jacob (24), 60 mg/kg dose in rats is equivalent to 583 mg/day in a 60 kg human.

The group given normal saline served as negative control. The AO group was given olive oil which was the vehicle for vitamin E. This group was to ensure that any effects seen in the vitamin E treated groups are due to vitamin E and not olive oil. Normal saline, olive oil, palm vitamin E and alpha-tocopherol were given daily via oral gavage according to the respective treatment regime.

At the end of the treatment period, rats from each treatment group were anesthetized with diethyl ether and sacrificed by cervical dislocation. The left and right tibias and 4th and 5th lumbar vertebrae were harvested and stored at -70 °C.

Table 1. Animal groupings and its treatments

Groups	Month 1	Month 2 and 3
I Control group (C)	No treatment	No treatment
II Alcohol normal saline (AN)	Alcohol 3 g/kg	Normal saline
III Alcohol olive oil (AO)	Alcohol 3 g/kg	Olive oil
IV Alcohol alpha-tocopherol (AA)	Alcohol 3 g/kg	Alpha-tocopherol 60 mg/kg
V Alcohol palm vitamin E (AE)	Alcohol 3 g/kg	Palm vitamin E 60 mg/kg

Biomechanical testing

The right tibia was subjected to three-point bending and breaking test using the Dynamic Microtester, Instron 5848 Microtester (Instron Corp, USA). A primary force of 1 N was used to fix the tibia on the device and the stress applied was recorded. The loading rate used was 50 mm/min and the automatic switch off pressure was set at 300 N.

The parameters measured and recorded included the maximum load, ultimate stress, Young's Modulus and yield stress. Maximum load (F_{max}) is the maximum force that can be withstood by the bone before breaking. Ultimate stress is the maximum stress the bone can sustain before breaking. Young's Modulus is a measurement of stiffness which represents the material's ability to resist deformation. Yield stress or yield point is the point at which the properties of the bone changes from elastic to plastic. It represents a gradual transition above which stresses begin to cause permanent damage such as trabecular microfracture. The three-point bending test has been developed to evaluate stiffness and strength of a bone (25). All parameters were analysed using Bluehill2 software.

Bone mineral measurement

After sacrifice, the left tibia and the 4th and 5th lumbar vertebrae were dissected out and cleansed of all soft tissues. Bones were then dried in the oven at 100ºC for 24 hr. Then, bones were ashed in a furnace at 800ºC for 14 hr and dissolved in 3 ml of nitric acid at 65% concentration. The solutions obtained were then diluted in lanthanum chloride and further diluted with deionized distilled water in which the dilution factor of tibial bones was 625000X while the lumbar vertebrae was 250000X. The calcium and magnesium content were measured with an atomic absorption spectrophotometer (Shimadzu AA-680, Shimadzu Corporation, Kyoto, Japan) in which calcium's wavelength was 422.7 nm while magnesium was 285.2 nm.

Statistical analysis

SPSS version 20 (SPSS Inc, Chicago, USA) was used for the statistical analysis. The data were tested for normal distribution using Shapiro-Wilk normality test. For parametric data, One Way ANOVA with Tukey's *post-hoc* test was used while Kruskal Wallis

Table 2. The effects of vitamin E supplementation on right tibia bone biomechanical parameters in alcohol treated rats

Groups	Maximum force (N) (n=8)	Ultimate stress (MPa) (n=8)	Young's modulus (MPa) (n=8)	Yield stress (MPa) (n=8)
Control group (C)	193.14 ± 24.66	17.46 ± 2.80	0.70 ±0.11	21.65 ±3.43
Alcohol normal saline (AN)	128.28 ± 9.16*	9.53 ± 0.68	0.36 ±0.04**,c	7.24 ±0.37**
Alcohol olive oil (AO)	111.97 ± 6.00*,a	8.31 ± 0.45*,b	0.24 ±0.02**,c,d	7.45 ±0.49**
Alcohol alpha-tocopherol (AA)	120.89 ± 5.80*	8.97 ± 0.43*	0.30 ±0.03*,e	7.46 ±0.47*
Alcohol palm vitamin E (AE)	152.68 ± 11.36a	11.31 ± 0.84b	0.43 ±0.04d,e	8.80 ±0.91*

Data presented as mean ± standard error of the mean (SEM)
C – control group; AN – rats induced with 3 g/kg alcohol followed by normal saline; AO – rats induced with 3g/kg alcohol followed by olive oil; AA – rats induced with 3 g/kg alcohol followed by 60 mg/kg alpha-tocopherol; AE – rats induced with 3 g/kg alcohol followed by 60 mg/kg palm vitamin E; *,** indicate significant difference compared to control group ($P<0.05$ and $P<0.01$ respectively); Groups which share the same alphabet indicate significant difference ($P<0.05$)

and Mann Whitney U-test were used for non-parametric data. The results were presented as mean±standard error of the mean and the level of significance was set at $P<0.05$.

This study has been approved by the institution's Animal Ethics Committee.

Results

Bone biomechanical

Alcohol caused a reduction in bone maximum force as seen in AN and AO groups compared to control group ($P<0.05$) (Table 2). Palm vitamin E (AE group) was able to increase the maximum force which was significantly higher than alcohol olive oil group ($P<0.05$). Alpha-tocopherol failed to increase the bone maximum force in alcohol treated rats ($P=0.57$ vs AN; $P=0.19$ vs AO). However there was no significant difference between AE and AA groups ($P=0.08$).

There was a significant reduction in ultimate stress for AO group compared to control ($P<0.05$). Palm vitamin E was able to increase the ultimate stress which was significantly different compared to AO group ($P<0.05$). This effect was not seen in alpha-tocopherol treated rats ($P=0.19$ vs AO group). In addition, there was no significant difference between alpha-tocopherol and palm vitamin E ($P=0.08$).

Alcohol decreased Young's Modulus in the two alcohol groups (AN and AO) compared to the control group ($P<0.01$). In addition, Young's Modulus of alcohol olive oil group was significantly lower than alcohol normal saline group ($P<0.05$). Palm vitamin E was able to reverse these effects and increase Young's Modulus significantly compared to alcohol olive oil ($P<0.05$). However, alpha-tocopherol did not produce the same effects ($P=0.81$ vs. AN; $P=0.11$ vs. AO). No significant difference was observed between AE and AA groups ($P=0.24$).

The alcohol treated groups (AN and AO) had lower yield stress value compared with the control ($P<0.01$). Both alpha-tocopherol and palm vitamin E were not able to increase the yield stress of alcohol treated rats

($P>0.05$). In fact, the values for the AE and AA groups were still lower than the control group ($P<0.01$).

Bone Minerals

Bone minerals analysis was done to measure the levels of major minerals found in bone i.e. calcium and magnesium. Figure 1 shows the results of calcium content in bones of the respective groups: a) tibia bones and b) lumbar bones. The AN group had a significant reduction in tibia calcium content compared to the control group ($P<0.01$). The AN group also had lower tibia calcium content than AO group ($P<0.01$). Both alpha-tocopherol and palm vitamin E were able to reverse the effect of alcohol and caused a significant increase in tibia calcium content compared to AN group ($P<0.01$). However, there was no significant difference between alpha-tocopherol and palm vitamin E treated groups ($P=0.43$).

In lumbar bones, alcohol caused a reduction in calcium content of AO group compared to the control group ($P<0.05$). Alpha-tocopherol was able to reverse alcohol's effect and cause a significant increase in lumbar calcium content compared to AO group ($P<0.01$). Palm vitamin E also showed a tendency to cause an increase in lumbar calcium content compared to AO group but the effect was not significant ($P=0.06$). There was no significant difference between alpha-tocopherol and palm vitamin E treated rats ($P=0.89$).

The results of magnesium content in bones are shown in Figure 2: a) tibia bones and b) lumbar bones. For magnesium content in tibia bones, both alcohol groups (AN and AO) had lower values compared to control group ($P<0.01$). In addition, magnesium content of tibia in AN group was significantly lower than AO group ($P<0.05$). Both alpha-tocopherol and palm vitamin E were able to cause a significant increase in tibia magnesium content compared to alcohol normal saline and alcohol olive oil groups ($P<0.05$). However, the tibia magnesium content for alcohol alpha-tocopherol (AA) group was still significantly lower than the control group ($P<0.05$). There was no significant
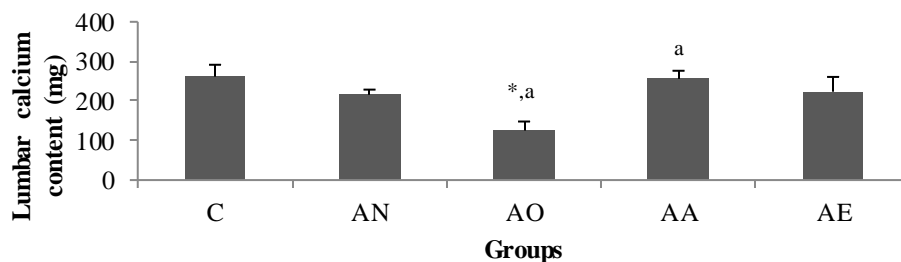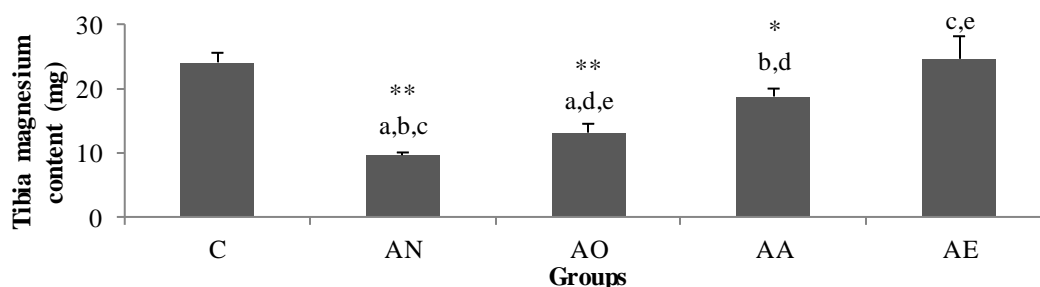
a) Tibia bones
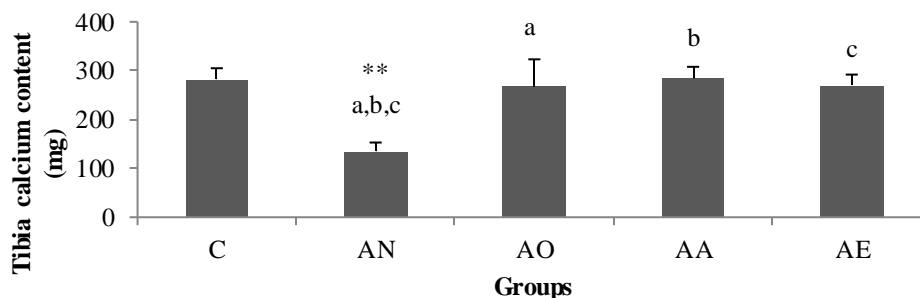
b) Lumbar bones

Figure 1. The effects of vitamin E supplementation on bone calcium content of a) tibia bones and b) lumbar bones in rats treated with alcohol
C – control group; AN – rats induced with 3 g/kg alcohol followed by normal saline; AO – rats induced with 3 g/kg alcohol followed by olive oil; AA – rats induced with 3 g/kg alcohol followed by 60 mg/kg alpha-tocopherol; AE – rats induced with 3 g/kg alcohol followed by 60 mg/kg palm vitamin E; Data presented as mean ± standard error of the mean (SEM); *,** indicate significant difference compared to control group ($P<0.05$ and $P<0.01$ respectively); Groups which share the same alphabet indicate significant difference ($P<0.01$)
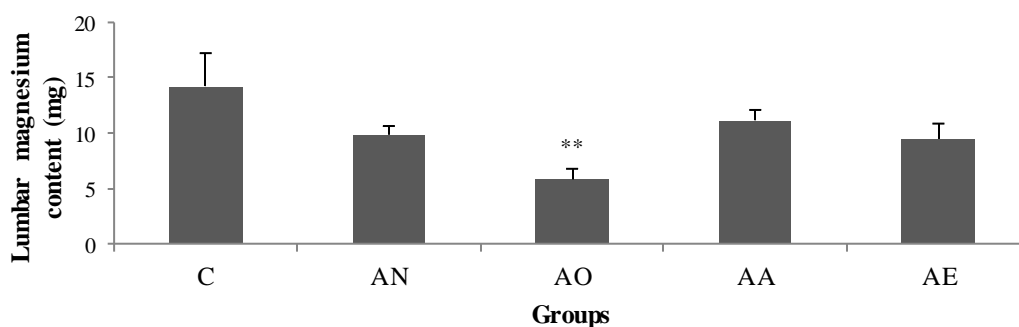
a)   Tibia bones

b) Lumbar bones

Figure 2. The effects of vitamin E supplementation on bone magnesium content of a) tibia bones and b) lumbar bones in rats treated with alcohol
C – control group; AN – rats induced with 3 g/kg alcohol followed by normal saline; AO – rats induced with 3 g/kg alcohol followed by olive oil; AA – rats induced with 3 g/kg alcohol followed by 60 mg/kg alpha-tocopherol; AE – rats induced with 3 g/kg alcohol followed by 60 mg/kg palm vitamin E; Data presented as mean ± standard error of the mean (SEM); *,** indicate significant difference compared to control group ($P<0.05$ and $P<0.01$ respectively); Groups which share the same alphabet indicate significant difference ($P<0.05$)

difference in the tibia magnesium content between alpha-tocopherol and palm vitamin E treated groups (*P*=0.29).

Magnesium content in lumbar bones was significantly lower in the AO group compared to control group (*P*<0.01). The groups treated with alpha-tocopherol and palm vitamin E showed an increase in lumbar magnesium content compared to AO group, however no significant differences were observed (*P*=0.07 and 0.36 respectively).

Discussion

This study, which employed the binge drinking rat model, showed adverse effects of alcohol on bone. Alcohol was found to significantly reduce the bone biomechanical properties which suggest weak bone. The two alcohol treatment groups, i.e. AO and AN, showed significantly lower value for all biomechanical parameters as compared to the control except for ultimate stress in AN group. Even though the ultimate stress parameter for AN group did not differ as compared to the control group, the decreasing trend similar to other parameters is noted. Alcohol may pose different effects when given for a short duration. One study showed that acute doses of alcohol increase bone strength but when the duration of alcohol treatment is increased, bone strength is decreased (26). The varying effects of alcohol according to its duration may contribute to the differences seen in our study.

The olive oil used in this study was regular olive oil which is also used as the solvent for palm vitamin E and alpha-tocopherol. Based on the present study, the AO and AN groups had comparable bone biomechanical parameter values except Young's modulus parameter in which the AN group had higher value than AO group. Comparable values indicate that the two alcohol groups did not differ and the olive oil did not cause any changes to the bone biomechanical parameters. However, difference was noted between the AO and AN groups in Young's modulus parameter. Olive oil contains several fatty acids such as oleic acid, linoleic acid and palmitic acid which exist in varying concentrations (27). The major fatty acid contained in olive oil is oleic acid. The composition of fatty acids especially the polyunsaturated fatty acids may be the reason for the difference in Young's modulus seen in the present study. Long chain polyunsaturated fatty acid has been shown to reduced bone turnover in a previous study (28) which may eventually affect bone strength. Further investigations are required to confirm this.

Despite the discrepancies outlined above, generally it was observed that alcohol induced a reduction in bone biomechanical properties. This was in line with the study done by Sampson (29) that reported alcohol had been shown to cause weak bones in adult. Another study stated that alcohol significantly reduced Young's Modulus of the tibia bones (30). In

the present study, alpha-tocopherol was not able to increase the bone biomechanical parameters. On the contrary, palm vitamin E was able to improve the biomechanical properties of the bone. This suggests that palm vitamin E is superior to alpha-tocopherol in improving bone biomechanical properties in rats given alcohol. Similar observations were reported by a previous study carried out in normal rats in which gamma-tocotrienol improved bone biomechanical properties better than alpha-tocopherol (15). In another study, it was noted that tocotrienols enriched fraction was superior to alpha-tocopherol in improving biomechanical properties in post-menopausal osteoporotic rats models (31). In an earlier study using acute pancreatitis model, both alpha-tocopherol and tocotrienols rich fraction exerted antioxidative actions and improved pancreatic inflammation. However, tocotrienols were superior with regards to its antioxidative effects (32).

The structural difference between these two types of vitamin E may be the reason for palm vitamin E being superior to alpha-tocopherol in this study. In one study, it was found that the unsaturated side chain of alpha-tocotrienol allows better penetration into tissues and renders it to be more potent in protecting against free radical-induced oxidative stress than alpha-tocopherol (33). In addition, tocotrienols also possess other properties such as anti-inflammatory (34) which may also contributed to the observations seen in this study.

Alcohol consumption has been associated with lower bone mineral density of the total femur and femoral neck in healthy young Korean women (35). Both men and women were affected by alcohol consumption which was seen as reduction in the volumetric bone mineral density (36). It was shown that ethanol administration caused a decrease in serum calcium level which disrupt calcium homeostasis and eventually lead to adverse effects on bone (37). Magnesium loss also occurs from several tissues due to alcohol consumption (38). This may be the reason for the reduction in bone calcium and magnesium in alcohol treated rats seen in this study.

However, this study showed some conflicting findings in terms of bone minerals. Rats treated with alcohol followed by normal saline (AN group) had the lowest tibial mineral content. Its levels were even lower than the rats receiving alcohol followed by olive oil (AO group). On the other hand, lumbar mineral content was lowest in the AO group. These conflicting findings did not reflect the biomechanical parameters which showed reduced bone strength by alcohol treatment. This suggests that reduction in bone strength seen in this study may be due to other factors other than mineral content. In one study, alcohol consumption by male rhesus macaques for 12 months showed no effects on bone mineral density but displayed reduction in intracortical bone remodeling

which may eventually lead to reduction in bone strength (39).

In addition, the AO group had significantly lower magnesium content than the control group in both tibia and lumbar bones (P<0.01). Calcium content was lower for AO group in the lumbar bones but not in the tibia bones. Previous studies showed conflicting findings. In one study rats which received liquid diet containing alcohol either for acute or chronic duration, did not show any changes in tibial bone magnesium content (40). In another study, liquid diet containing alcohol given to rats for 42 days caused a reduction in tibial bone minerals including calcium and magnesium (41). Different duration and different route of administration may be the reason for the variations seen in the mineral results.

With regards to the different types of bone, both cortical and trabecular were affected by alcohol consumption in men while in women, bone mineral density changes were seen more in trabecular (36). In the present study, minerals loss was observed in both tibia and lumbar vertebra bones. Distribution of cortical and trabecular bone varies between these two bones. The proportion of trabecular bone is higher compared to cortical bone in lumbar vertebra (42) while tibia is one of the bones which has high cortical content (43). Based on the present findings, we conclude that alcohol affect the mineral content of both cortical and cancellous bones.

In terms of vitamin E supplementation, the results of the present study were in accordance with the previous reported literature (23, 44). However, it was also shown that palm vitamin E increased lumbar magnesium content in vitamin E deficient rats (45). This was not observed in the present study which showed that palm vitamin E did not affect lumbar magnesium content but only affected tibia magnesium content. In addition, palm vitamin E did not affect lumbar calcium content as opposed to alpha-tocopherol which increased lumbar calcium content compared to the AO group. The differences could be due to different rat models that were used i.e. vitamin E deficient rats and alcohol-induced rats. Different model may exert different mechanisms in causing bone loss and the effects of vitamin E may also differ depending on the models used. In one study, bone changes differ according to different bone sites and depend on the mechanical needs and metabolic demands of the bone (46). This may also explain the differences observed in this study between the tibia and lumbar bones which possess different bone composition.

Alcohol consumption has been associated with oxidative stress which inhibits Wnt signaling in bone which leads to inhibition of bone formation (47). In addition, alcohol also affects bone related gene expression such as osteocalcin, alkaline phosphatase and bone morphogenetic proteins (48). These effects

of alcohol may lead to impairment of bone formation thus causing bone mineral loss and weakening of the bones as seen in this study. In human, it was found that low alcohol drinkers had lower risk of hip fracture as opposed to high alcohol drinkers which showed higher risk of hip fracture (49).

Oxidative stress is associated with low bone mineral density, a condition which seemed to be worsen if low serum levels of vitamin E was also present (50). Low levels of serum vitamin E have also been associated with osteoporosis which was expressed by lower bone mineral density (51). In another study, low intake and low serum concentrations of alpha-tocopherol were associated with an increased fracture risk in elderly men and women (52). Vitamin E may confer the bone protective effects via its antioxidant properties. In addition to its antioxidant properties, palm vitamin E has been shown to regulate bone related gene expression in nicotine-induced rats (53). The same mechanism may also be involved in alcohol-induced rats. However, this needs to be ascertained further.

The relationship between alcohol consumption and inflammation process has been noted in which alcohol is associated with increased cytokines and production of reactive oxygen species (10). This inflammatory condition may lead to various systemic diseases including bone disorder. Tocotrienols have been shown to exert anti-inflammatory effects (54) and may exert protective effects on bone via this property. In another study, vitamin E was shown to have anabolic effects on bone (15) which may also improve the bone parameters in rats treated with alcohol as observed in the present study.

This study reported on bone strength and bone minerals of rats treated with alcohol. Even though alcohol has been shown to be detrimental to bone in numerous previous studies, this study highlighted the beneficial effects of vitamin E in reversing the effects of alcohol on bone. Further investigations need to be carried out, such as bone histomorphometry, bone biochemical markers and other major bone mineral such as phosphate and citrate, which can complement the findings of this study.

Conclusion

Both palm vitamin E and alpha-tocopherol reversed bone mineral loss induced by alcohol consumption. However, only palm vitamin E was able to improve bone strength in the alcohol-treated rats.

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