Journal of Clinical Toxicology
ISSN: 2161-0495

Shen et al., J Clinic Toxicol 2014, S7
DOI: 10.4172/2161-0495.S7-004

Research Article Open Access

Alcohol-induced Bone Loss and Quality during Adolescence is Improved by Green Tea Polyphenols

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Abstract

Our previous studies have shown significant osteo-protective effects of green tea polyphenols (GTP, green tea extract) in various bone loss models. To test the hypothesis that green tea supplementation would protect against binge alcohol-induced deterioration of bone quality in adolescent drinkers, we used a similar approach with green tea supplementation in drinking water. Using a six week, 2 × 3 factorial design of treatment × dose in male Sprague Dawley (SD) adolescent rats, bone parameters [femoral and lumbar vertebrae-4 (LV-4) area (BMA), bone mineral content (BMC), bone mineral density (BMD)], bone turnover biomarkers [serum osteocalcein (OC) and tartrate-resistant acid phosphatase-5b (TRAP-5b)] and blood chemistry were measured. The blood chemistry results showed that alcohol administration significantly decreased albumin, glucose, alkaline phosphatase, and amylase levels; increased globulin, phosphorus, creatine kinase, cholesterol, and potassium levels; and had no effect on protein, calcium, blood urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, sodium, and chloride. GTP supplementation significantly suppressed alkaline phosphatase levels and had no impact on other blood chemistry parameters. Alcohol administration lowered BMA, BMC, and BMD of the femur and LV-4, as well as serum TRAP-5b, but had no impact on serum OC. Supplementation of GTP into the drinking water increased BMD for the femur and BMC for the LV-4. GTP had no effect on scanned BMA or serum OC concentration. There was an interaction between the alcohol administration and GTP dosage in serum TRAP-5b and several parameters of bone strength, which reduced the negative effect on alcohol-induced bone modeling. In summary, our results show that a 6-week binge alcohol administration lowered bone mineral content, density, and strength in femura, reduced proximal tibial trabecular bone volume and lumbar vertebrae, and decreased cortical thickness at the tibial mid-diaphysis. Supplementation of GTP into the drinking water increased femoral bone mineral density and tibial cortical thickness at the mid-diaphysis. Importantly, GTP supplementation into drinking water improved overall bone quality in young binge-alcohol treated male rats through suppressing bone turnover rate.

Keywords: Adolescent; Alcohol consumption; Binge drinking; Blood chemistry; Bone mineral density; Bone quality; Dietary polyphenols; Green tea; Rat

Introduction

The relation between the consumption of alcohol and bone mineral density (BMD) in humans is a J-shaped dose response, with low-to-moderate consumption reported to increase BMD [1,2], and high, chronic alcohol consumption reducing BMD [3,4]. Bone health parameters in addition to BMD are also negatively affected by chronic alcohol consumption [5] resulting in an increased risk of fractures, osteopenia, and osteoporosis [6,7]. While most human studies have focused on alcohol use in adults, adolescence is an important period in bone development and is often a time when harmful drinking habits are initiated [8,9]. Binge drinking, a common practice during adolescence, does not appear to provide the same benefit to bone as non-binge drinking of equivalent monthly amounts [10] and, therefore, may be harmful to life-long bone development. Thus, it is important to understand how binge drinking in adolescence affects bone health, and to devise approaches to combat the negative consequences during this critical period of bone development.

Alcohol exposure in adolescent and adult rodents is known to negatively affect several bone health parameters such as suppression of bone formation, elevation of bone resorption, and loss of cancellous bone [11]. The rodent model parallels many of the effects seen in human alcoholics and heavy drinkers regardless of whether the alcohol is administered by intraperitoneal injection [12], intragastric gavage [13], a liquid diet [13], or as vapor [14]. The mechanisms underlying ethanol effects on bone physiology are not completely understood, but a dysregulation of bone formation and resorption is a common finding in humans [4] and rats [12,15]. Other contributing mechanisms may include ethanol-induced apoptosis and lipid droplet formation in osteocytes [16], vitamin deficiencies [17] and additional genetic changes [18]. As noted above, adolescence is a critical period for the attainment of peak bone mass and bone development. The effects of ethanol on bone during adolescent exposure can have life-long consequences [19]. Therefore, treatments that prevent or restore bone loss as well as micro structural deterioration during adolescence are urgently needed.

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Received December 20, 2013; **Accepted** January 09, 2014; **Published** January 20, 2014

Citation: Shen CL, Syapin PJ, Graef JL, Smith BJ, Brackee G, et al. (2013) Alcohol-induced Bone Loss and Quality during Adolescence is Improved by Green Tea Polyphenols. J Clinic Toxicol S7:004. doi:10.4172/2161-0495.S7-004

Green tea is one of the most popular beverages in the world, which importantly contains bioactive polyphenols that exert several beneficial effects on bone metabolism, including increasing bone formation and suppressing bone resorption [20-22]. As such, green tea polyphenols (GTP, extract of green tea) may be useful as a supplement to help prevent or reverse the detrimental effects of ethanol on bone. Our previous studies suggest the protective roles of GTP in bone loss and micro structural deterioration in a variety of bone loss models. Such an osteo-protective effect of green tea is probably due to its capacity in suppressing oxidative stress damage and chronic inflammation, or in increasing antioxidant capacities [21,23-26]. However, the effects of GTP on bone health in adolescence with chronic binge exposure are still unknown.

Therefore, the present study was designed to investigate the effects of GTP supplementation on bone turnover biomarkers, bone mass, microstructure, and quality (bone strength) in adolescent rats treated with repeated binge-like alcohol exposure. In addition, the blood chemistry profiles were also evaluated. We hypothesized that supplementation of GTP in drinking water would mitigate alcohol-induced bone loss, micro structural deterioration, and bone strength, without negatively affecting the blood chemistry profile. Studying the effects of GTP on bone health in adolescent rats with chronic binge exposure will potentially advance the understanding of their effects on bone health of binge-drinking adolescent humans.

Materials and Methods

Experimental design

A 2 × 3 factorial design was used and included two treatment groups [control (saline) and alcohol (3 g/kg, i.p.) and three doses of GTP supplementation (0.0, 0.1, and 0.5%)]. Intraperitoneal (i.p.) administration of alcohol is a well-established chronic binge alcohol exposure model [12,27] and was employed in this study. Alcohol-treated animals were given 3 g/kg (i.p.) treatments using 20% (vol./vol.) ethanol/saline solution on three consecutive days/week for 6 weeks. The dose of alcohol was chosen to achieve a peak blood alcohol concentration (BAC) of approximately 300 mg/dL [28]. Control animals were similarly treated with 0.9% saline in the same volume per body weight. No injections were given on the remaining 4 days each week in order to mimic a chronic binge alcohol-drinking pattern previously shown to induce bone loss and microstructure deterioration in male rats [12,27]. All blood and tissue collection/analyses were blinded to the experimenter.

Animals and GTP treatment

Forty-eight 30-day-old male Sprague-Dawley rats (Harlan Corp., Indianapolis, IN) were randomly assigned in one of six group treatments: (1) placebo (P) saline-i.p. injected; (2) alcohol-i.p. injected (A); (3) placebo+0.1% GTP (PLG) saline-injected plus 0.1% GTP (w/v) in drinking water; (4) alcohol+0.1% GTP (ALG); (5) placebo+0.5% GTP (PHG) saline-injected plus 0.5% GTP and; (6) alcohol+0.5% GTP (AHG) for 6 weeks. This 2 (placebo vs. alcohol) × 3 (0, 0.1%, and 0.5% (w/v) GTP in drinking water) factorial design allowed evaluation of the effects of alcohol, GTP levels, and any alcohol × GTP interaction. The doses of GTP were chosen based on our previous work, which demonstrated the significant osteo-protective effects of GTP [23,21].

All animals were fed, ad libitum, the pelleted AIM-93 G diet (DYETS, Bethlehem, PA) during the 6-week testing period. Rats had free access to double distilled water containing GTP at the appropriate level for each of the three doses. GTP water was prepared fresh daily and the amount of water consumed was recorded for each rat. Rats were housed in individual stainless steel cages under a controlled temperature of 21 ± 2°C with a 12 h light-dark cycle. Rats were weighed weekly with a final body weight just prior to termination of the study and collection of samples. All procedures were approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee.

Green tea polyphenols were purchased from Zheijan Yuxin Pharmaceutical Co., Ltd., China) with a purity higher than 98%. Every 1000 mg of GTP contained 653 mg of EGCG, 191 mg of epicatechin gallate (ECG), 99 mg of epicatechin (EC), 41 mg of epigallocatechin (EGC), and 16 mg of Catechin according to the HPLC-ECD and HPLC-UV analyses.

Sample preparation

After anesthetization, blood samples were drawn from the heart into vacutainer tubes and serum samples were isolated and stored at -80°C for later analyses. Femora and tibiae were harvested and cleaned of adhering soft tissue. The femur, tibia and lumbar vertebrate - 4 samples were stored in 70% ethanol at 4°C until analyzed.

Blood chemistry analyses

Blood chemistry analyses were determined using an automatic chemical analyzer at the College of Veterinary Medicine, Texas A&M University, College Station, TX. The parameters included total serum protein, albumin, globulin, albumin/globulin (A/G), calcium (Ca), inorganic phosphorus (Pi), glucose, blood urea nitrogen (BUN), alkaline phosphatase (ALP), creatinine phosphokinase (CK), aspartate transferase activity (AST), alanine transferase activity (ALT), amylase, cholesterol, sodium (Na), potassium (K), and chloride (Cl).

Serum bone biomarkers

Osteocalcin (OC) and tartrate resistant acid phosphatase-5b (TRAP-5b) in serum were quantitatively measured using commercial ELISA kits from Biomedical Technologies, Inc. (Stoughton, MA) and Immunodiagnostic Systems Ltd (Fountain Hills, AZ), respectively, following the manufacturer's instruction.

Femoral and vertebral bone densitometry

Total bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) of the whole left femur and LV-4 of each rat were determined by dual-energy X-ray absorptiometry (DXA) (HOLOGIC, Waltham, MA). Samples were scanned using the ultra-high resolution mode designed for small animal specimens. To simulate bone in soft-tissue, specimens were place in a weighing boat submerged in Millipore purified water (EMD Millipore, Billerica, MA). BMD and BMA were measured, and BMD was calculated by dividing BMC by BMA. The coefficient of variation was less than 1.0% [29].

Trabecular and cortical bone microarchitecture

Trabecular and cortical bone microarchitecture in tibia were assessed using micro-computed tomography (μCT) (MicroCT40, SCANCO Medical, Switzerland) as previously reported [29]. For evaluation of the proximal tibial metaphysis, 100 slices (16 μm each of 1.6 mm) distal to the proximal tibial growth plate were identified as the volume of interest (VOI), and analyses were performed in a 1024 × 1024 matrix resulting in an isotropic voxel resolution of 22 μm^3. An integration time of 70 milliseconds per projection was used. Trabecular bone morphometric parameters of the proximal tibial metaphysis included trabecular bone volume fraction (BV/TV), number (Tb.N), thickness (Tb.Th), and separation (Tb.Sp), structure model index (SMI), and trabecular connectivity density (Conn Dens) by analyzing

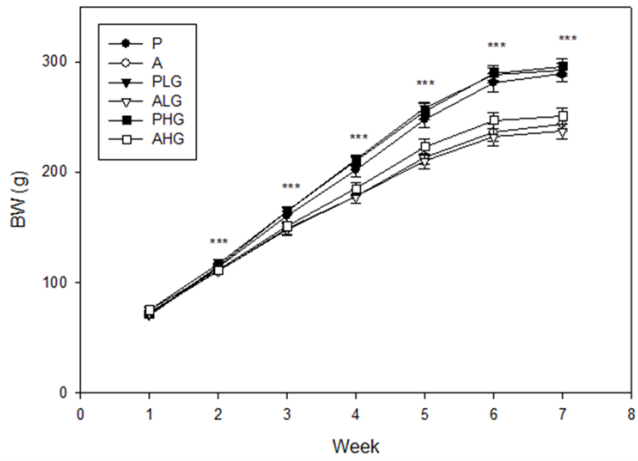
the VOI. Cortical bone evaluation was performed in the mid-diaphysis region by acquiring 30 images (16 µm each or 0.48 mm). Cortical bone morphometric parameters in tibiae included cortical thickness, cortical area, medullary area, and cortical porosity.

Bone quality assessment

Femoral quality (bone breaking strength) was evaluated by a three-point bending test using a custom-designed and built apparatus according to the procedures of Nielsen [30]. Descriptions of the terms used for the assessment of bone strength have been described previously [31]. The parameters of bone breaking strength included maximum force, energy to maximal force, slope, deflection, moment of inertia, bending moment, stress, strain, and modulus of elasticity [32]. Deformation was a measure of deflection. Bending moment was a measure of the amount of force withstood by the bone, whereas stress was a measure of force per unit area of bone. Stress allowed comparisons to be made between bones that differ in size and shape. The moment of inertia was a measure not only of the area over which the force was applied, but also of the shape in which the area was distributed. Stain was a measure of the amount of bending per unit of length that occurs as the bone was tested. The modulus of elasticity was a measure of the rigidity of the bone or, more simply, it was the stress to strain ratio [32].

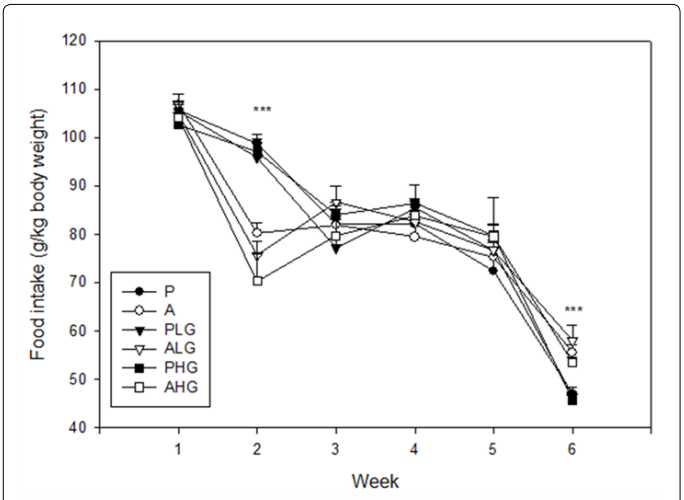
Statistical analysis

Data were analyzed with SigmaStat software (version 2.03, CA) and values were expressed as mean ± standard error of the mean (SEM). Normality of distribution and homogeneity of variance were tested. Body weight, food intake, and water consumption data were analyzed by three-way analysis of variance (ANOVA) (alcohol × GTP dose × time) followed by Fisher protected least significant difference (Fisher's LSD) *post-hoc* test to evaluate the effect of alcohol, GTP dose, time (week), or interaction. Data of blood chemistry, serum OC and TRAP-5b, and bone parameters were analyzed by two-way analysis of ANOVA (alcohol × GTP dose) followed by Fisher's LSD *post-hoc* test to evaluate the effect of alcohol, GTP dose, or interaction. The level of significance was set at $P<0.05$ for all statistical tests, and statistical trend ($P<0.10$) was indicated in some cases.



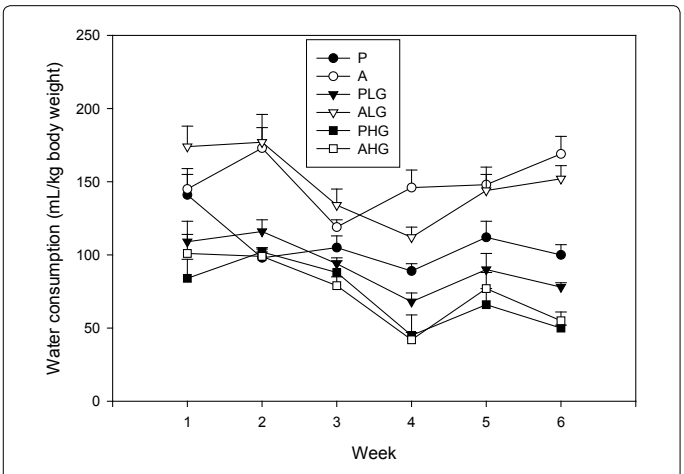
Alcohol Treatment Modestly Reduced the Rate of Body Weight Gain

Figure 1: Binge-alcohol treatment modestly reduced the rate of body weight gain over the six-week time-period of the experiment as shown by 2-way ANOVA and Fisher's LSD *post-hoc* analyses. No interaction effects were seen. Abbreviations are: P=placebo control, A=alcohol only treatment, PLG=placebo low GTP (0.1% green tea polyphenol), ALG=alcohol low GTP, PHG=placebo high GTP (0.5% GTP), AHG=alcohol high GTP. Statistics shown are mean ± SEM (standard error of the mean) and *p<0.05, **p<0.01, also need to change Y-AXIS as it makes the negative effect look larger.



Alcohol Treatment Modestly Affected Food Consumption

Figure 2: Binge-alcohol treatment reduced food consumption during the initial phase of the experiment as shown by 2-way ANOVA and Fisher's LSD *post-hoc* analyses. No interaction effects were seen. Abbreviations are: P=placebo control, A=alcohol only treatment, PLG=placebo low GTP (0.1% green tea polyphenol), ALG=alcohol low GTP, PHG=placebo high GTP (0.5% GTP), AHG=alcohol high GTP. Statistics shown are mean ± SEM (standard error of the mean) and *p<0.05, **p<0.01, ***p<0.001, NEED POST HOC data on graph.



Alcohol Treatment and Green Tea Supplementation Affected Fluid Intake

Figure 3: Binge-alcohol treatment affected fluid consumption during the initial phase of the experiment as shown by 2-way ANOVA and Fisher's LSD *post-hoc* analyses. Interaction effects were seen with water reduction. Abbreviations are: P=placebo control, A=alcohol only treatment, PLG=placebo low GTP (0.1% green tea polyphenol), ALG=alcohol low GTP, PHG=placebo high GTP (0.5% GTP), AHG=alcohol high GTP. Statistics shown are mean ± SEM (standard error of the mean) and *p<0.05, **p<0.01, ***p<0.001, NEED POST HOC data on graph.

Results

Body weight, food intake and water consumption

There was no difference in initial body weight (Figure 1), food intake (Figure 2), and water consumption (Figure 3) among all treatment groups. Over the course of the 6-week study, all animals

gained body weight. Alcohol-treated rats showed a modest, but significantly lower body weight gain than saline-treated rats at the 2nd week of experiment, and this trend was still evident throughout the rest of study period (Figure 1). GTP supplementation did not affect body weight, regardless of GTP concentrations used throughout the study period. No interaction between alcohol administration and GTP levels in body weight was observed.

In terms of food intake, at week 2, alcohol-treated animals had lower food intake than those treated with saline. Such a negative impact of alcohol was not observed from week 3 to week 5. Instead, at week 6 alcohol-administered animals increased food intake compared placebo-treated animals (Figure 2). In addition, both alcohol administration and GTP supplementation significantly affected water consumption throughout the study period. Except for week 1 and week 6, there were significant interactions between alcohol and GTP resulting in both the PHG and AHG groups having the lowest water consumption (Figure 3).

Blood chemistry

The 6-week alcohol administration resulted in a decrease in the values for albumin, A/G ratio, glucose, ALP activity, amylase activity, and Na/K ratio, while it caused an increase in the values for globulin, Pi, CK, and K (Table 1). In general, GTP supplementation did not affect any parameters of blood chemistry with the exception of suppressing serum ALP activity. An interaction (alcohol × GTP) effect was observed for serum cholesterol levels showing: (i) alcohol significantly increased serum cholesterol levels; (ii) GTP supplementation reduced the alcohol-related increase with the 0.5% GTP supplementation returning cholesterol to normal levels; and (iii) there was no difference among the AHG, P, PLG, and PHG groups (Table 1).

Bone densitometry and bone biomarkers

The effects of alcohol administration and GTP supplementation on bone mass and bone biomarkers are shown in Table 2. After 6 weeks of treatment, alcohol administration significantly compromised bone mass as indicated by decreased BMA, BMC, and BMD of femur and LV-4. GTP supplementation significantly mitigated the alcohol-induced loss of bone mass, in terms of BMC and BMD; while it did not affect BMA. In terms of bone biomarkers, neither alcohol administration nor GTP levels significantly affected serum OC after 6 weeks. There was an interaction between alcohol administration and GTP levels in TRAP-5b (P=0.004), but not in OC (P=0.258), showing increased TRAP-5b in the non-alcohol treated group with increasing GTP, while decreasing TRAP-5b in the alcohol treated groups with increasing GTP. Furthermore, alcohol administration increased the OC/TRAP-5b ratio and GTP supplementation decreased the ratio, indicating GTP preserving bone loss.

Trabecular and cortical bone analyses using μCT

In order to further characterize the effects of alcohol administration and GTP supplementation on the trabecular bone compartments, the proximal tibia metaphysis was evaluated using μCT imaging. At the proximal tibia, alcohol administration decreased trabecular BV/TV, Tb.N, and Tb.Th, and increased trabecular Tb.Sp (Table 3). Trabecular connectivity density was significantly lower in the alcohol-treated groups than those in the placebo-treated groups. Analysis of the SMI of trabecular bone revealed that alcohol administration increased SMI to a more rod-like structure (Table 3). Supplementation of GTP into drinking water for 6 weeks did not influence any trabecular bone parameters, nor were any interactions between alcohol and GTP observed.

	Placebo			Alcohol			Two-way ANOVA		P value
Parameters	**0% GTP Alcohol × GTP (P)**	**0.1% GTP (PLG)**	**0.5% GTP (PHG)**	**0% GTP (A)**	**0.1% GTP (ALG)**	**0.5% GTP (AHG)**	**Alcohol**	**GTP**	
Total serum protein (g/dL)	5.50 ± 0.15	5.20 ± 0.15	5.50 ± 0.15	5.60 ± 0.16	5.60 ± 0.16	5.40 ± 0.14	0.223	0.768 0.327	0.327
Albumin (g/dL)	3.95 ± 0.08^{x}	3.95 ± 0.08^{x}	3.91 ± 0.07^{x}	3.68 ± 0.08^{y}	3.86 ± 0.08^{y}	3.60 ± 0.07^{y}	0.001	0.162	0.377
Globulin (g/dL)	1.55 ± 0.12^{y}	1.52 ± 0.12^{y}	1.58 ± 0.11^{y}	1.95 ± 0.13^{x}	1.81 ± 0.12^{x}	0.83 ± 0.11^{x}	0.003	0.797	0.794
A/G ratio	2.56 ± 0.13^{x}	2.47 ± 0.13^{x}	2.46 ± 0.13^{x}	2.02 ± 0.14^{y}	2.22 ± 0.13^{y}	2.06 ± 0.13^{y}	<0.001	0.833	0.571
Ca (mg/dL)	10.6 ± 0.2	9.8 ± 0.2	10.3 ± 0.2	10.5 ± 0.2	10.4 ± 0.2	10.1 ± 0.2	0.589	0.207	0.190
Pi (mg/dL)	7.7 ± 0.3^{y}	7.6 ± 0.3^{y}	8.1 ± 0.3^{y}	9.4 ± 0.3^{x}	9.7 ± 0.3^{x}	9.6 ± 0.3^{x}	<0.001	0.759	0.616
Glucose (mg/dL)	208 ± 12^{x}	184 ± 12^{x}	186 ± 11^{x}	170 ± 12^{y}	157 ± 12^{y}	166 ± 11^{y}	0.005	0.305	0.742
BUN (mg/dL)	19.6 ± 7.1	18.7 ± 7.1	22.4 ± 6.7	36.4 ± 7.6	22.6 ± 7.1	21.6 ± 6.7	0.258	0.561	0.448
Creatinine (mg/dL)	0.24 ± 0.02	0.23 ± 0.02	0.25 ± 0.02	0.26 ± 0.02	0.28 ± 0.02	0.26 ± 0.02	0.115	0.977	0.533
ALP (U/L)	157.5 ± 8.5^{xa}	132.5 ± 8.5^{xc}	153.8 ± 3.0^{xb}	141.0 ± 9.1^{ya}	115.0 ± 8.5^{yc}	124.6 ± 8.1^{yb}	0.004	0.019	0.698
CK (U/dL)	481 ± 292^{y}	600 ± 315^{y}	761 ± 257^{y}	750 ± 386^{x}	1199 ± 273^{x}	1506 ± 292	0.038	0.261	0.745
AST(SGOT) (U/dL)	91.4 ± 19.4	116.7 ± 19.4	104.0 ± 17.1	157.4 ± 19.4	109.8 ± 19.4	127.3 ± 18.1	0.081	0.832	0.182
ALT (SGPT) (U/dL)	33.1 ± 4.7	45.5 ± 4.7	33.8 ± 4.4	39.8 ± 5.0	37.1 ± 4.7	36.0 ± 4.4	0.968	0.374	0.285
Amylase (U/dL)	2717 ± 103^{x}	2498 ± 96^{x}	2541 ± 91^{x}	2398 ± 111^{y}	2490 ± 96^{y}	2137 ± 91^{y}	0.004	0.080	0.105
Cholesterol (mg/dL)	68.1 ± 4.6^{B}	68.5 ± 4.3^{B}	71.7 ± 4.3^{B}	91.6 ± 5.0^{A}	78.4 ± 4.6^{AB}	71.8 ± 4.3^{B}	0.005	0.200	0.050
Na (meq/dL)	143.3 ± 3.0	137.3 ± 3.0	146.2 ± 2.8	144.4 ± 3.2	145.2 ± 3.0	144.5 ± 2.8	0.335	0.393	0.272
K (meq/dL)	4.6 ± 0.2^{y}	4.9 ± 0.2^{y}	4.7 ± 0.1^{y}	5.5 ± 0.2^{x}	5.4 ± 0.1^{x}	5.7 ± 0.1^{x}	<0.001	0.723	0.476
Na/K	30.6 ± 1.0^{x}	27.5 ± 1.0^{x}	30.7 ± 0.9^{x}	26.6 ± 1.0^{y}	26.6 ± 1.0^{y}	25.5 ± 0.9^{y}	<0.001	0.300	0.091
Cl (meq/dL)	101.7 ± 2.1	97.5 ± 2.1	102.7 ± 2.1	102.3 ± 2.2	102.2 ± 2.2	102.3 ± 2.0	0.317	0.427	0.446

Alcohol negatively affected blood chemistry markers of liver and pancreas function with GTP restoring cholesterol levels to normal

Table 1: Blood chemistry values in placebo- and alcohol-treated rats supplemented with green tea polyphenols (GTP) in drinking water are shown. Results are expressed as mean values ± standard error of mean (SEM). All dietary treatment groups were analyzed by two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (Fisher's LSD) *post-hoc* test to evaluate the effect of alcohol, GTP dose, or interaction. Within a row with different superscripts (x and y for alcohol effect; a and b for GTP; A, B and C for interaction) are significantly different by two-way ANOVA and Fisher's LSD test (P<0.05). Abbreviations are: A/G, albumin/globulins ratio; Ca, calcium; Pi, phosphate; BUN, blood urea nitrogen; ALP, alkaline phosphatase; CK, creatinine phosphokinase; AST, aspartate transferase activity; ALT, alanine transferase activity; Na, sodium; K, potassium; Cl, chloride.

	Placebo			Alcohol			Two-way ANOVA P value		
Parameters	**0% GTP (P)**	**0.1% GTP (PLG)**	**0.5% GTP (PHG)**	**0% GTP (A)**	**0.1% GTP (ALG)**	**0.5% GTP (AHG)**	**Alcohol**	**GTP**	**Alcohol×GTP**
Femur									
Bone area (cm^2)	1.560 ± 0.043^{x}	1.519 ± 0.043^{x}	1.548 ± 0.047^{x}	1.282 ± 0.052^{y}	1.360 ± 0.043^{y}	1.351 ± 0.043^{y}	<0.001	0.825	0.431
BMC (g)	0.269 ± 0.009^{x}	0.280 ± 0.009^{x}	0.290 ± 0.010^{x}	0.200 ± 0.011^{y}	0.214 ± 0.009^{y}	0.226 ± 0.009^{y}	<0.001	0.091	0.978
BMD(g/cm^2)	0.172 ± 0.006^{xc}	0.184 ± 0.006^{xb}	0.196 ± 0.006^{xa}	0.155 ± 0.007^{yc}	0.158 ± 0.005^{yb}	0.167 ± 0.005^{ya}	<0.001	0.030	0.608
LV-4									
Bone area (cm^2)	0.365 ± 0.011^{x}	0.397 ± 0.011^{x}	0.397 ± 0.011^{x}	0.302 ± 0.010^{y}	0.308 ± 0.011^{y}	0.314 ± 0.010^{y}	<0.001	0.111	0.510
BMC (g)	0.068 ± 0.002^{xc}	0.071 ± 0.002^{xb}	0.075 ± 0.002^{xa}	0.045 ± 0.002^{yc}	0.051 ± 0.012^{yb}	0.054 ± 0.002^{ya}	<0.001	0.016	0.805
BMD (g/cm^2)	0.184 ± 0.004^{x}	0.178 ± 0.004^{x}	0.187 ± 0.004^{x}	0.152 ± 0.004^{y}	0.166 ± 0.004^{y}	0.170 ± 0.004^{y}	<0.001	0.068	0.072
Serum									
OC (ng/mL)	588.3 ± 47.6	582.5 ± 47.5	605.5 ± 44.5	568.2 ± 47.5	656.3 ± 47.5	522.9 ± 47.5	0.804	0.483	0.258
TRAP-5b (U/L)	10.1 ± 1.4^{C}	14.9 ± 1.3^{AB}	17.4 ± 1.1^{A}	12.5 ± 1.5^{AB}	8.7 ± 1.4^{C}	10.8 ± 1.2^{C}	0.003	0.092	0.004
OC/TRAP-5b	44.5 ± 5.5^{ya}	32.0 ± 5.5^{yb}	30.1 ± 5.2^{yc}	57.6 ± 5.5^{xa}	53.9 ± 5.5^{yb}	40.3 ± 5.5^{xc}	0.002	0.023	0.547

Alcohol negatively affected bone mass and turnover biomarkers

Table 2: Bone mass and bone turnover biomarkers in placebo- and alcohol-treated rats supplemented with green tea polyphenols (GTP) in drinking water are shown. Results are expressed as mean values ± standard error of mean (SEM). All dietary treatment groups were analyzed by two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (Fisher's LSD) *post-hoc* test to evaluate the effect of alcohol, GTP dose, or interaction. Within a row with different superscripts (x and y for alcohol effect; a and b for GTP; A, B and C for interaction) are significantly different by two-way ANOVA and Fisher's LSD test (P<0.05). Within the Placebo groups (Placebo control, Placebo+0.1% GTP, and Placebo +0.5% GTP) or Alcohol groups (Alcohol control, Alcohol +0.1% GTP, Alcohol +0.5% GTP), data were analyzed by one-way ANOVA followed by Fisher's LSD test to determine the effect of GTP levels (P<0.05). Abbreviations are: BMC, bone mineral content; BMD, bone mineral density; OC, osteocalcin; TRAP-5b, tartrate-resistant acid phosphatase-5b.

	Placebo			Alcohol			Two-way ANOVA P value		
Parameters	**0% GTP (P)**	**0.1% GTP (PLG)**	**0.5% GTP (PHG)**	**0% GTP (A)**	**0.1% GTP (ALG)**	**0.5% GTP (AHG)**	**Alcohol**	**GTP**	**Alcohol × GTP**
BV/TV (%)	8.41 ± 0.49^{x}	7.51 ± 0.49^{x}	8.41 ± 0.46^{x}	5.98 ± 0.52^{y}	5.83 ± 0.49^{y}	5.35 ± 0.46^{y}	<0.001	0.572	0.356
Tb.N ($1/mm^3$)	2.356 ± 0.141^{x}	2.087 ± 0.141^{x}	2.368 ± 0.133^{x}	1.743 ± 0.151^{y}	1.509 ± 0.141^{y}	1.521 ± 0.133^{y}	<0.001	0.222	0.567
Tb.Th (mm)	0.056 ± 0.001^{x}	0.055 ± 0.001^{x}	0.055 ± 0.001^{x}	0.052 ± 0.001^{y}	0.054 ± 0.001^{y}	0.052 ± 0.001^{y}	0.004	0.524	0.417
Tb.Sp (mm)	0.421 ± 0.041^{y}	0.468 ± 0.041^{y}	0.427 ± 0.039^{y}	0.686 ± 0.045^{x}	0.660 ± 0.041^{x}	0.669 ± 0.039^{x}	<0.001	0.921	0.678
Conn Dens (1/mm)	31.82 ± 3.05^{x}	25.21 ± 2.85^{x}	31.49 ± 2.69^{x}	21.89 ± 3.05^{y}	21.82 ± 0.30^{y}	16.95 ± 2.69^{y}	<0.001	0.508	0.156
SMI	2.55 ± 0.05^{y}	2.60 ± 0.05^{y}	2.53 ± 0.04^{y}	2.76 ± 0.05^{x}	2.73 ± 0.03^{x}	2.82 ± 0.04^{x}	<0.001	0.916	0.240

Alcohol negatively affected all parameters of trabecular bone in the Tibia

Table 3: Trabecular bone parameters of the tibia measured in placebo - and alcohol-treated rats supplemented with green tea polyphenols (GTP) in drinking water are shown. Results are expressed as mean values ± standard error of mean (SEM). All dietary treatment groups were analyzed by two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (Fisher's LSD) *post-hoc* test to evaluate the effect of alcohol, GTP dose, or interaction. Within a row with different superscripts (x and y for alcohol effect; a and b for GTP; A, B and C for interaction) are significantly different by two-way ANOVA and Fisher's LSD test (P<0.05). Max force, maximal force. Abbreviations are: BV/TV, bone volume/total volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Conn Dens, connectivity density; SMI, structural model index.

	Placebo			Alcohol			Two-way ANOVA P value		
Parameters	**0% GTP (P)**	**0.1% GTP (PLG)**	**0.5% GTP (PHG)**	**0% GTP(A)**	**0.1% GTP (ALG)**	**0.5% GTP (AHG)**	**Alcohol**	**GTP**	**Alcohol×GTP**
BV/TV (%)	0.967 ± 0.001	0.968 ± 0.001	0.969 ± 0.001	0.969 ± 0.001	0.966 ± 0.001	0.967 ± 0.001	0.640	0.952	0.304
Cortical thickness (mm)	0.521 ± 0.009^{xb}	0.550 ± 0.008^{xa}	0.545 ± 0.008^{xa}	0.474 ± 0.009^{yb}	0.482 ± 0.008^{ya}	0.494 ± 0.008^{ya}	<0.001	0.032	0.443
Cortical Area (mm^2)	9.090 ± 0.223^{x}	9.608 ± 0.223^{x}	9.414 ± 0.210^{x}	7.848 ± 0.258^{y}	7.894 ± 0.223^{y}	8.480 ± 0.238^{y}	<0.001	0.168	0.276
Medullary Area (mm^2)	0.118 ± 0.005^{x}	0.118 ± 0.005^{x}	0.115 ± 0.004^{x}	0.093 ± 0.005^{y}	0.102 ± 0.005^{y}	0.107 ± 0.005^{y}	<0.001	0.528	0.301
Porosity (%)	3.234 ± 0.123	3.096 ± 0.103	3.190 ± 0.115	3.085 ± 0.133	3.426 ± 0.123	3.538 ± 0.133	0.092	0.280	0.097

Alcohol negatively affected tibial mid-diaphysis parameters.

Table 4: Parameters of tibial mid-diaphysis in placebo- and alcohol-treated rats supplemented with green tea polyphenols (GTP) in drinking water are shown. Results are expressed as mean values ± standard error of mean (SEM). All dietary treatment groups were analyzed by two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (Fisher's LSD) *post-hoc* test to evaluate the effect of alcohol, GTP dose, or interaction. Within a row with different superscripts (x and y for alcohol effect; a and b for GTP; A, B and C for interaction) are significantly different by two-way ANOVA and Fisher's LSD test (P<0.05). Abbreviations are: BV/TV, bone volume/total volume.

In this study, we also evaluated the effects of treatments on cortical bone in the mid-diaphyseal region of the tibia, using μCT imaging. The results of two-way ANOVA analyses (Table 4) show that (i) the alcohol-treated animals had decreased cortical thickness, cortical area, and medullary area composed to those in the placebo-treated group (P<0.001), (ii) GTP supplementation for 6 weeks significantly increased cortical thickness (P=0.032), but (iii) no interaction between alcohol and GTP was observed.

Bone quality assessment

The effect of alcohol administration and GTP supplementation on bone strength was determined by a 3-point bending test (Table 5). Two-way ANOVA results demonstrated that alcohol administration significantly decreased maximal force, slope, moment of inertia, and bending moment. Significant interactions between alcohol

	Placebo			Alcohol			Two-way ANOVA P value		
Parameters	**control**	**0.1% GTP**	**0.5% GTP**	**control**	**0.1% GTP**	**0.5% GTP**	**Alcohol**	**GTP**	**Alcohol×GTP**
Max force (N)	112.05 ± 2.56^{x}	113.4 ± 2.5^{x}	105.1 ± 2.4^{x}	87.2 ± 3.2^{y}	86.9 ± 2.5^{y}	90.0 ± 2.5^{y}	<0.001	0.568	0.064
Energy to max force (mJ)	26.7 ± 2.2^{B}	27.7 ± 2.2^{B}	25.4 ± 2.1^{B}	21.6 ± 2.8^{A}	34.6 ± 2.2^{A}	35.7 ± 2.2^{A}	0.044	0.013	0.009
Slope (N/mm)	260.0 ± 12.2^{x}	263.0 ± 12.2^{x}	254.3 ± 11.5^{x}	184.1 ± 15.5^{y}	168.7 ± 12.2^{y}	179.8 ± 12.2^{y}	<0.001	0.872	0.663
Deflection (μm)	179.5 ± 4.1^{x}	181.7 ± 4.1^{x}	168.4 ± 3.8^{x}	139.8 ± 5.2^{y}	139.3 ± 4.1^{y}	144.3 ± 4.1^{y}	<0.001	0.568	0.064
Bending moment (N*mm)	179.5 ± 4.1^{x}	181.7 ± 4.1^{x}	168.4 ± 3.8^{x}	139.8 ± 5.2^{y}	139.3 ± 4.1^{y}	144.3 ± 4.1^{y}	<0.001	0.568	0.064
Stress (MPa)	45.8 ± 2.1	46.1 ± 2.1	44.0 ± 1.9	46.5 ± 2.6	46.0 ± 2.1	39.4 ± 2.1	0.451	0.063	0.415
Moment of inertia (mm^3)	6.49 ± 0.40^{x}	6.62 ± 0.40^{x}	6.42 ± 0.38^{x}	4.62 ± 0.51^{y}	4.82 ± 0.40^{y}	6.06 ± 0.40^{y}	<0.001	0.243	0.130
Strain	0.221 ± 0.015^{B}	0.226 ± 0.015^{B}	0.217 ± 0.014^{B}	0.218 ± 0.019^{B}	0.303 ± 0.015^{A}	0.325 ± 0.015^{A}	<0.001	0.007	0.005
Modulus of elasticity (Mpa)	208.2 ± 11.7^{A}	207.1 ± 11.7^{A}	208.0 ± 11.0^{A}	216.1 ± 14.7^{A}	155.2 ± 11.7^{B}	126.2 ± 11.7^{B}	<0.001	0.003	0.003

Alcohol reduced bone strength and GTP supplementation increased bone quality.

Table 5: Bone strength in placebo- and alcohol-treated rats supplemented with green tea polyphenols (GTP) in drinking water. Results are expressed as mean values ± standard error of mean (SEM). All dietary treatment groups were analyzed by two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (Fisher's LSD) *post-hoc* test to evaluate the effect of alcohol, GTP dose, or interaction. Within a row with different superscripts (x and y for alcohol effect; a and b for GTP; A, B and C for interaction) are significantly different by two-way ANOVA and Fisher's LSD test ($P<0.05$). Max force, maximal force.

administration and GTP supplementation were observed in several parameters of bone quality, such as energy to maximal force, deflection, strain, and modulus of elasticity (Table 5). After 6 weeks, both ALG and AHG groups had greater energy to max force, deflection, and strain, but lower modulus of elasticity than other groups (P, PLG, PHG, and A). Neither alcohol administration nor GTP supplementation affected stress results.

Discussion

In the current investigation, a model of binge alcohol administration to adolescent male rats was successfully utilized to investigate the impact of GTP supplementation in drinking water in chronic binge alcohol exposure-induced bone loss and micro structural deterioration. Our results of bone densitometry and μCT show that chronic binge alcohol exposure produced a detrimental effect on bone mass and microstructure compared to a placebo-treated group, a result in agreement with previous studies where bone mass and microstructure were accessed [12,27].

This study demonstrated potent effects of GTP supplementation in preserving bone mass and micro architecture in adolescent male rats during chronic binge alcohol exposure. Supplementation of GTP in drinking water attenuates alcohol-induced decrease in BMD of femur, BMC of LV-4, and cortical thickness at tibial mid-diaphysis. The effect of GTP in mitigating the influence of chronic binge alcohol exposure was modest on BMC and BMD. Interestingly, these effects of GTP on cortical bone (i.e., thickness), were of a magnitude that significantly improved the quality of femura (as shown by higher levels of energy to max force, deflection, and strain). The findings that GTP supplementation in drinking water enhanced bone mass (i.e., BMC, BMD) and bone strength in alcohol-treated rats support our proposed hypothesis that 6-week GTP supplementation improves bone quality as determined by bone strength breaking test. Such a beneficial impact of GTP on bone properties agree with our previous works using various bone loss models due to aging [21,33,34], sex hormone deficiency [21,23,34], chronic inflammation [25,26,35], and obesity [22,36]. These changes in bone mass, especially as they relate to the cortical bone of GTP-supplemented rats was mediated primarily through a suppression of bone turnover rate, as shown by a lower OC/TRAP-5b ratio, resulting in a larger bone mass.

Intriguingly, the impact of GTP supplementation on bone resorption (i.e., TRAP-5b) was dependent on exposure to alcohol. For example, in the absence of alcohol exposure, GTP supplementation increased the serum TRAP-5b concentrations of rats (i.e., bone resorption) in a dose-dependent manner. The detrimental effect of large quantities of green tea extract on bone properties has previously been observed in growing male mice [37]. Although our study differs somewhat, and showed an elevation of bone resorption compared to the previous data showing suppression of bone formation, both indicate that high levels of GTP in young animals can be detrimental. Specifically, Iwaniec 2009 [37] reported 5-week-old lean and *ob/ob* mice fed powder diets containing 0.0, 1.0 or 2.0% (wt/wt) of green tea extract (GTE) (~7 to 14 servings/day) for 6 weeks experienced a decrease in bone length, volume and BMC of femur, but increased trabecular bone volume of lumbar vertebra compared to wild-type mice, suggesting that dietary GTE supplementation inhibited rate of bone accumulation during growth. Green tea has been considered a relatively safe beverage, a rich source of antioxidants, and showed no serious side effects for up to 8 servings per day in humans [38]. However, a higher dosage of green tea, such as those by Iwaniec [37], may become a source of pro-oxidants that could be detrimental to bone matrix. In the present study, we used growing male rats with an approximate 1-4 servings/day GTP concentration treatment, and also observed some negative effects of GTP on bone parameters in placebo-treated rats, especially for increased TRAP-5b levels. Although we did not collect data for oxidative stress markers in this study, we speculate that for our adolescent, growing animals, alcohol treatment resulted in chronic oxidative stress while the control animals were under much less oxidative stress. As a consequence, GTP may have had a ceiling effect as a pro-oxidant in the saline-treated group, while serving as a potential anti-oxidant in the alcohol-treated rats.

On the other hand, in the presence of alcohol exposure, GTP supplementation suppressed TRAP-5b levels. The finding that GTP supplementation tended to influence bone resorption ($P=0.092$, Table 2), instead of bone formation ($P=0.483$) on alcohol-treated animals, is in the agreement with the reports of Shen et al. [25,26] that mitigating bone loss in chronic inflammation-treated rats by GTP supplementation was due to the suppression of bone resorption (as shown in lower serum TRAP), but not due to bone formation (as shown by no change in serum osteocalcin, $P>0.05$) [24,35]. The ability of GTP to inhibit bone resorption in the alcohol treated animals can be explained when considered in conjunction with previous reports on EGCG's effect on osteoclast activity. *In vitro* cellular studies demonstrated that EGCG (i) significantly inhibited the survival of differentiated osteoclasts [39] and increased the apoptosis of osteoclasts [40-42]; (ii) inhibited the differentiation of osteoclasts [33] and the formation of osteoclasts by

inhibiting the expression of matrix metalloproteinase-9 in osteoblasts [39,41,42] or via decreasing nuclear factor-κB activation [43], (iii) induced cell death of osteoclasts in terms of single strand DNA damage, without affecting osteoblastic cells in a co-cultured system of osteoblasts and osteoclasts [40,42] via the Fenton reaction [40,44] and caspase activation [45]; and (iv) (+) catechin inhibited bone resorption and prevented osteoclast activation by acting on bone collagen that could well render bone tissue less prone to resorption [46]. The present result of GTP's suppression of bone resorption, as shown by lower TRAP-5b, seems to be supported by previous studies that EGCG inhibits osteoclastogenesis by suppressing the nuclear factor-kappaB signal [33,47] and by inhibiting the matrix metalloproteinase-9 expression [39].

Consistent with data from clinical studies [48-50], we found that alcohol administration modulated several parameters of blood chemistry indicating an effect on both the liver and pancreas. Interestingly, no effect of hypocalcemia was present suggesting that the effects on bone were not directly from a change in overall blood calcium. As expected, alcohol changed both fluid and food intake resulting in a difference in body weight (~7%) between the alcohol-treated and control groups over the 6-week period, with alcohol-treatment modestly reducing the rate of growth. Alcohol generally increases overall fluid consumption due to its anti-diuretic effect. It usually reduces food consumption due to the overall caloric value of alcohol, and reduced activity during intoxication, which is consistent with our results.

Although GTP was shown in this study to improve tibial BMC and BMD by enhancing cortical bone, it is important to note that no beneficial effects were observed in trabecular bone compartment of the proximal tibia. Detrimental effects of alcohol treatment were demonstrated on trabecular bone volume (i.e., 29% decrease), other morphometric parameters (e.g., decreased TbN and TbTh) as well as the structural model index of the trabeculae. Treatment with GTP was not able to prevent these negative effects on any of the trabecular bone parameters. The results of the cortical and trabecular bone micro architectural assessment show that in the animal model of binge-drinking, GTP provides protects bone but these benefits are confined to the cortical bone.

The present study is limited by the fact that GTP bioavailability data was not measured. However, we have previously demonstrated an elevation of urinary GTP metabolites due to GTP supplementation in various rat models, and the amount of GTP consumption in our previous studies was comparable to that in the present study [21,23]. Regardless, the potential difference in study design (i.e., species, age, study duration) should be noted.

In this study, GTP was evaluated as an alternative treatment option for mitigating bone loss and micro architecture deterioration due to chronic binge exposure. Our data show that GTP supplementation has preservative effects on BMC, BMD, and cortical bone in alcohol-treated male rats. These effects may be mediated in part through reduced bone turnover rate along with modulated endocortical bone compartments, resulting in a larger net bone volume. Beyond our previous GTP studies using other animal models [21-23,34-36], the current study using alcohol-treated male rats shed more light on GTP supplementation's possible benefit to the improvement of skeletal health in adolescent males with chronic binge exposure, in terms of GTP's impact on osteoblast and osteoclast activity during bone modeling. Interestingly, significant positive alcohol × GTP interactions were observed for bone biomechanical properties. These results are encouraging and provide incentive for further studies.

Conclusions

We demonstrated several beneficial effects of GTP supplementation on bone mass, microstructure, and quality during skeletal modeling in a chronic binge alcohol exposure model. In general, 6 week GTP supplementation of an adolescent male rat model of binge alcohol consumption provided an osteo-protective effect on cortical bone and its biomechanical properties, with evidence supporting mechanisms involving suppression of bone turnover.

Acknowledgements

This study was supported by Laura W Bush Institute for Women's Health (CLS, PJS and SEB) and NIH/NCCAM (U01AT006691) (CLS). We thank Dr. Jay J Cao for bone strength analysis, and Jia-Jia Chen and Raul Y Dagda for animal care involved in this study.

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This article was originally published in a special issue, **Drug & Alcohol Abuse** handled by Editor(s). Dr. Asok Dasmahapatra, University of Mississippi, USA