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SHORT COMMUNICATION

Subacute Exposure to Alcohol in Relation to Bone Microstructure of Mice

A. SAROCKA[1], V. KOVACOVA[1], R. OMELKA[2], M. BAUEROVA[2], E. KAPUSTA[3], Z. GOC[3], G. FORMICKI[3], M. MARTINIAKOVA[1]

[1]Department of Zoology and Anthropology, [2]Department of Botany and Genetics, Faculty of Natural Sciences, Constantine the Philosopher University in Nitra, Slovak Republic, [3]Department of Animal Physiology and Toxicology, Institute of Biology, Faculty of Geography and Biology, Pedagogical University in Cracow, Poland

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Summary
Our study aimed to investigate subacute exposure to alcohol in relation to bone microstructure of mice. Animals from experimental (E) group drank a solution composed of 15 % ethanol and water for 14 days (one remodeling cycle), while those from control (C) group drank only water. In the compact bone of E group, decreased bone formation and increased porosity were observed which corresponds with lower levels of serum alkaline phosphatase and glutathione. Alcohol significantly increased sizes of primary osteon's vascular canals and decreased those of secondary osteons, Haversian canals. Relative bone volume, bone mineral density (BMD), relative bone volume without marrow cavity were also lower in E group. On the contrary, trabecular bone microstructure did not differ significantly between E and C groups. Liver function test showed higher levels of alanine aminotransferase, aspartate aminotransferase in alcohol-fed mice. Serum calcium, phosphate were significantly lower in E group. According to our study, only changes in compact bone microstructure of mice following one remodeling cycle were observed due to both direct and indirect effects of alcohol.

Key words
Alcohol • Bone • Microstructure • Mice

Corresponding author
A. Sarocka, Department of Zoology and Anthropology, Faculty of Natural Sciences, Constantine the Philosopher University in Nitra, Nábrežie mládeže 91, 949 74 Nitra, Slovak Republic. E-mail: sarocka.anna@gmail.com

There are many reports demonstrating various toxic effects of alcohol on bone, including osteoporosis, osteomalacia, aseptic necrosis, fracture incidence, low bone mass, decreased bone mineral content and BMD (Seeman 2001, Chakkalakal 2005, González-Reimers *et al.* 2015).

However, there are no studies describing its influence on 2D characteristics of the compact bone after one remodeling cycle. Therefore, the aim of current study was to analyze bone microstructure of mice using 2D and 3D imaging methods after subacute exposure to alcohol.

In our experiment, ten clinically healthy 12-weeks-old Swiss mice (males) were used. Animals were segregated into two groups, of 5 animals each. Mice were fed with standard diet (Agropol, Motycz, Poland) and water *ad libitum* and grown in 12/12 light photoperiods. In E group, mice received a solution composed of 15 % ethanol and water (1.7 g 100 % ethanol/kg b.w. per day) for 14 days. The solution of alcohol in water has been made every day and it was administered orally to mice by a syringe in known doses (6 doses of 50 µl 15 % ethanol daily). It corresponds to a consumption of six 50 ml of 40 % ethanol or 2.5 l of 12° beer for 75 kg male adults. The second group without alcohol administration served as control. All the applied

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Fax +420 241 062 164, e-mail: physres@fgu.cas.cz, www.biomed.cas.cz/physiolres

procedures were approved by the First Local Ethic Committee on Experiments on Animals in Cracow (resolution number 175/2012).

At the end of treatment period, mice were killed and their femurs were used for microscopical analyses. Thin sections (70-80 μm) were prepared according to Martiniaková *et al.* (2008). The qualitative 2D characteristics of the compact bone were determined according to Enlow and Brown (1957) and Ricqlés *et al.* (1991). The quantitative (morphometrical) 2D parameters of the compact bone were assessed using the software Motic Images Plus 2.0 ML (Motic China Group Co., Ltd., Xiamen, China). The sizes of vascular canals of primary osteons, Haversian canals and secondary osteons were measured in all views (anterior, posterior, medial, lateral) of thin sections.

Quantitative 3D analysis of compact and trabecular bone tissues were determined using microcomputed tomography (μCT 50, Scanco Medical, Switzerland). Compact bone structure was analysed in a region of interest starting 5.2 mm from the end of the growth plate (distal epiphysis) and extending 1.5 mm at femoral midshaft. Following parameters were measured: relative bone volume, BMD, relative bone volume without marrow cavity, bone surface without marrow cavity and cortical bone thickness. Trabecular bone structure was analysed in a region of interest starting 1.2 mm from the end of the growth plate (distal epiphysis) and extending 1.5 mm. We measured bone surface, relative bone volume, trabecular number, thickness, separation. The activity of plasma bone alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutathione (GSH), calcium (Ca), phosphate (P) were measured using commercially available ELISA tests and spectrophotometrically.

Statistical analysis was performed using SPSS 8.0 software. All data were expressed as mean ± standard deviation. The unpaired t-test was used for establishing statistical significance ($P<0.05$) between both groups.

Endosteal and periosteal surfaces of femurs in mice from C group consisted of non-vascular bone tissue. In lateral parts near endost, irregular Haversian bone tissue has also been identified. In middle parts of the compact bone, several secondary osteons were observed. Non-vascular bone tissue was found only in medial parts (Fig. 1). These results are in accordance to those of other researchers (Enlow and Brown 1957, Reim *et al.* 2008).

Mice from E group displayed differences in compact bone microstructure compared to C group. Primary vascular radial bone tissue was observed in posterior parts of endosteal surface. Also, more secondary osteons (about 46 %; increased endocortical remodeling) and more resorption lacunae (four times more) mainly in anterior parts were found (Fig. 1b).

These changes can be caused by inhibition of periosteal and endosteal bone formation due to alcohol administration which corresponds with a lower level of serum ALT in E group (Table 1). Similarly, Broulik *et al.* (2010) reported lower ALP in alcohol-fed rats. Alcohol can also be responsible for a formation and production of reactive oxygen species (ROS) (Rocco *et al.* 2014) which enhance bone resorption and osteoclastogenesis (Bai *et al.* 2005). Among biological antioxidants, GSH is the most important and it acts as direct ROS scavenger (Domazetovic *et al.* 2017). Decrease of GSH level in our study (Table 1) indicates a presence of oxidative stress, which would be consistent with an increased cortical porosity.

Altogether, 392 vascular canals of primary osteons, 37 Haversian canals and 37 secondary osteons were measured (Table 1). All variables of primary osteons' vascular canals had significantly higher values in E group. On the contrary, sizes of Haversian canals and secondary osteons were significantly decreased in this group.

Vasodilation of primary osteons' vascular canals and vasoconstriction of Haversian canals in mice exposed to alcohol could be associated with deleterious effect of alcohol on blood vessels, which are present in primary and secondary osteons (Pries *et al.* 2005). However, Haversian canals in secondary osteons are delimited by a cement line which is not found in primary osteons. Therefore, the cement line could be the main reason for different results in histomorphometry of both canals (Martiniaková *et al.* 2013).

Quantitative 3D analysis of the compact bone discovered significantly decreased values of relative bone volume, BMD and relative bone volume without marrow cavity in E group (Table 1).

Similar results were also obtained by other authors (Garcia-Valdecasas-Campelo *et al.* 2006, Trevisiol *et al.* 2007, Mercer *et al.* 2012). Alcohol consumption stimulates a metabolism of lipids resulting in their accumulation in diaphyseal marrow of the bone (Wezeman *et al.* 1999). Also, other cellular changes in bone marrow and endocortical bone surface caused by alcohol consumption lead to a disruption of bone

remodeling involving reduction of the number and activity of basic multicellular units (Chakkalakal 2005). Therefore, decreased bone formation rate followed by a low bone mass and decreased BMD are generally identified in alcoholics.

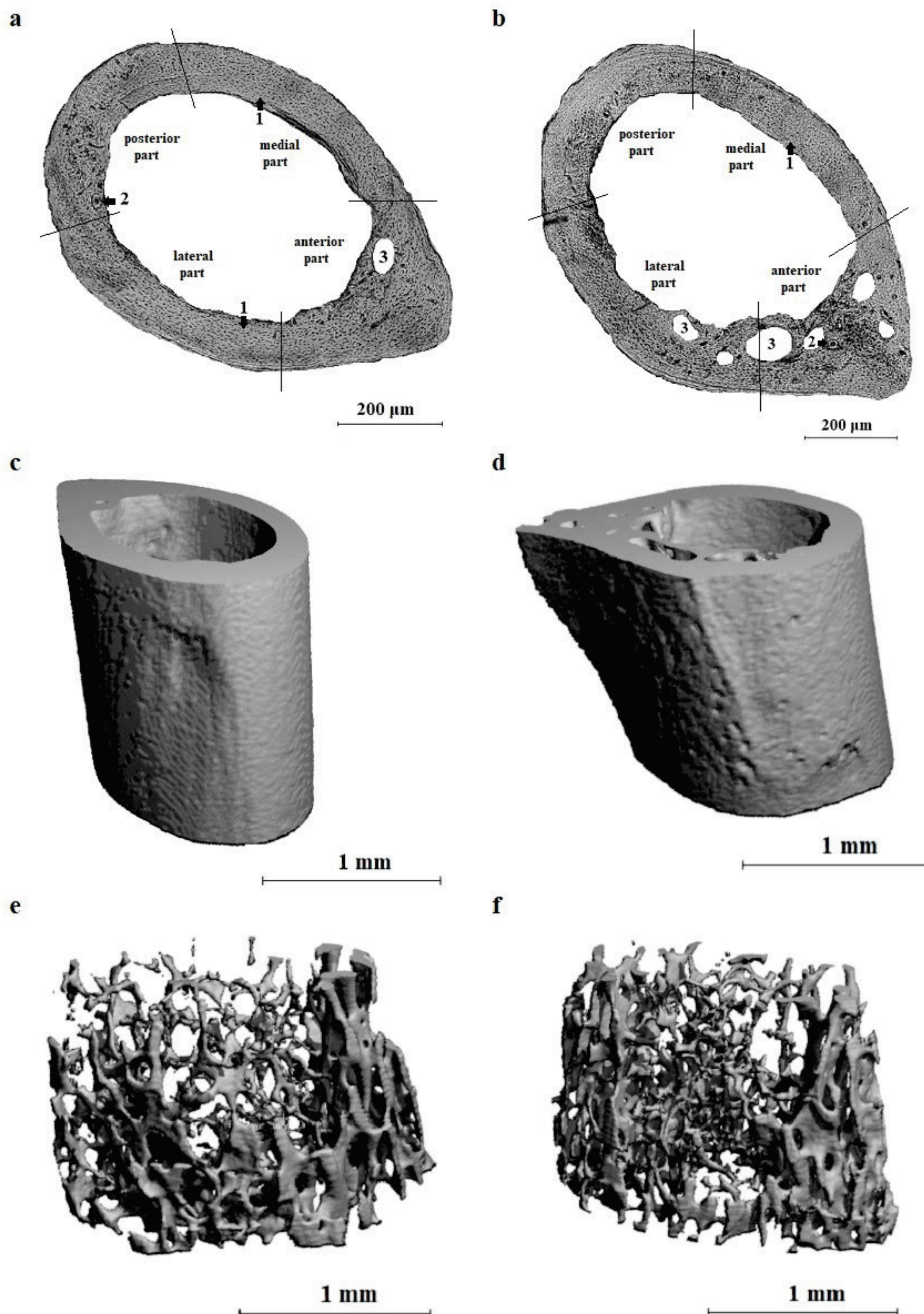

Fig. 1. Representative 2D and 3D images of compact and trabecular bone tissues in mice. (**a**) – Microscopic structure of the compact bone in control mice. (**b**) – Microscopic structure of the compact bone in mice receiving 15 % ethanol. 1 – non-vascular bone tissue, 2 – intact secondary osteon, 3 – resorption lacunae. (**c**) – Representative reconstructed 3D image of the compact bone in control mice. (**d**) – Representative reconstructed 3D image of the compact bone in mice receiving 15 % ethanol. (**e**) – Representative reconstructed 3D image of the trabecular bone in control mice. (**f**) – Representative reconstructed 3D image of the trabecular bone in mice receiving 15 % ethanol.

Table 1. Morphometrical and biochemical results.

A. Quantitative 2D analysis of compact bone tissue

Measured structures	Group	n	Area (μm^2)	Perimeter (μm)	Max. diameter (μm)	Min. diameter (μm)
Vascular canals of primary osteons	C	202	31.24 ± 4.45	19.84 ± 1.39	3.28 ± 0.29	3.02 ± 0.26
	E	190	37.82 ± 5.45	21.81 ± 1.57	3.63 ± 0.32	3.32 ± 0.30
	t-test		P<0.05	P<0.05	P<0.05	P<0.05
Haversian canals	C	15	25.39 ± 4.08	18.03 ± 1.41	3.10 ± 0.29	2.58 ± 0.31
	E	22	22.01 ± 3.64	16.68 ± 1.38	2.76 ± 0.26	2.49 ± 0.29
	t-test		P<0.05	P<0.05	P<0.05	NS
Secondary osteons	C	15	296.01 ± 43.93	61.23 ± 4.36	10.29 ± 0.72	9.16 ± 1.07
	E	22	248.96 ± 55.88	56.04 ± 6.32	9.55 ± 1.14	8.2 ± 1.08
	t-test		P<0.05	P<0.05	P<0.05	P<0.05

B. Quantitative 3D analysis of compact bone tissue

Group	n	BV/TV (%)	BMD (mg HA/cm^3)	BV/TV* (%)	Bs (mm^2)	Ct Th (mm)
C	5	0.53 ± 0.03	579.54 ± 61.01	0.95 ± 0.01	4.29 ± 2.09	0.18 ± 0.01
E	5	0.47 ± 0.02	490.33 ± 23.23	0.92 ± 0.02	4.56 ± 0.96	0.15 ± 0.01
t-test		P<0.05	P<0.05	P<0.05	NS	NS

C. Quantitative 3D analysis of trabecular bone tissue

Group	n	BV/TV (%)	TbN (1/mm)	TbTh (mm)	TbSp (mm)	Bs (mm^2)
C	5	0.11 ± 0.03	4.71 ± 0.59	0.04 ± 0.01	0.21 ± 0.03	16.47 ± 4.35
E	5	0.08 ± 0.03	4.41 ± 0.74	0.04 ± 0.01	0.23 ± 0.05	12.55 ± 4.29
t-test		NS	NS	NS	NS	NS

D. Biochemical analyses

Group	n	ALP (U/l)	ALT (U/l)	AST (U/l)	GSH (μmol/mg protein)	Ca (mg/l)	P (mg/l)
C	5	152.44 ± 9.90	7.12 ± 0.56	32.81 ± 5.48	3.51 ± 0.21	83.94 ±3.05	40.97 ± 2.2
E	5	44.29 ± 2.60	17.51 ± 1.74	42.32 ± 5.72	2.67 ± 0.21	73.66 ± 2.86	34.24 ± 5.8
t-test		P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05

n: number of measurements; C – control mice; E – experimental mice receiving 15 % ethanol; P<0.05 (+); NS: non-significant differences; BV/TV – relative bone volume; BMD – bone mineral density; BV/TV* – relative bone volume without marrow cavity; Bs. – bone surface; Ct. Th. – cortical bone thickness; Tb. N. – trabecular number; Tb. Th. – trabecular thickness; Tb. Sp. – trabecular separation; ALP – alkaline phosphatase; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GSH – glutathione; Ca – calcium; P – phosphorus.

Surprisingly, trabecular bone microstructure did not differ significantly between mice from E and C groups. Due to much larger surface to volume ratio, this bone is more actively remodeled than compact one, with remodeling rates that can be up to 10 times higher (Clarke 2008). Therefore, it cannot be excluded that in trabecular bone there are periods of intense bone resorption followed by resting metabolic periods with little or no change in bone remodeling (Maurel *et al.* 2012).

Liver function test showed higher levels of ALT, AST in E group (Table 1). High AST/ALT ratio suggests

advanced alcoholic liver disease (Nyblom *et al.* 2004). Generally, liver dysfunction is associated with vitamin D deficiency (Quintero-Platt *et al.* 2015). Alcohol's toxic skeletal effects have been suggested to involve impaired vitamin D/calcium homeostasis (Mercer *et al.* 2012). Actually, serum calcium, phosphate were significantly lower in E group (Table 1). On the other hand, no differences in ALT, AST, Ca, P were observed in alcohol-fed rats after long-term treatment (Broulik *et al.* 2010).

In summary, only changes in compact bone microstructure of mice following one remodeling cycle were observed due to direct and indirect effects of alcohol.

Conflict of Interest

There is no conflict of interest.

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