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Creatine supplementation exacerbates ethanol-induced hepatic damage in mice

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ABSTRACT

Objective: The aim of this study was to investigate the effects of creatine supplementation on early stages of ethanol-induced hepatic damage.
Methods: Male Swiss mice were divided into three groups (n = 12/group): control (C), ethanol (E), and ethanol supplemented with creatine (EC). The control group received a diet containing 15.8% of total calories from proteins, 46.3% from carbohydrates, and 37.9% from lipids. The ethanol and ethanol and creatine groups received diets containing 15.8% of total calories from proteins, 16.2% from carbohydrates, and 34.5% from lipids; the remaining calories were obtained from the addition of 5% of 95% ethanol. Creatine (1%; weight/vol) was added to the diet of EC mice. After 14 and 28 d, six animals from each group were sacrificed, generating subdivisions in each group: C14 and C28, E14 and E28, EC14 and EC28. After sacrifice, the liver was removed, weighed, and prepared for histologic, biochemical, and molecular analysis, and blood was collected.
Results: Ethanol intake induced mild cell degeneration, liver damage, oxidative lesions, and inflammation. Surprisingly, ethanol intake combined with creatine exacerbated cell degeneration and fat accumulation, hepatic expression of genes related to ethanol metabolism, oxidative stress and inflammation, and promoted oxidative stress and elevated plasma alanine aminotransferase ($P < 0.05$).
Conclusion: Creatine supplementation associated with ethanol is able to interfere in the alcohol metabolism and oxidative stress and to exacerbate ethanol-induced hepatic damage. These new findings are opposite to those observed in several studies where protective effects of creatine in a wide variety of injury models, including non-alcoholic fatty liver disease, were described.

Introduction

Complications related to abusive ethanol consumption significantly affect the health of people worldwide. Studies have demonstrated that heavy drinkers had higher rates of overall mortality and of mortality from complications related to alcohol abuse, such as alcoholic liver disease, than the general population [1,2]. Alcohol consumption is responsible for ~4% of all deaths annually and 5% of all disabilities worldwide [3] and it has a significant economic effects on the health sector. The total estimated cost per year of diseases related to alcohol risk consumption in the Brazilian Unified Health System was US $8,262,762, including outpatient and hospital care [4].

The pathogenesis of alcohol-mediated liver injury is related to its metabolism [5–7]. Ethanol metabolism in the liver is mainly attributed to the action of two major enzyme systems: alcohol dehydrogenase and the microsomal enzyme oxidation system, including cytochrome P450 2E1 (CYP2E1) [5,6]. These two metabolizing pathways induce the generation of toxic metabolites like acetaldehyde and reactive oxygen species (ROS), antioxidant depletion, and cellular lesions that impair fatty acid oxidation, favoring its accumulation in the liver [8,9]. Fatty liver induced by ethanol abuse can quickly progress to irreversible lesions when alcohol abuse is persistent, stimulated by an exacerbated inflammatory response and accumulation of fibrous tissue in the liver, all characteristics of alcoholic fatty liver disease (AFLD) [10].

Over the past few years, consistent data have demonstrated that creatine supplementation may prevent the accumulation of

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fat in the liver and the progression of non-alcoholic fatty liver disease (NAFLD) in different situations (for more information, see the comprehensive review by Deminice et al. [11]). Data from our group and others previously demonstrated that creatine supplementation can modulate the expression of key genes related to the fatty acid oxidation pathway and prevent liver fat accumulation using in vitro and in vivo NAFLD models [12–14]. Despite this promising therapeutic approach to NAFLD and related metabolic disturbances, the effects of creatine supplementation in AFLD models have been poorly investigated. To our knowledge, Ganesan et al. [15] was the only study to date aimed at investigating the effects of creatine supplementation in a model of AFLD. Surprisingly, these authors demonstrated that creatine supplementation was unable to prevent the development of alcoholic fat accumulation in the liver. These data contrast with all previous studies demonstrating the protective effects of creatine on NAFLD [12,14,16]. Indeed, in addition to the histopathologic analysis of the liver, Ganesan et al. did not evaluate the effects of creatine supplementation regarding ethanol metabolism, inflammation, and oxidative stress.

Considering the limited and ambiguous research available, the objective of this study was to investigate the effects of creatine supplementation on the early stages of ethanol-induced liver damage, evaluating the effects of supplementation on ethanol metabolism, liver inflammation, and oxidative stress, an important mechanism affecting ethanol-induced liver toxicity. The present study is relevant because a better understanding of the effects of creatine supplementation associated with ethanol ingestion can assist in the development of future strategies to help patients with alcoholic liver disease. We hypothesized that creatine supplementation may protect alcohol-induced hepatic injury similarly to what has already been demonstrated in NAFLD models.

Material and Methods

Experimental model and diet composition

Sample size calculations of five animals per group were based on the quantification of liver creatine levels, obtained in a pilot study, with an effect size of 0.88, a power of 80%, and significance of 5%. Based on these calculations and to avoid losses, we decided to use six animals per group in each experimental time, 14 and 28 d. Male swiss mice (*SWR/J*; ~25 g body weight) obtained from the animal house of the Biological Sciences Centre of the State University of Londrina (Brazil) were randomly divided into three groups (n = 12/group): control (C), ethanol (E), and ethanol supplemented with creatine (EC). Animals from latter two groups were fed with Lieber-De Carli diet (Rhoster, São Paulo, Brazil), diluted in mineral water containing 5% (vol/vol) of 95% ethanol. Control mice received an isocaloric diet without ethanol, as previously described by Mandrekar et al. [17]. Animals from different groups were pair-fed in relation to the ethanol group. The Lieber-De Carli diet of the control group contained 15.8% of total calories from proteins, 46.3% from carbohydrates, and 37.9% from lipids; the diet of the ethanol group contained 15.8% of total calories from proteins, 16.2% from carbohydrates, and 34.5% from lipids, the remaining calories were obtained from the addition of ethanol. Creatine monohydrate (1%; weight/vol; Sigma Aldrich, St. Louis, MO, USA) was added to the diet of the EC group; this concentration was chosen based on previously data demonstrating 1%; weight/vol is able to increase liver creatine concentration [11]. Animals were individually housed and maintained on a 12-h light/dark cycle and body weight and diet consumption were assessed daily. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the State University of Londrina. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

After 14 and 28 d of experimentation, six animals from each group were randomly chosen and anesthetized with 30 μL of 10% ketamine and 2% xylazine mixture (2:1). The blood was collected by cardiac puncture and animals were sacrificed by cervical dislocation. Sacrifice was performed between 09:00 h and 12:00 h. The liver was removed and weighed, and a piece from each lobe (5 mm^3) was collected and fixed in 4% paraformaldehyde for paraffin inclusion following routine procedures. The remaining tissue was frozen for biochemical analysis. Liver weight was presented as percentage of body weight (%BW). To identify animals that were sacrificed after 14 and 28 d of experimentation, the numbers 14 and 28 were added in the name of the group: C14 and C28, E14 and E28, and EC14 and EC28. The subdivision 14 corresponded to animals that were sacrificed after 14 d of experimentation and the subdivision 28 from to animals that were sacrificed after 28 d. The schematic representation of experimental design is illustrated in Figure 1.

Histologic characterization of liver architecture

Liver fragments were embedded in paraffin and cut in 5 μm thick slices. The tissue architecture and collagen deposition were blindly evaluated after hematoxylin and eosin and Picrosirius Red staining, respectively. Tissue sections were blind analyzed to evaluate the occurrence of the following parameters: cellular degeneration, interstitial inflammatory cell infiltration, necrotic zones, and alteration of tissue organization [18]. For the severity of cellular degeneration, the number of cells demonstrating any alterations (dilation, vacuolization, and pyknotic nuclei) was visually determined, such as grade 0 = no change from normal histology; grade 1 = a limited number of isolated cells (until 5% of the total cell number); grade 2 = groups of cells (5–30% of total cell number); and grade 3 = diffuse cell damage (>30% of total cell number). The inflammatory activity was graded into the following grades: grade 0 = no cellular infiltration; grade 1 = mild leukocyte infiltration (1–3 cells per visual field); grade 2 = moderate infiltration (4–6 leukocytes per visual field); and grade 3 = heavy infiltration by neutrophils (>6 leukocytes per visual field). The necrotic level was determined as grade 0 = no necrosis; grade 1 = dispersed necrotic foci; grade 2 = confluent necrotic areas; and grade 3 = massive areas of necrosis. The severity of tissue disorganization was scored as 0 = normal structure; 1 = less than one-third of tissue; 2 = more than one-third and less than two-thirds; 3 = more than two-thirds of tissue.

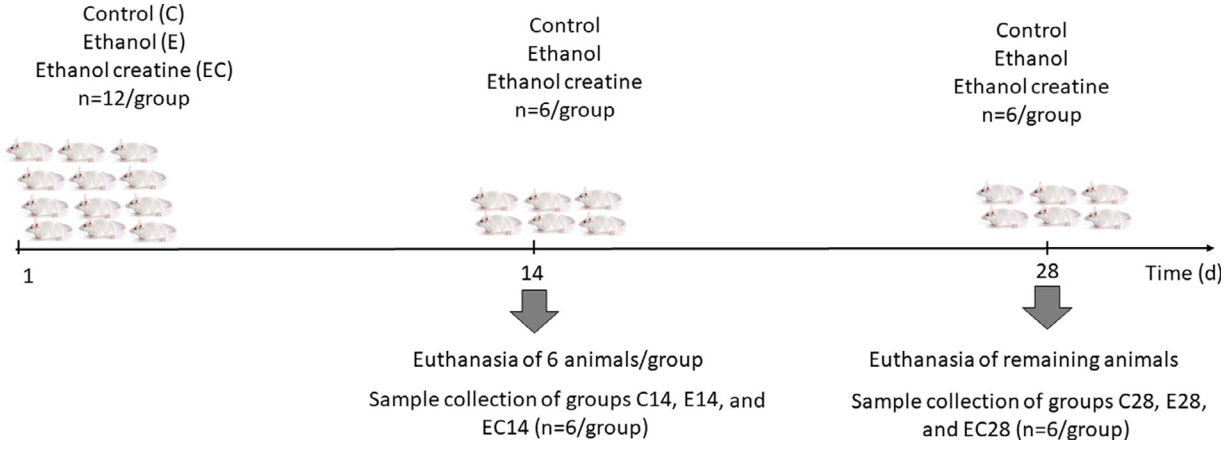

Fig. 1. Schematic representation of experimental design. Animals were randomly divided into three groups (n = 12/group): Control (C), ethanol (E), and ethanol supplemented with creatine (EC). After 14 d of experimentation, six animals from each group were randomly chosen and sacrificed and samples collected. After 28 d of experimentation, the remaining animals were sacrificed, and samples collected. C14, E14, and EC14: animals from control, ethanol, and ethanol creatine groups, respectively, sacrificed after 14 d of experimentation. C28, E28, and EC28: animals from control, ethanol, and ethanol creatine groups, respectively, sacrificed after 28 d of experimentation.

Fibrous tissue accumulation was evaluated using 30 images per group stained with Picrosirius Red and analyzed using Image J software (National Institute of Health, Bethesda, MD, USA). The liver tissue area occupied by collagen (stained red) was quantified for each visual field and expressed in % of total field, as described elsewhere [19].

Gene expression

Total RNA was isolated from liver (80 mg) using a RiboPure Kit (Ambion, Foster City, CA, USA). Total RNA was quantified by spectrophotometer at OD 260/280 (NanoDrop2000 c, ThermoFisher Scientific, Durham, NC, USA). Additional DNase I treatment (DNA-free Kit, Ambion) was employed to remove contaminating DNA from the isolated RNA. cDNA was synthesized from 1000 ηg of total RNA using high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Quantitative real-time polymerase chain reaction (PCR) was performed using 7500 Fast Real-time PCR System (Applied Biosystems). The following Taqman Gene Expression Assays (Applied Biosystems) were used in this study: *CYP2E1*, Mm00491127_m1; aldehyde dehydrogenase 2, mitochondrial (*ALDH2*) Mm00477469_m1; tumor necrosis factor (*TNFa*) Mm00443258_m1; interleukin-6 (IL-6) Mm00446190_m1; IL-1β Mm00434228_m1; superoxide dismutase 1, soluble (*SOD1*) Mm01344233_g1; NADPH oxidase 4 (*NOX4*) Mm00479246_m1. β-actin was used as the reference gene for the normalization of the reaction. Relative quantification was determined by $2^{-\Delta\Delta CT}$ method.

Determination of liver fat accumulation

Liver fat was determined as previously described by Deminice et al. [14]. Briefly, pieces with 0.5 g from different lobes of liver were homogenized in 1 mL of distilled water. Subsequently, 5 mL of chloroform-methanol (2:1) was added, and the tubes were thoroughly mixed and centrifuged to separate fat. After centrifugation, the organic phase was transferred to a preweighed tube. Then, the chloroform-to-methanol extraction was repeated twice, and the organic phases combined. The extracts were evaporated to dryness under a stream of nitrogen and weighed again. The results were expressed as fat liver (g)/g of tissue.

Quantification of liver creatine

Hepatic creatine concentration was determined by Jaffe's reaction using a method adapted from Taussky [20] and De Saedeleer and Marechal [21], as previously described by Deminice et al. [16]. Total protein was quantified using a commercially available kit by biuret method (Labstest; Labtest Diagnostica, Minas Gerais, Brazil), and the results were expressed as μM liver creatine/g total protein.

Evaluation of biochemical oxidative and inflammatory damage

Lipoperoxidation was determined by the quantification of malondialdehyde (MDA) in liver homogenate (10 mg/mL), using a high-performance liquid chromatography method, as previously described [22]. The readings were taken at 535 nm in an 11-min isocratic run and the results were expressed as nM liver MDA/g total protein.

To evaluate the presence of oxidized protein formation from inflammatory nature, liver advanced oxidation proteins products (AOPPs) were determined using a semiautomatic method described previously [23]. AOPP concentrations were expressed as μM/g total protein of chloramines-T equivalents.

Reduced and oxidized glutathione levels (GSH and GSSG, respectively) were measured as described previously [24]. The assay involved spectrophotometric measurement of reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid by GSH at 412 nm, by the formation of yellow-colored 5-thio-2-nitrobenzoic acid. GSSG was converted to GSH by the addition of glutathione reductase and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH; Sigma Aldrich). Results were expressed as μM GSH/g total protein or as a percentage ([GSSG / GSH] × 100).

Plasma determination of alanine aminotransferase levels

The plasma determination of alanine aminotransferase (ALT) levels was performed spectrophotometrically using a commercially available kit (Labtest; Labtest Diagnóstica), and the results were expressed as enzymatic unit/L of plasma.

Statistical analysis

The normality of the data and the presence of outliers were investigated by Shapiro–Wilk and Rout test, respectively. Data shown as mean ± SD or as median and interquartile range when nonparametric. One-way analysis of variance followed by Tukey's post hoc test were used for parametric data analysis. The nonparametric data were tested using Kruskal–Wallis followed by Dunn's post hoc. Data analysis was conducted using GraphPad Prism (version 6; San Diego, CA, USA) and SPSS version 22 (IBM, Armonk, NY, USA) and significance was assigned for $P < 0.05$.

Results

Association of creatine supplementation and ethanol intake increased liver weight and did not change general parameters such as body weight and diet intake

There were no differences in body weight or diet consumption between the groups within either experimental times, 14 or 28 d ($P > 0.05$). Ethanol did not change liver or body weight after either 14 or 28 d of intake. However, when associated with creatine, 14 d of ethanol intake increased (0.04 ± 0.006) the relation of liver weight to body weight compared with control animals (0.03 ± 0.005), which was not observed at 28 d (Table 1). As expected, creatine supplementation increased hepatic creatine concentration in the EC group at 14 d (1000% increase) and 28 d (2700% increase; Table 1) compared with control mice.

Creatine supplementation exacerbates ethanol-induced hepatic cell degeneration

The evaluation of liver hematoxylin and eosin staining revealed a preserved liver architecture in the control group, with absence of cell degeneration (Fig. 2). In contrast, ethanol consumption for 14 d induced a diffuse mild cell degeneration, characterized by the presence of lipid microvesicles (Fig. 2). After 28 d of ethanol intake, the degeneration was more evident, with the presence of both micro- and macrovesicular vacuolization (Fig. 2). Creatine supplementation, when combined with ethanol at 14 d, enhanced the liver degeneration promoted by ethanol intake alone, with the presence of both micro- and macrovesicular fat accumulation (Fig. 2). This exacerbation was worse after 28 d of ethanol intake and creatine, with a high density of macrovesicular fat infiltration (Fig. 2).

Table 1
Body weight, diet consumption, liver weight (percent body weight), and liver creatine content in mice fed for 14 or 28 d with Lieber-DeCarli diet containing ethanol supplemented or not with creatine

	C (n = 12)		E (n = 12)		EC (n = 12)	
	14 d (n = 6)	28 d (n = 6)	14 d (n = 6)	28 d (n = 6)	14 d (n = 6)	28 d (n = 6)
Body weight (g)	24.5 ± 2.8 a	24.1 ± 3.9 a	22.3 ± 1.8 a	26.8 ± 2.8 a	24.6 ± 2.9 a	25.6 ± 2.9 a
Diet consumption (mL/d)	22.5 ± 3.1 a	21.0 ± 2.2 a	21.7 ± 2.5 a	22.2 ± 0.9 a	22.0 ± 3.1 a	20.9 ± 1.0 a
Liver weight (% body weight)	0.03 ± 5 e-3 a	0.05 ± 3 e-3 a	0.04 ± 4 e-4 a	0.06 ± 5 e-3 a	0.04 ± 6 e-3 b	0.06 ± 8 e-3 a
Liver creatine (μM/g protein)	20.4 ± 2.0 a	19.0 ± 1.2 a	13.2 ± 1.0 a	18.3 ± 0.6 a	225.2 ± 9.5 e+1 b	565.5 ± 1.2 e+2 c

C, control; E, ethanol; EC, ethanol creatine
Data are expressed as mean ± SD. Intergroup differences were assessed by one-way analysis of variance using the Tukey post hoc test, with $P < 0.05$ considered significant. Diet concentration of ethanol: 5% (vol/vol). Diet concentration of creatine: 1% (weight/vol) Different letters (a, b, c)represent statistical difference ($P < 0.05$).

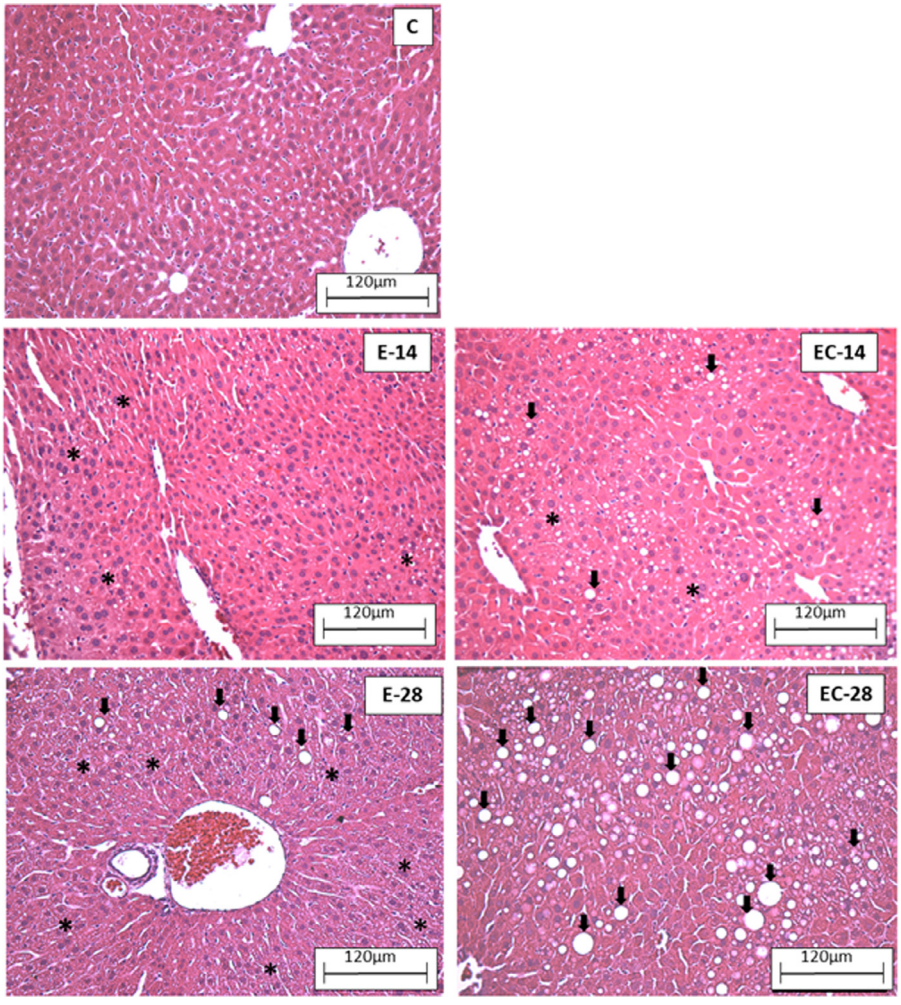

Fig. 2. Microscopical evaluation of liver architecture after H&E staining. Illustrative panel with representative light micrographs of experimental groups (200 × magnification). C, control; E-14, ethanol group at 14 d; EC-14, ethanol + creatine group at 14 d; E-28, ethanol group at 28 d; EC-28, ethanol creatine group at 28 d. Asterisk indicates microvesicular lesions and arrow indicates macrovesicular lesions.

Table 2
Histologic alterations used to assess hepatic damage

	C		E		EC	
	14 d	28 d	14 d	28 d	14 d	28 d
Cellular degeneration	0 (0) a	0 (0) a	2 (0) b	3 (0) c	3 (0) c	3 (1) c
Necrosis	0 (0) a	0 (0) a	1 (1) a	0 (1) a	1 (0) a	1 (1) a
Inflammatory activity	0 (1) a	1 (1) a	1 (1) a	1 (1) a	1 (1) a	2 (0) b
Tissue disorganization	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (1) a	1 (1) a

C, control; E, ethanol; EC, ethanol creatine
Values presented as a median (IQR). Intergroup differences were assessed by Kruskal–Wallis using the Dunn post hoc test. Different letters (a, b, c) represent statistical difference ($P < 0.05$).

The creatine-exacerbated ethanol-induced liver damage was confirmed using liver damage histologic grade (Table 2). When creatine supplementation was combined with ethanol intake, cell degeneration was significantly increased at 14 d. Similar results were demonstrated for inflammatory cell infiltration (28 d; EC28: 2[0]) when compared with ethanol intake alone (E28: 1[1]). Data also demonstrated that 14 d of ethanol ingestion induced mild cell degeneration compared with the control group. After 28 d of ethanol intake, cell degeneration was more evident in the ethanol group (Table 2).

Creatine supplementation worsened ethanol-induced expression of genes related to ethanol metabolism, inflammation, and oxidative stress

Fourteen days of ethanol intake increased the hepatic *Aldh2* gene expression ($P = 0.06$; Fig. 3A) by 600% but did not alter *Cyp2 e1*. Moreover, a significant increase in liver *Cyp2 e1* (~500%) and in *Aldh2* (800%) was observed compared with the control group when ethanol intake was combined with creatine supplementation for 14 d (Fig. 3A). Regarding inflammation-related genes, ethanol intake

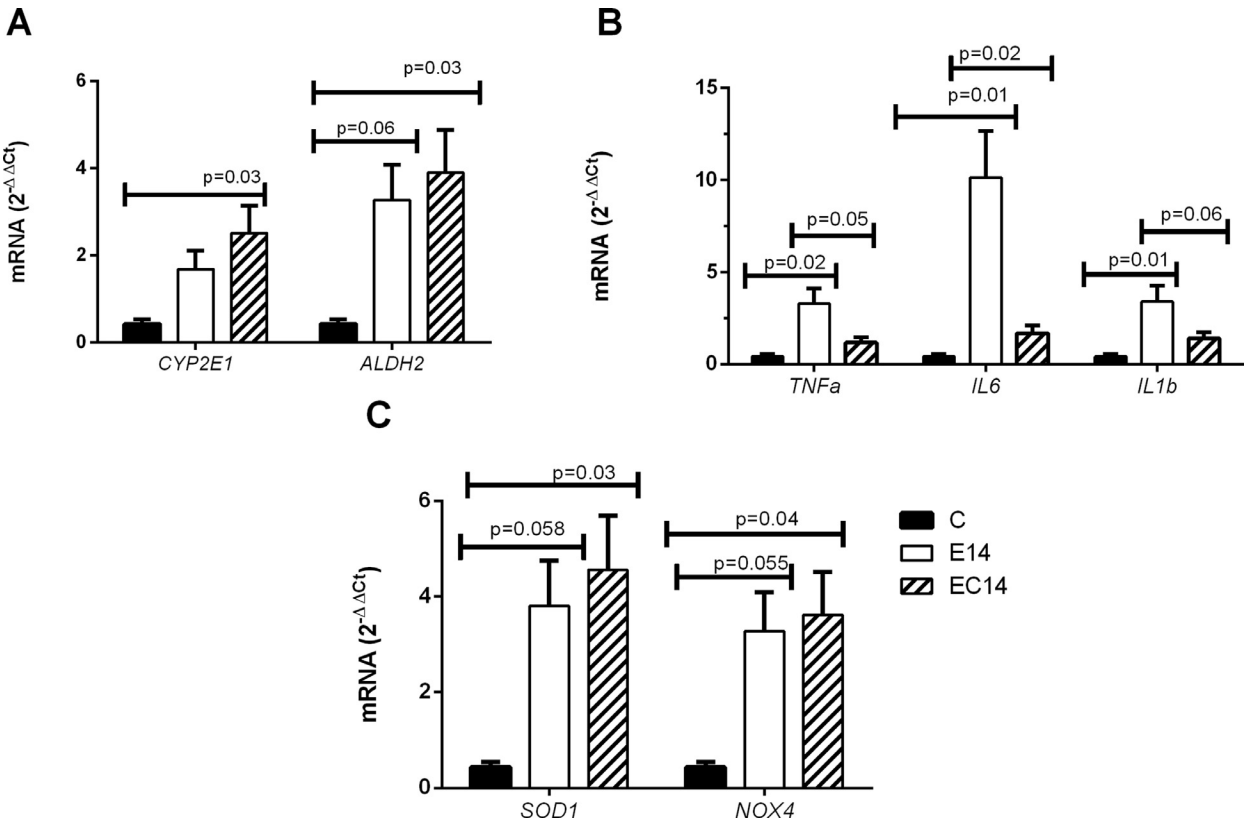

Fig. 3. Expression of key genes involved in ethanol metabolism, inflammation, and oxidative stress in mice fed for 14 d with Lieber-De Carli diet containing ethanol 5% (vol/vol) supplemented or not with creatine 1% (weight/vol). Data are expressed as mean ± SD. Intergroup differences were assessed by one-way analysis of variance using the Tukey post hoc test. *ALDH2*, aldehyde dehydrogenase 2; C, control; *CYP2E1*, cytochrome P450, family 2, subfamily e, polypeptide 1; E, ethanol; EC, ethanol creatine.; *IL1b*, interleukin 1 beta; *NOX4*, NADPH oxidase 4; *SOD1*, superoxide dismutase 1; *TNFa*, tumor necrosis factor.

increased the hepatic gene expression of *Tnfa* (600%), *Il6* (2200%) and *Il1* β (700%) after 14 d (Fig. 3B). The association of creatine supplementation and ethanol, however, significantly decreased the hepatic mRNA levels of these three genes (*Tnfa:* $P = 0.05$; *Il6:* $P = 0.02$; *Il1* β: $P = 0.06$), in this experimental time, when compared with the ethanol group (Fig. 3B). Ethanol intake also upregulated the expression of *Sod1* (800%) and *Nox4* (650; $P = 0.05$ for both; Fig. 3C). Similar results were observed when ethanol was associated with creatine supplementation after 14 d (Fig. 3C).

Twenty-eight days of ethanol intake promoted no significant changes in *Cyp2 e1, Aldh2, Tnfa, Il6, Il1* β, *Sod1,* or *Nox4* gene expression. However, ethanol intake, when combined with creatine supplementation, significantly increased liver mRNA concentration of all these genes (Fig. 4). When compared with control, the EC28 group presented increases in expression as follows: 300% in *Cyp2 e1,* 2000% in *Aldh2,* 450% in *Tnfa,* 500% in *Il6,* 1000% in *Il1* β, 1100% in *Sod1,* and 1000% in *Nox4.*

Creatine supplementation worsened ethanol-induced liver fat accumulation and oxidative damage

The histologic data suggesting that creatine supplementation exacerbates ethanol-induced hepatocyte vacuolization was confirmed by biochemical liver fat extraction that demonstrated a 200% increase in liver fat accumulation after 14 d of ethanol intake combined with creatine ($P < 0.01$), which was not evident when ethanol was ingested alone (Fig. 5A). Twenty-eight days of ethanol intake promoted a 300% increase in liver fat accumulation compared with control, the same magnitude observed when ethanol was combined with creatine for 28 d (Fig. 5A). The present results also demonstrated that creatine supplementation favors ethanol-induced hepatic oxidative damage. Ethanol intake alone did not alter liver concentration of MDA, AOPP (Fig. 5B and C), or GSH and GSSG/GSH (Fig. 5D and E) after 14 d ($P > 0.05$). In contrast, when ethanol intake was combined with creatine supplementation, it promoted elevated hepatic MDA compared with the control (400% increase) and ethanol (200% increase) groups after 14 d. After 28 d of ethanol intake, lipoperoxidation (MDA levels) significantly increased in comparison with controls (73% increase); a further increase was observed when creatine supplementation was combined with ethanol compared with control (142%) and ethanol (39%) groups (Fig. 5B). Creatine supplementation also promoted elevated hepatic AOPP at 14 (100%) and 28 d (77% of increase), although no changes were demonstrated when ethanol was ingested alone (Fig. 5C). The association between ethanol and creatine intake promoted an increase of 56% in GSH levels after14 d of ingestion (Fig. 5D). However, both ethanol alone and ethanol combined with creatine caused reduced hepatic GSH levels at 28 d (Fig. 5D). The GSSG-to-GSH relation did not change between groups (Fig. 5E).

Ethanol intake did not change ALT levels after either 14 or 28 d (Fig. 6A). However, when combined with creatine supplementation, ethanol intake increased ALT plasma levels by 200% compared with the control group and 94% compared with the ethanol group (Fig. 6A). All animals in each group (n = 6/group) were included in the analysis. As demonstrated in Figure 6, creatine supplementation did not induce hepatic fibrosis. No change in collagen deposition was demonstrated after ethanol intake alone

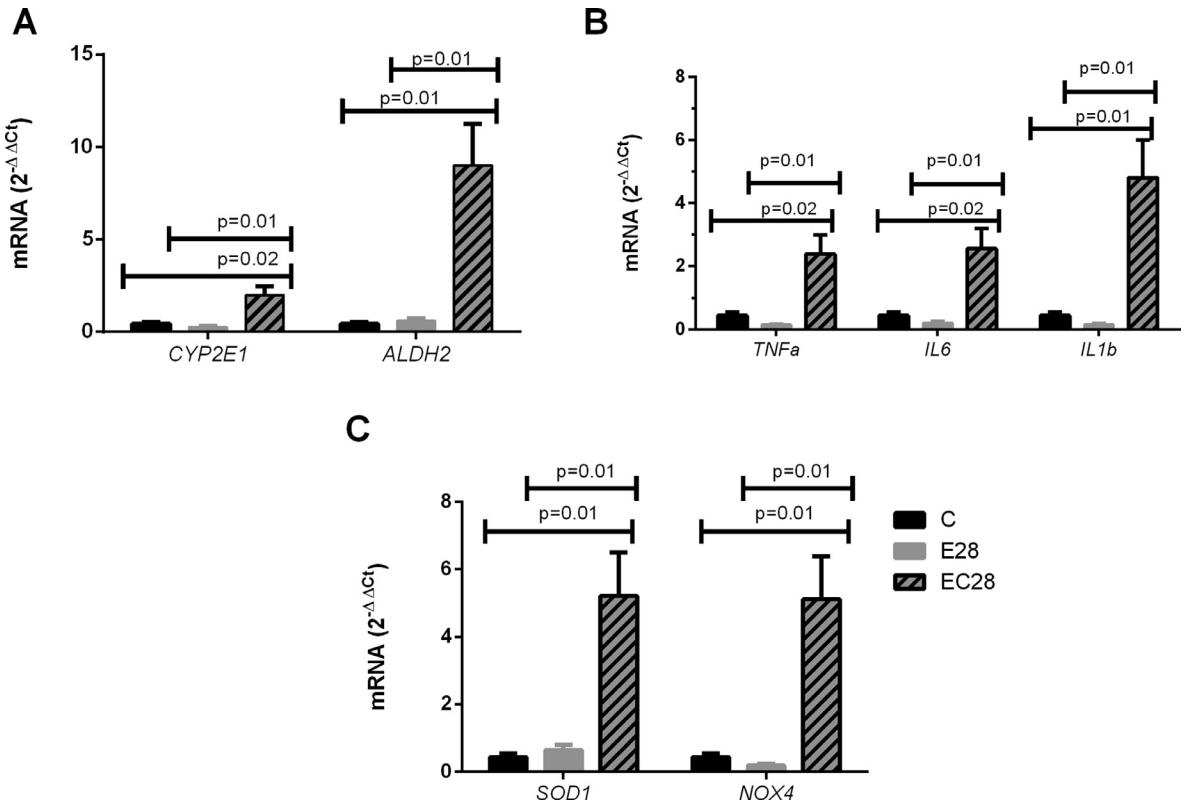

Fig. 4. Expression of key genes involved in ethanol metabolism, inflammation, and oxidative stress in mice fed for 28 days with Lieber-De Carli diet containing ethanol 5% (vol/vol) supplemented or not with creatine 1% (weight/vol). Data are expressed as mean ± SD. Intergroup differences were assessed by one-way analysis of variance using the Tukey post hoc test. *ALDH2*, aldehyde dehydrogenase 2; C, control; *CYP2E1*, cytochrome P450, family 2, subfamily e, polypeptide 1; E, ethanol; EC, ethanol creatine.; *IL1b*, interleukin 1 beta; *NOX4*, NADPH oxidase 4; *SOD1*, superoxide dismutase 1; *TNFa*, tumor necrosis factor.

or in association with creatine supplementation compared with control (Fig. 6B and C).

Discussion

The main findings of the present study are that creatine supplementation exacerbates the hepatic toxic effects of ethanol by interfering in the ethanol metabolism and causing ethanol-induced inflammation and oxidative stress. This is evidenced by an increase in liver fat accumulation and cell degeneration, activated hepatic inflammatory signaling, and increased hepatic oxidative damage and elevated plasmatic ALT. These data together makes us refute our study hypothesis that creatine supplementation could protect the liver against alcohol-induced injury.

Our hypothesis was firstly formulated based on results from our and other groups' studies demonstrating that creatine supplementation prevented the accumulation of fat in the liver and the progression of NAFLD in different situations [11–14]. Indeed, creatine has been considered a promising molecule for use in several disease conditions (see comprehensive reviews [25–27]). These studies were based on earlier results in which the antioxidant and anti-inflammatory effects of creatine were confirmed in several conditions [28–34]. Lawler et al. [31] showed that creatine has direct antioxidant effects because of its ability to directly scavenge superoxide and peroxynitrite anions. Sestili et al. [33] compiled a series of works demonstrating the trophic, prosurvival, and prodifferentiation effects of creatine in pathologic muscle and brain conditions.

The present study demonstrated that 14 and 28 d of ethanol consumption promoted mild hepatic cell degeneration and liver injury, characterized by the presence of lipid vesicles and liver fat accumulation, enhanced oxidative stress, oxidative damage, and moderated grades of inflammation without significant necrosis or fibrosis, all symptoms of the early stages of AFLD [8,35]. These results were expected considering the experimental protocol adopted and the period of ethanol exposure (14–18 d). Bertolla et al. [36] compared different experimental protocols of induction of alcoholic liver injury and concluded that prolonged times of ethanol exposure and the administration of ethanol doses by gavage can promote more intense lesions.

In contrast and surprisingly, the present study demonstrated that creatine supplementation promoted expressive changes in the profile of liver toxicity induced by ethanol administration. Although some differences were found between the effects of the association of creatine supplementation and the consumption of ethanol in the two experimental times, in general it was observed that creatine supplementation upregulated the expression of important genes involved in ethanol metabolism, inflammation (predominantly after 28 d), and oxidative stress. The association also exacerbated ethanol-induced liver fat accumulation and hepatic injury, with increased grades of cell degeneration and oxidative damage. A study by Ganesan et al. [15] (as far as we know, is the only study to date that examined the effects of creatine supplementation on AFLD) demonstrated that dietary creatine supplementation did not prevent alcoholic liver injury. These authors exposed rats to 5 wk of a Lieber-De Carli ethanol diet and concluded that creatine supplementation was ineffective in preventing examined signs of alcoholic liver injury such as steatosis, in contrast to the present study, which demonstrated that creatine supplementation worsens the ethanol liver toxicity profile compared

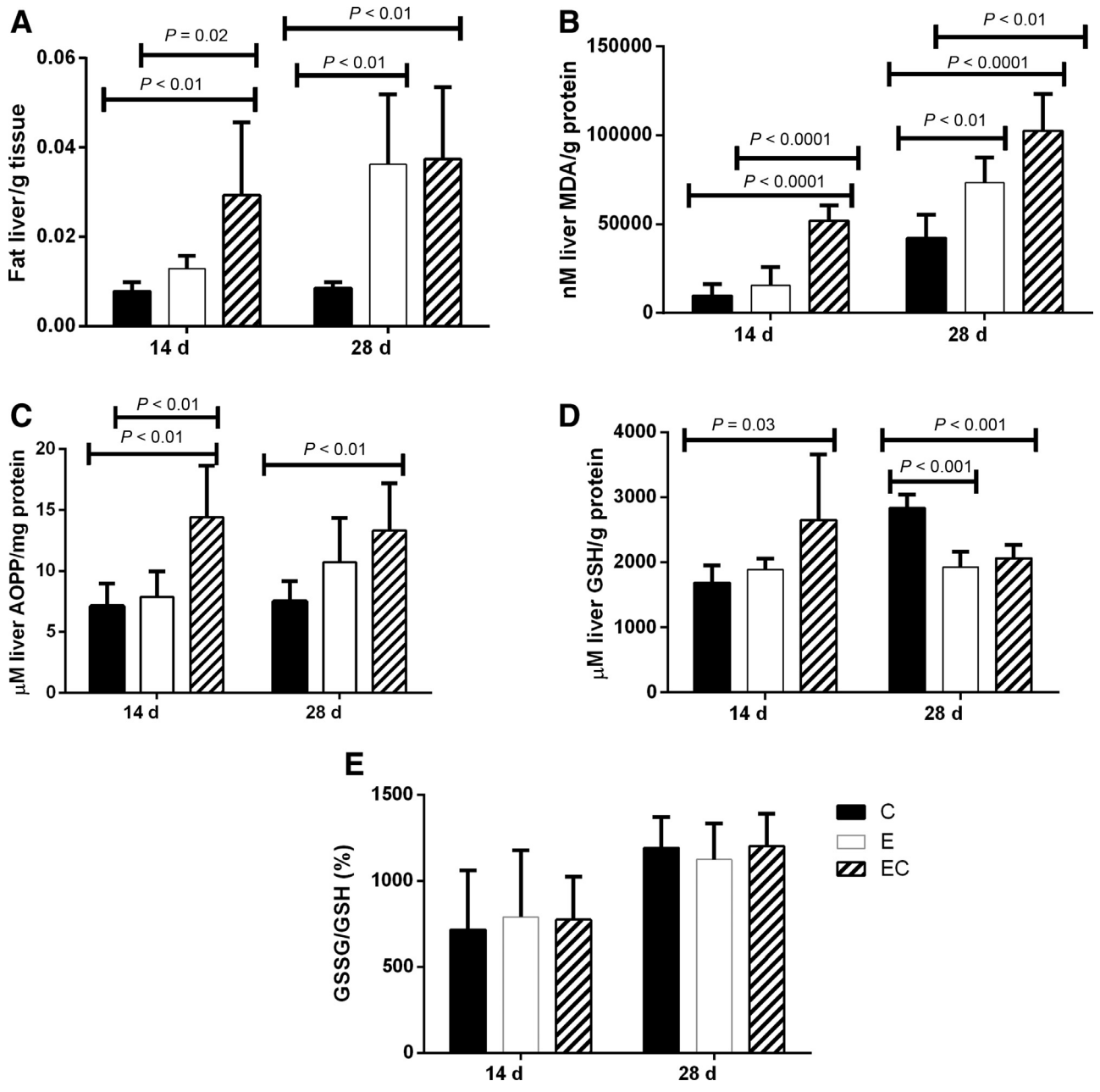

Fig. 5. Total fat liver (g/g tissue) (**A**), hepatic malondialdehyde (**B**), AOPP (**C**), GSH (**D**), and GSSG/GSH (%) in mice fed for 14 or 28 d with Lieber-De Carli diet containing ethanol 5% (vol/vol) supplemented or not with creatine 1% (weight/vol). Data are expressed as mean ± SD. Intergroup differences were assessed by one-way analysis of variance using the Tukey post hoc test. AOPP, advanced oxidation proteins products; C, control; E, ethanol; EC, ethanol creatine; GSH, reduced glutathione; GSSG, oxidized glutathione.

with consumption of ethanol alone. The present data demonstrated that creatine supplementation further increased the levels of fat in the liver, hepatic oxidative stress, and inflammation, and it promoted ALT elevation compared with the groups that received ethanol alone. Our results also suggested that creatine supplementation anticipates ethanol-induced liver toxicity because at 14 d, EC animals demonstrated the same levels of liver fat accumulation and oxidative damage as mice receiving ethanol alone at 28 d.

Considering the effects of creatine supplementation in ethanol-induced liver toxicity found here, the present data clearly supported the existence of different pathophysiologic mechanisms for the occurrence of AFLD and NAFLD. The data also allow speculation that the mechanism by which creatine exacerbates alcoholic lesions might be associated to creatine-worsening oxidative damage when combined with ethanol intake. This can be understood when the effects of creatine supplementation combined with ethanol in gene expression is analyzed. The association of ethanol and creatine was able to increase the expression of *ALDH2* and *CYP2 E1*, especially after 28 d of intake, when cellular degeneration and oxidative lesions were more pronounced. These results indicate that creatine can modulate two different pathways of ethanol metabolism. *ALDH2* plays an important role in the metabolism of acetaldehyde, an oxidized metabolite of ethanol produced by the action of alcohol dehydrogenase in ethanol metabolism [37]. *CYP2 E1* belongs to a superfamily of heme enzymes involved in oxidation of numerous endogenous substrate such as steroids, fatty acids, and xenobiotics [38]. This enzyme can be induced by ethanol ingestion and catalyzes ethanol oxidation at rates much higher

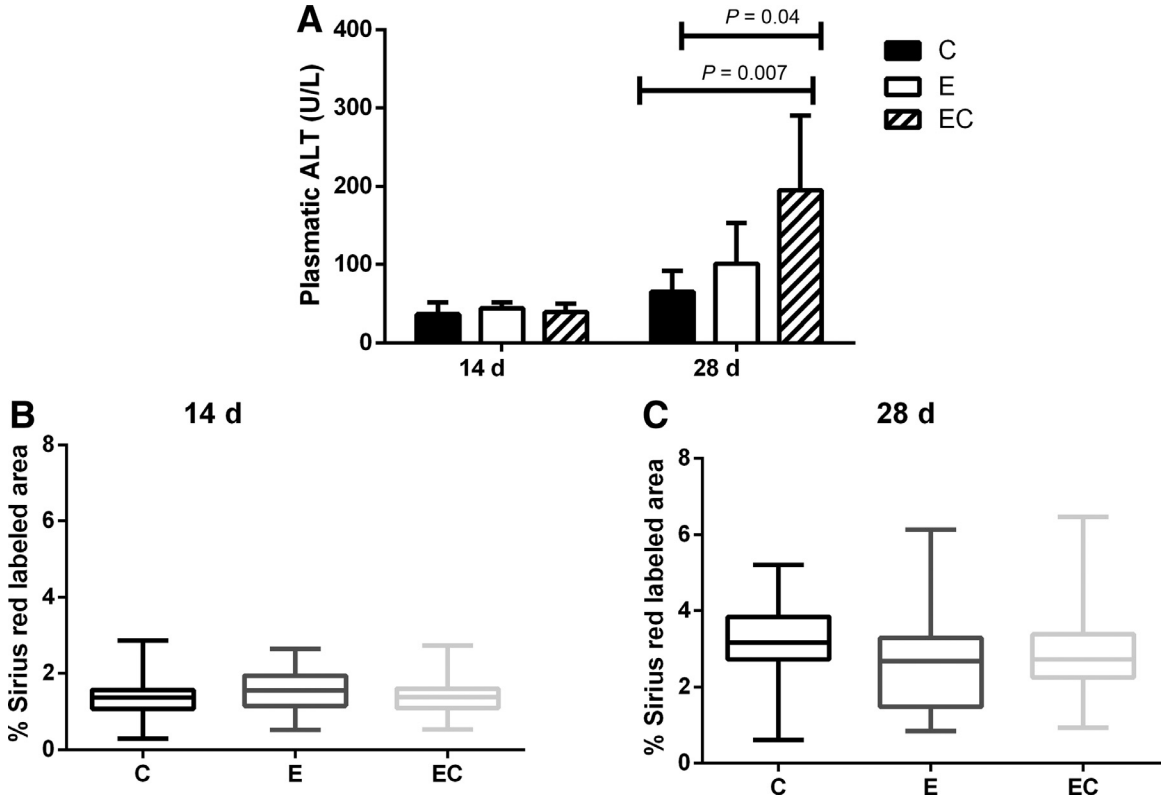

Fig. 6. Evaluation of plasma ALT (**A**), liver collagen deposition (picrosirius red staining) after 14 (**B**) or 28 d (**C**) in mice fed with Lieber-DeCarli diet containing ethanol 5% (vol/vol) supplemented or not with creatine 1% (weight/vol). Data are expressed as median or as mean ± SD (plasmatic ALT). Intergroup differences were assessed by Kruskal–Wallis using the Dunn post hoc test or by one-way analysis of variance using the Tukey post hoc test. ALT, alanine aminotransferase; C, control; E, ethanol; EC, ethanol creatine.

than other CYP enzymes [38]. The catalytic reaction of CYP2 E1 is known to generate elevated amounts of ROS [39]. In addition to altering these enzymes, the combined intake of creatine supplementation and ethanol increased the expression of genes responsible for the production of proinflammatory cytokines like TNF-α, IL-6, and IL-1β in the same experimental time (28 d). It has been demonstrated that Kupffer cells are the major source of cytokines in the liver [40]. Studies indicate that the proinflammatory cytokines, especially TNF-α, are closely related with liver injury, increasing fatty acid synthesis, and inhibiting fatty acid oxidation in hepatocytes [41,42]. Chronic ethanol intake can activate Kupffer cells and induce CYP2 E1, cytokines production, and ROS generation [43].

Creatine supplementation associated with ethanol intake also upregulated the *SOD1* and *NOX4* genes. These effects indicate an elevated production of superoxide anion and could have contributed to the oxidative stress–induced lesions observed. Oxidative stress has been considered a key event in the pathogenesis and progression of alcoholic liver disease [44] because ethanol increases the cytosolic and mitochondrial prooxidant state with the reduction of the ratio of NAD+ to NADH, which intensifies the cellular production of ROS by increasing the flow of electrons down the respiratory chain in mitochondria [44]. Furthermore, the reduction in NADH availability is associated with fat accumulation because it favors the synthesis of fatty acids and opposes their oxidation [45]. The oxygen species promote liver injury by different mechanisms, most of them involved in alcoholic liver disease. The main mechanisms include the induction of DNA damage, inhibition of mitochondrial and peroxisomal β-oxidation enzymes, and lipoperoxidation and oxidative damage to rough and smooth endoplasmic reticulum and golgi complex that impairs apoprotein synthesis, lipoprotein formation, and its excretion of the hepatocyte, leading to fat accumulation in the cells [44]. The present data demonstrated that when combined with ethanol, creatine promotes hepatic lipoperoxidation, as evidenced by MDA quantification, after 14 and 28 d of creatine association with ethanol. The intracellular levels of GSH also increased with the association between creatine supplementation and ethanol. GSH could increase in response to the variations in intracellular stresses, for example, MDA-induced oxidative lesions [46]. However, GSH levels were reduced after 28 d of ethanol administration alone and combined with creatine supplementation, indicating its consumption by the ROS generated. Creatine supplementation and ethanol intake also induced increased AOPP levels compared with ethanol intake alone. These results reinforce a relation between inflammation and oxidative stress in the liver injury induced by the association between ethanol and creatine because AOPP consists, in oxidative modifications, of proteins that predominantly occur through myeloperoxidase-derived hypochlorite [47]. This seems paradoxical because several previous studies have demonstrated the antioxidant and anti-inflammatory effects of creatine [31–33].

Conclusion

Based on the data presented, we concluded that creatine supplementation exacerbates ethanol-induced liver injury characterized by increased cellular degeneration, fat accumulation, and oxidative damage. The alteration in ethanol metabolism, elevated oxidative stress, and inflammation are probably the causes involved, at least in part, in these ethanol-creatine injury effects. This opposes several studies demonstrating protective effects of creatine in a wide variety of injury models and several target organs and tissues of the body, including NAFLD. Although adverse,

our data must be transferred to humans with caution because there are no studies comparing alcoholic and non-alcoholic liver disease with creatine supplementation in humans.

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