

Oxidative stress in lymphoid tissues and complement activation in alcoholic mother rats and their newborns

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Abstract. Pregnant Wistar rats were exposed to ethanol under chronic conditions using the gavage method to assess the complement activation and effects of oxidative stress on fetus lymphoid organs and liver. The effects were monitored on both the 10th (G10) and the 30th (G30) day of the offspring of alcoholic mother rats. Maternal ethanol caused a significant decrease in the glutathione level, whereas malondialdehyde and carbonyl levels increased in the liver and lymphoid tissues. Na⁺,K⁺-ATPase, glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase activities in these organs also decreased. Furthermore, complement C3 and C5 activities of G10 and G30 groups were significantly higher compared with those of the control group. In conclusion, the results demonstrated that alcohol was capable of triggering damage to the membranes of the liver and lymphoid tissues of G10 and G30 groups, and C3 and C5 contributed to the development of alcohol-induced fetal tissue injury.

Key words: Maternal ethanol — Lymphoid tissue injury — Complement activation — Oxidative stress

Abbreviations: AT, acetaldehyde; CAT, catalase; CL, chemiluminescence assay; FASD, fetal alcohol spectrum disorder; GP, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; LP, lipid peroxidation; MDA, malondialdehyde; PO, protein oxidation; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Maternal alcohol consumption during pregnancy can have toxic effects on the mother and the developing fetus depending on the acute or chronic use and the dose. This is because alcohol rapidly diffuses through the placental barrier, and the alcohol content in the fetal blood emerges as equal to that in the maternal blood within minutes of the onset of a drinking episode. Evidence suggests that in utero embryonic and fetal exposure to ethanol leads to morphological and behavioral or neuro developmental anomalies (Paintner et al. 2012; Guevara et al. 2018). At present, this spectrum is collectively

termed fetal alcohol spectrum disorder (FASD). FASD includes alcohol-related birth defects, alcohol-related neurological disorders, and fetal alcohol syndrome (FAS) (May et al. 2009). FAS causes mental retardation and growth deficits together with facial anomalies in newborns of chronic alcohol-consuming mothers (Miller et al. 2013). Several studies have demonstrated that one of the mechanisms implicated in ethanol teratogenicity is oxidative stress (Mancinelli et al. 2014). When ethanol is consumed, it is readily absorbed from the gastrointestinal tract into the blood. Most of the ingested alcohol is metabolized by adult and fetal alcohol dehydrogenase, which converts ethanol into its corresponding aldehydes using nicotinamide adenin dinucleotide (NAD⁺) as a cofactor (Crabb et al 2004). Acetaldehyde (AT), the product of ethanol oxidation, is a highly unstable and reactive compound that has a high affinity for sulfhydryl groups within cells or tissues, and is also capable of reacting covalently with

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nucleophiles, including nucleic acids, lipids, carbohydrates, amino acids, peptides, and proteins (Deitrich et al. 2006). Ethanol-induced protein adducts have been postulated to alter cellular function by direct toxicity as well as producing immune-mediated tissue damage. Excess production of AT promotes lipid peroxidation, protein oxidation, mobilization of ferritin irons and also reactive oxygen species (ROS) production. As a result, membrane and enzyme function may be disrupted via overproduction of ROS and, therefore, alter tissue glutathione (GSH) and antioxidant enzymes such as glutathione peroxidase (GP), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) (Cohen et al. 2003; Brocardo et al. 2011). Recent studies have shown that maternal ethanol induces oxidative stress; increases lp in fetal liver, brain, kidneys, and testes; and causes oxidative DNA damage in mouse embryos (Dong et al. 2010; Ceni et al. 2014), leading to severe birth defects (Greenberg 2003). Lymphoid tissues are responsible for regulating immune responses and function. Also, the lymphoid tissue cells are sensitive to changes in the antioxidant status because they carry out important functions by generating a high number of oxygen free radicals (Ozerkan et al. 2014). Moreover, the cells of the immune system have a high percentage of polyunsaturated fatty acids in their plasma membranes, and therefore, it is not surprising that these cells usually contain a higher concentration of antioxidants compared with other cells (Hatam and Kayden 1979). Research using animal models has shown that prenatal ethanol exposure negatively affects lymphoid tissue development, immune cell function, humoral immunity, and cytokine secretion (Terasaki and Schwarz 2016). Interestingly, no study concerning maternal alcohol administration in lymphoid tissue on FASD exists.

Another key hallmark is inflammation; toxic compounds produced in excess and antioxidant deficiencies or oxidative stress might be pathogenic factors in the alcoholic injury of lymphoid tissues in chronic alcoholism. The complement cascade has three different pathways: classical, lectin, or alternative. Complement activation has been shown to be associated with oxidative stress in numerous studies. ROS (such as H_2O_2) has been reported to be activated *via* the nonenzymatic pathway directly, and the inhibition of ROS formation decreases complement activation and deposition. The initiators of the three pathways are different, but all three ultimately lead to the activation of complement component 3 (C3). Complement activation leads to increased expression of inflammatory cytokines and chemokines and is involved in host defense as well as wound healing and response to tissue injury (Collard et al. 1998; Cohen et al. 2011). Increasing evidence suggests that alcohol-induced liver injury may be associated with increased oxidative stress and complement activation. The C1q can independently interact with a multiplicity of biological structures, including pathogen-associated and cell-associated molecules (danger-associated

oligosaccharide structures or lipids; Ghebrehiwet et al. 2017), occurring *via* the opsonization of immune complexes and cellular debris by the C1/classical pathway and production of C3 fragments (Mevorach et al. 1998). Recently, C1q and C3 have been shown to contribute to the pathogenesis of ethanol-induced liver injury (Shen et al. 2014). Currently available data suggest that C3 or C5 protects from chronic ethanol-induced liver injury in C3- and C5-deficient mice (Pritchard et al. 2007). Furthermore, the inhibition of the complement cascade has been reported to produce a better outcome in the heart, liver, and brain after ischemic damage (Mocco et al. 2006; Ducruet et al. 2009). Therefore, together, the C1q family of proteins is able to not only modulate the inflammatory and adaptive responses but also enhance the clearance of apoptotic cells by macrophages. Although increasing evidence indicates that complement is a convincing contributor to the pathophysiology of ethanol-induced tissue injury (especially in the liver and brain), the mechanisms by which ethanol activates the complement in lymphoid tissues have not been investigated. On the basis of the experimental studies, using biochemical approaches, the present study aimed to investigate the alcohol-induced oxidative stress and associated C3 and C5 activities on the lymphoid tissues in excess in rats fed chronically with alcohol and their newborn on the 10th and 30th days.

Materials and Methods

Experimental procedure

All experimental protocols were approved by the Cerrahpasa Medical Faculty Animal Care and Use Committee and we followed the National Institutes of Health (Washington, DC) Guide for the Care and Use of Laboratory Animals. Ten adult male and 30 adult female Wistar rats (aged 6–7 months; Cerrahpasa Medical Faculty Experimental Research Centre, Istanbul/Turkey) weighing 180–200 g were used for this experimental study. For mating, a male rat was randomly picked up and placed into a female's cage. They were placed in a secluded, temperature- and humidity-controlled room ($22 \pm 3^\circ C$ and $60 \pm 5\%$, respectively) in which a 12/12-h light-dark cycle was maintained.

Pregnant rats were obtained by mating overnight with a sexually experienced male. Pregnancy was confirmed by vaginal smear and embryonic day zero was determined by the presence of sperm in a vaginal smear (Marcondes et al. 2002).

Pregnant rats were assigned into three groups: the control group, which received isotonic sodium chloride (isc) intraperitoneally (i.p.) during pregnancy from 1 to 21 days gestation and during lactation until postnatal day 10 (C10) and 30 (C30), alcoholic mother's 10-day-old newborn (G10)

and alcoholic mother's 30-day-old newborn (G30). When pregnancy was established, 40% v/v ethanol (4 g/kg) was administered intragastrically. The treatment was carried out during pregnancy and during lactation until postnatal day 10 and 30.

All animals were also fed a standard laboratory diet (carbohydrate, 50%; lipid, 22%; protein, 25%).

On days 15, 18, and 20 of pregnancy, a 20- μ l blood sample was taken from the tails of the rats every morning 6 and 22 h after the administration of fresh diets. Plasma was immediately separated following centrifugation and quantified at 340 nm to determine the blood alcohol concentration (BAC; NAD-ADH Reagent Multiple Test Vial; Sigma-Aldrich, MO, USA) using a Shimadzu UV-1201 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). For each group, on days E15, E18, and E20, a total of six values were obtained as a result of two applications *per* day (Lundquist 1959). The highest values of these were defined as the peak BAC for each group.

Biochemical analyses

The rats were fasted overnight. The next morning, under light ether anesthesia (inhaled 4000 ppm/h), the heart was punctured using an injector, and the blood was collected into biochemical test tubes. Blood samples were also collected from the heart of the offspring on the day lactation ceased (days 15 and 30). Blood test tubes were centrifuged at $2000 \times g$ for 10 min at room temperature, and the serum was separated. After removing the blood samples, the rats were killed by decapitation, and lymphoid (thymus, spleen, and lymph nodes) and nonlymphoid (liver) tissues were surgically removed. Tissues used for biochemical studies were frozen in liquid nitrogen and kept at -80°C until further use. Tissue samples were excised and weighed, and a part of it was minced and homogenized well in liquid nitrogen. Then, the homogenate was sonicated for 60 s at 220 V (Sonic Dismembrator, Model 300 (Fisher, Germany) in each cold assay buffer. All chemicals used for the experiments were of analytical grade and purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (MO, USA).

Lipid peroxidation measurement

Tissue samples were estimated spectrophotometrically by the thiobarbituric acid-reactive substance method with Singh et al (2000) and expressed in terms of malondialdehyde (MDA) formed *per* mg protein (nmol MDA/mg protein). Briefly, 0.2 ml of tissue homogenate supernatant was mixed with 0.8 ml of 0.15 M Tris KCl buffer to which 0.25 ml of 30% trichloroacetic acid (TCA) was added and then 0.25 ml of 52 mM thiobarbituric acid (TBA) was added and placed in a water bath for 1 h at 90°C , cooled in ice and centrifuged at room

temperature for 15 min at 3200 rpm. The absorbance of the clear supernatant and standards (0.01–10 μM) were measured against the reference blank of distilled water at 532 nm spectrophotometer (UV-160A Shimadzu). Protein was determined according to the method of Lowry et al (1951).

Protein oxidation measurement

Measurements of protein (carbonyl) oxidation in stomach samples and serum were made by the method of Evans et al (1999) with slight modifications. The homogenized lymphoid tissues were transferred to an Eppendorf tube, and then streptomycin sulfate solution (10% w/v) was added to a final concentration of 1% to precipitate any extracted DNA which could react with 2,4 dinitrophenylhydrazine (DNPH) and contribute to the carbonyl level. The mixture was mixed and left to stand for 10 min at room temperature, after which it was centrifuged at $3000 \times g$ for 10 min at room temperature. The supernatant was removed in 0.3 ml aliquots into two tubes. DNPH (1.2 ml, 10 mM in 2 M HCl) was added to one tube and 1.2 ml of 2 M HCl to the other tube. The tubes were then incubated for 1 h on a shaker at room temperature and then the protein was precipitated by adding 0.3 ml of 20% (w/v) trichloroacetic acid to each tube and left for 10 min. Protein was spun down at $5000 \times g$ for 5 min, the supernatant was discarded and the pellet was washed with 1.25 ml of an ethyl acetate: ethanol mixture (1: 1, v/v) to remove excess DNPH. This procedure was repeated three times. The final pellet was dissolved in 0.938 ml of 6 M guanidine hydrochloride and the absorbance of both solutions (DNPH and HCl) measured at 370 nm from which the carbonyl content could be evaluated (carbonyl concentration in nM: $\Delta A_{370} \times 45.45$, where ΔA_{370} equals A_{370} of DNPH solution – A_{370} of HCl solution). The protein concentration was calculated from the A_{280} of the HCl samples ($A_{280} \times 1.8$ gives the protein concentration in mg/ml). Carbonyl concentration (nmol/mg of protein) = nmol/ml of carbonyl groups/protein concentration in mg/ml.

Na⁺,K⁺ ATPase activity measurement

It is based on the measurement of inorganic phosphate that is formed from 3 mM Na_2ATP added to the medium during the incubation period (Reading and Isbir 1980). The medium incubated in a 37°C water bath for 5 min with a mixture of 100 nM Na_2Cl , 5 mM KCl, 6 mM MgCl_2 , 0.1 mM EDTA, 30 mM Tris-HCl (pH 7.4). Following the preincubation period, Na_2ATP , at final concentration of 3 mM, was added to each tube and incubated at 37°C for 30 min after the incubation, the tubes were placed in an ice bath and the reaction was stopped. Subsequently, the level of inorganic phosphate was determined in a spectrophotometer at an excitation wavelength of 690 nm. The specific activity of the enzyme is expressed as nanomoles Pi *per* milligram of protein *per* hour.

Tissue chemiluminescence assay

Measurements were made at room temperature using a junior LB 9509 luminometer (EG&G Berthold, Germany). Specimens (0.1 g) were put into vials containing PBS and 4-(-2-hydroxyethyl) piperazine-1-ethanesulphonic acid (HEPES) buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantified after the addition of either lucigenin or luminol for a final concentration of 0.2 mM. Luminol detected a group of reactive species, i.e. hydroxyl radical (OH[•]), hydrogen peroxide (H₂O₂), while lucigenin is selective for super oxide radicals (O₂^{•-}) (Davies et al. 1992). Counts were obtained at 1 min intervals and the results were given as the area under curve for a counting period of 5 min. Counts were corrected for wet tissue weight and expressed as relative light units (rlu/mg tissue) (Haklar et al. 1998).

Total GSH measurement

GSH was measured by an adaptation of the method of Beutler et al (1963). For this, 0.2 ml of homogenate was vortexed in 0.2 ml of 0.5 M perchloric acid to precipitate protein. After centrifugation, 300 µl of the supernatant was added to 600 µl 0.3 M Na₂HPO₄ and 150 µl Ellman's reagent prepared by combining 200 mg 5,5' dithiobis-2-nitrobenzoic acid and 5 g sodium citrate in 1 l HPLC grade water. After incubation at 37°C for 15 min, the yellow colour formed was measured against a reagent blank at 412 nm in a spectrophotometer (UV-160A Shimadzu). A daily standard curve was prepared with glutathione (1–100 µM).

GP measurement

GP activity was measured by the methods of Paglia and Valentine (1967). In this method, GP catalyzes the oxidation of glutathione in the presence of tert-butyl hydroperoxide. Oxidized glutathione is converted to the reduced form in the presence of glutathione reductase and NADPH, while NADPH is oxidized to NADP. The reduction in absorbance of NADPH at 340 nm is measured. The absorbance change *per min* and the molar extinction coefficient of NADPH are used to calculate GP activity. GP activity expressed as µmol NADPH/min/g tissue.

GR measurement

GR enzyme activity was analyzed according forth method of Beutler (1985). Fifty of NADPH (2 mM) in 10 mM Tris-HCl buffer (pH 7.0) was added to a cuvette containing 50 µl of GSSG (20 mM) in phosphate buffer (0.5 M, pH 7.0) and 850 µl phosphate buffer and was then incubated at 37°C for 10 min. 10 µl homogenate was added to the NADPH-GSSG buffered solution and measured at 340 nm for 30 min. GR

activity expressed as µmol NADPH/min/g tissue. The results were expressed in nmol/mg protein.

SOD measurement

SOD activity was determined according to the method of Winterbourn et al. (1975). In this method, a xanthine-xanthine oxidase complex produces superoxide radicals, which react with nitrobluetetrazolium (NBT) to form the farmasone compound. The SOD activity is measured at 560 nm by detecting the inhibition of this reaction. One unit of SOD activity is defined as the activity that causes 50% inhibition of NBTH2 reduction rate. One unit of SOD expressed in U/mg protein was designated as the amount of enzyme that inhibits the reduction of nitrobluetetrazolium reduction by 50%.

CAT measurement

CAT activity was determined according to the method of Aebi (1985). H₂O₂ solution (10 mM), enzyme extract, and 50 mM phosphate buffer (pH 7.0) were pipetted into a cuvette. The reduction of H₂O₂ was followed at a wavelength of 240 nm for 4 min against a blank containing 50 mM phosphate buffer and enzyme extract. The activity was expressed as U/mg protein.

Complement 3 (C3) and complement 5 (C5) measurement

ELISA kit (C3, bio search cat no: MBS724829; C5, bio search cat. no: MBS721993 USA) was used to obtain, complement C3 and C5 assays.

Statistical analysis

SPSS software, version 15.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis of biochemical data. In order to, assess differences between groups, one-way analysis of variance (ANOVA) and Turkey's multiple comparison test were used. Results were presented as mean ± SD and *p* < 0.05 was considered to indicate a statistically significant result.

Results

As shown in Figure 1A, chronic administration of ethanol in pregnant rats resulted in a massive increase in the MDA content in liver and lymphoid tissue of the G10 and G30 groups compared with the C group (*p* < 0.001). The levels of another oxidative marker protein oxidation in the liver and lymphoid tissues were significantly higher in the G10 and G30 groups than in the C group (*p* < 0.001; Figure 1B). Na⁺/

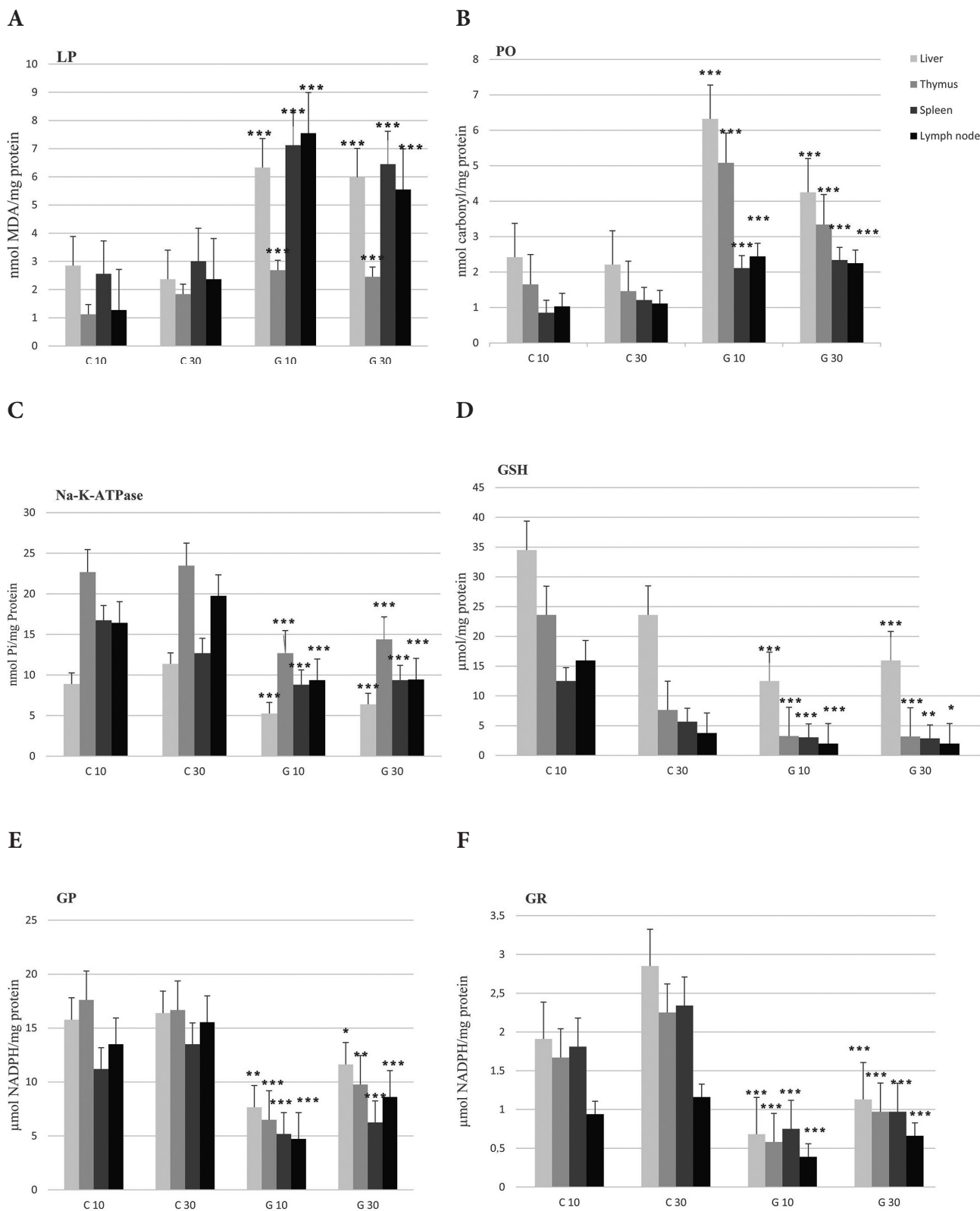


Figure 1. Effect of chronic alcohol consumption on lipid peroxidation (LP) levels (A), protein oxidation (PO) levels (B), Na^+/K^+ -ATPase activities (C), reduced glutathione (GSH, D), glutathione peroxidase (GP, E) and glutathione reductase (GSR, F) in liver and lymphoid tissues of 10 and 30 day old offsprings. *** $p < 0.001$ vs. control group. For more details, see Materials and Methods.

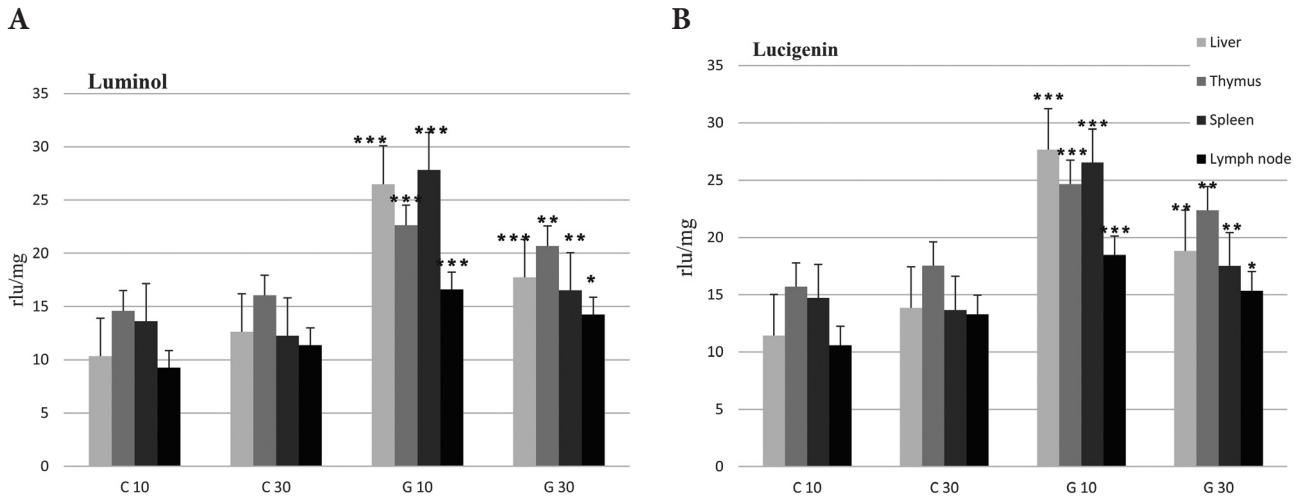


Figure 2. Effect of chronic alcohol consumption on luminol (A) and lucigenin (B) chemiluminescence in liver and lymphoid tissues of 10 and 30 day old offsprings. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs, control group.

K^+ -ATPase activities significantly decreased in the G10 and G30 groups than in the C group ($p < 0.001$; Figure 1C). The chemiluminescence levels in the liver and lymphoid tissue samples detected using both luminol and lucigenin probes showed significant increases in the G10 and G30 groups than in the C group ($p < 0.05$ – $p < 0.001$; Figures 2A,B). In accordance with this, ethanol caused a significant decrease in liver and lymphoid GSH levels in the G10 and G30 groups compared with the C group ($p < 0.05$ – $p < 0.001$; Figure 1D). The enzymatic GP activities significantly decreased in the liver and lymphoid tissue of G10 and G30 groups compared

with C group ($p < 0.05$ – $p < 0.001$; Figure 1E). As shown in Figure 1F, the GR activity in the studied tissues of G30 and G10 markedly decreased significantly compared with the C group ($p < 0.001$). As shown in Figure 3A, the SOD activation significantly decreased in the liver and lymphoid tissue of G10 and G30 groups compared with the C group ($p < 0.01$, $p < 0.001$). Similarly, CAT activation in the liver and lymphoid tissues significantly decreased compared with the C group ($p < 0.001$; Figure 3B). C3 and C5 activities were significantly higher in the G10 and G30 groups than in the C group ($p < 0.01$, $p < 0.001$; Figure 4).

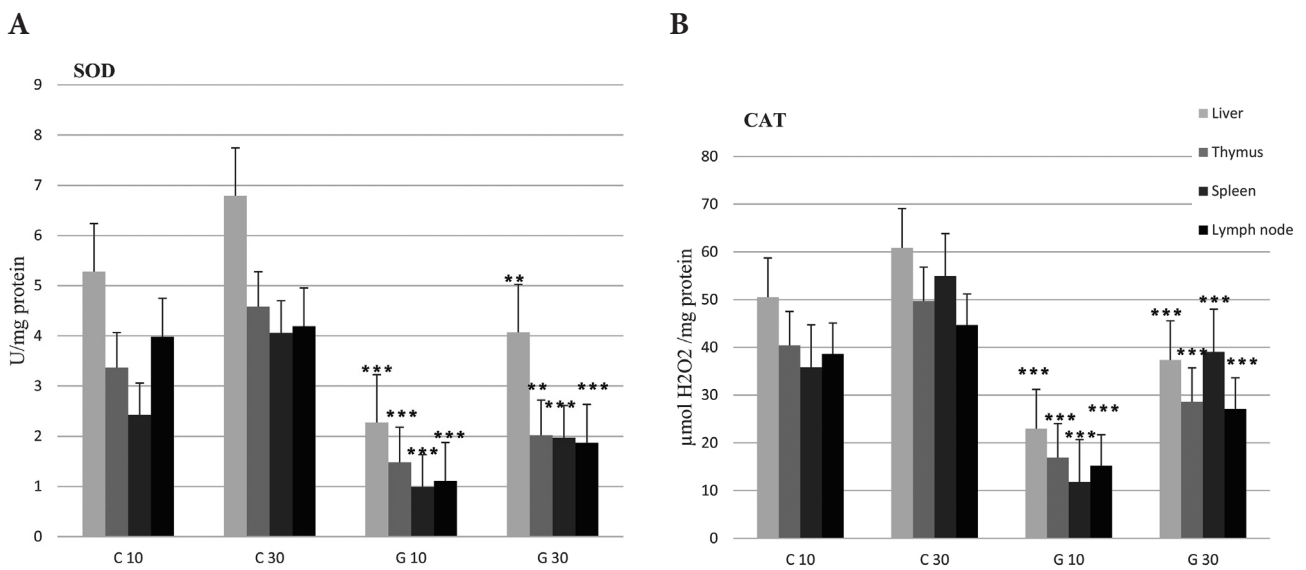


Figure 3. Effect of chronic alcohol consumption on super oxide dismutase (SOD, A) and catalase (CAT, B) activities in liver and lymphoid tissues of 10 and 30 day old offsprings. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs, control group.

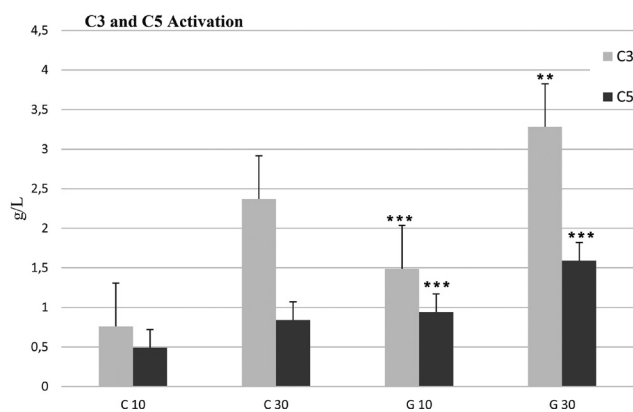


Figure 4. Effect of chronic alcohol consumption on complement C3 and C5 activities in 10 and 30 day old offsprings. ** $p < 0.01$, *** $p < 0.001$ vs, control group.

Discussion

This novel study examined maternal alcohol administration in lymphoid tissue on FASD. The results of the present study demonstrated that maternal alcohol caused oxidative tissue damage in the liver and lymphoid tissues (thymus, spleen, and lymph nodes) of the newborn rats (10 and 30 days old), as assessed by increased MDA, protein oxidation, CL levels; concomitant reduction in GSH levels and Na^+/K^+ -ATPase, GP, GR, SOD and CAT activities. Furthermore, the alcohol-induced ROS evaluation in plasma C3 and C5 was increased. Both clinically and experimentally, chronic excessive alcohol consumption impaired the function of a wide range of cells and tissues (Molina et al. 2003), but the mechanism and the extent of damage on different cell and organ systems have not been explained well. As implied in many studies, a major indicator of the cellular and molecular mechanisms of alcohol-induced tissue injury is the formation of ROS and the metabolite AT generation of ethanol. As a result of the formation of protein adducts (producing immune-mediated tissue damage), the activities of mitochondria and restorative enzymes were disrupted. Furthermore, both human and animal experimental studies reported that AT caused a reduction in the number of GSH groups and hydrophobic regions on the cell membrane and mitochondrial membranes (Wu and Cederbaum 2003). In the present study, MDA, a marker of lipid peroxidation, increased in the liver and lymphoid tissues, indicating the presence of maternal alcohol – induced oxidative damage. In accordance with the present findings, previous fetotoxicity studies showed that lipid peroxidation increased in the liver and brain tissues (Shirpoor et al. 2008). Moreover, luminol and lucigenin enhanced CL data supported, that tissue injury induced by maternal alcohol involved the generation of toxic oxygen metabolites. Protein (carbonyl) oxidation and lipid

peroxidation levels were widely used as biomarkers of tissue damage or oxidative stress (Augustyniak and Skrzydlewska 2010). As shown in Figure 1B, the protein oxidation levels significantly increased ($p < 0.001$) in G10 and G30 compared with the controls. Recent studies have shown that oxidized proteins are often functionally inactive, and their unfolding is associated with enhanced susceptibility to proteinases (Henderson et al. 1999). Sulfhydryl-containing proteins are considerable targets for free radicals and AT as well (Abraham et al. 2002). Na^+/K^+ -ATPase is an enzyme located in the cell membrane responsible for the active transport of various ions, thus maintaining the ionic and osmotic balance of the cells. Membrane-bound enzymes, such as Na^+/K^+ -ATPase, require phospholipids for the maintenance of their activity and are susceptible to structural changes due to LP (Prabhu and Balasubramanian 2003). Therefore, the assessment of Na^+/K^+ -ATPase activity can be used as an index for oxidant-induced tissue injury and lipid peroxidation (Tuna et al. 2001). This can explain why liver and lymphoid tissue injuries were accompanied by a simultaneous increase in MDA and protein oxidation levels and a decrease in Na^+/K^+ -ATPase activity in the present study.

Recent studies have indicated reduced GSH level as one of the mechanisms involved in the pathogenesis of alcohol-induced tissue injury. Thiol agents, such as GSH, are involved in preventing the damage resulting from alcohol-induced ROS (Schlorff et al. 1999). Thus, the decrease in liver and lymphoid tissue GSH levels may be due to its consumption during the alcohol-induced oxidative stress. Specifically, the present study demonstrated an increased level of MDA and PO and a decreased level of GSH and Na^+/K^+ -ATPase activity in the liver and lymphoid tissues of newborns for the first time. In accordance with the present results, previous reports also showed that the depletion of tissue GSH might permit LP and subsequent tissue damage (Cano et al. 2001).

Another noticeable finding in this study was that the GP and GR activities significantly decreased in G10 and G30 with respect to controls. The decrease could be attributed to diminishing GSH pool and regulation of redox balance and might generally be expected due to a possible increase in the formation of oxidized glutathione (GSSG) and the subsequent export of GSSG out of the cell (Aydin et al. 2007). This study also reported that both SOD and CAT activities were lower compared with those in the C group. The GP and CAT protected SOD against inactivation by H_2O_2 . Reciprocally, SOD protected GP and CAT against superoxide anions (Vidyasagar et al. 2004).

The reduction in the activity of these enzymes and GSH levels and increased lipid peroxidation and protein oxidation could reflect the adverse effect of maternal alcohol on this finely balanced antioxidant system in the liver and lymphoid tissues of 10- and 30-day old newborns. The present study indicated that the fluid of the fetal compartment retained

a peak alcohol concentration for a longer period of time due to the inability of the fetus to metabolize alcohol (Paintner et al. 2012), resulting in impairment in the oxidant/redox system in FAS, which was specific to the newborn liver and lymphoid tissues.

The activation of complement is required for the development of ethanol-induced tissue injury in mice (Cowell et al. 2003). However, the specific pathways by which ethanol exposure activates complement have not been identified. Ethanol feeding induces both complement activation and oxidative stress, even apoptosis (Higuchi et al. 1996). Furthermore, previous studies have shown the relationship between complement cascade and antioxidants (Lockwood and Gross 2005). Therefore, this study hypothesized that ethanol would activate the complement pathway, and C3 and C5 would contribute to ethanol-induced oxidative damage in lymphoid tissues. Recently, a close relationship was reported between C3 activation and excessive superoxide radicals. In this study, the natural free-radical scavenger, SOD, reduced ischemic brain injury and, simultaneously, the C3 level. These data implied that complement activation was directly related to oxidative stress (Yang et al. 2013). Moreover, endogenously generated C5 also exacerbated neuronal apoptosis under ischemic conditions (Pavlovski et al. 2012). In this study, the results showed that prenatal stress induced oxidative damage in lymphoid tissue of newborns (10- and 30-day old). Furthermore, plasma C3 and C5 levels significantly increased ($p < 0.01$, $p < 0.001$; Figure 4), implying that higher ROS levels showed higher C3 and C5 levels.

In conclusion, this study identified a causal relationship between ROS and C3 and C5 activation. The complement cascade was activated after ethanol induced tissue injury and had a deleterious role, and oxidative stress was closely associated with C3 and C5 activation. An antioxidant that can inhibit complement C3 activation and also possible C5 may offer a new strategy for therapeutic approaches in oxidative stress-related disorders such as alcoholic liver disease and stroke.

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