ORIGINAL PAPER

Ethanol exposure in prenatal and early postnatal induced cardiac injury in rats: involvement of oxidative stress, Hsp70, ERK 1/2, JNK, and apoptosis in a 3-month follow-up study

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Abstract

Alcohol exposure during pregnancy induces a wide range of structural and functional abnormalities in the fetal heart. However, the underlying mechanism of this phenomenon is not well known. This study was undertaken to elucidate probable mechanisms of myocardial damage induced by prenatal and early postnatal ethanol treatment. Pregnant Wistar rats received ethanol 4.5 g/kg BW once per day from the seventh day of gestation (GD7) throughout lactation. The oxidative stress injury of the myocardium in pups was evaluated by measuring levels of oxidative stress biomarkers. Histopathological examinations and Western blot were performed to evaluate histological features, apoptosis, and molecular alterations in the myocardial tissue of male pups on the postnatal day 21 (PN-21) and postnatal day 90 (PN-90). The results showed that maternal ethanol consumption caused oxidative stress (impaired total antioxidant capacity and malondialdehyde), histological changes, and apoptosis of the myocardium in the pups on PN-21 and PN-90. At the molecular levels, Western blot analysis revealed that ethanol modulated the protein expression of p-ERK1/2, p-JNK, and Hsp70 in the myocardial tissue of the pups after 21 and 90 days of birth compared with the controls. These findings revealed that maternal ethanol intake induced cardiac toxicity in part, mediated by oxidative stress and apoptosis in the pups. A further mechanism study revealed that ethanol enhanced ERK1/2 and JNK phosphorylation and Hsp70 protein expression.

Keywords Ethanol · Offspring · ERK1/2 · JNK · Hsp70 · Heart

Introduction

Maternal ethanol consumption during pregnancy or lactation causes serious health problems and is considered a spectrum of disorders, namely fetal alcohol syndrome (FASD) (Nogales et al. 2017; Seleverstov et al. 2017; Shirpoor et al. 2015). Several cardiovascular disorders are often visible in individuals with FASD including structural and functional deficits (Denny et al. 2017; Sun et al. 2015a; Tobiasz et al. 2018). In spite of the past few decades of research, our understanding of the pathogenesis of alcohol cardiotoxicity in pups is still limited. Important progress in this field has been the appreciation

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of the role of oxidative stress and apoptosis in the pathogenesis of alcohol heart damage (Coll et al. 2017; Shirpoor et al. 2015).

There is growing evidence indicating that ethanol-induced oxidative stress has been widely associated with mitogen-activated protein kinase (MAPK) pathways (Cui et al. 2011; Zhao et al. 2015). Emerging evidence considers a pivotal role for the MAPK family in several aspects of ethanol's hazardous effects (Aroor and Shukla 2004; Muniz et al. 2018). MAPK superfamily plays a crucial role in regulating cell growth, proliferation, and apoptosis in the heart. The JNK transduction pathway is correlatively propelled by oxidative stress, which may trigger apoptosis and cell mortality (Shen and Liu 2006; Zhu et al. 2018), while the extracellular signal-regulated kinase (ERK1/2) transduction pathway may promote a double-edged role in determination of cell survival or death, leading to apoptosis or necrosis (Lu and Xu 2006). Several studies reported that ethanol treatment modulated the MAPK signaling pathway–induced cell death (Kalluri and Ticku 2002; Sanna et al. 2002; Wang et al. 2017). Hsp70 is the best-

studied heat shock protein and a ubiquitous protein highly expressed in tissues to protect cells from stress situations (Sun et al. 2018; Wang et al. 2019). Numerous findings proved that the overexpression of Hsp70 increased cardiac resistance to stressors (Ronchi et al. 2004; Wang et al. 2016; Williamson et al. 2008; Yao et al. 2011). Hsp70 works as a molecular chaperone and has a crucial role in conserving and repairing cellular homeostasis or disorders under thermal, I/R (ischemia/reperfusion), and oxidative stress (Sun et al. 2015b). We, therefore, hypothesized that fetal exposure to ethanol during pregnancy and lactation would induce myocardial apoptosis, probably by modulating the ERK1/2 and JNK signaling pathways. We also expected increased Hsp70 in the myocardium because, as mentioned above, ethanol increases oxidative stress in the heart tissue.

Methods

Animals and experimental design

All animal procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care Committee, the Urmia University of Medical Sciences. The adult female Wistar rats (200–250 g) were kept on a 12:12-h light/dark cycle, with a controlled temperature (22 ± 1 °C). Food and water were made available ad libitum. The rats were bred overnight with males and tested for fertility the following morning. The presence of a vaginal plug was used as an indicator to confirm the mating event, which often leads to pregnancy. Day 0 of gestation (GD0) was determined by the presence of a vaginal copulatory plug. On gestation day 7 (GD7), female rats were singly housed and randomly divided into four groups ($n = 8$): (1) control-PN21, (2) ethanol-PN21, (3) control-PN90, (4) ethanol-PN90.

Ethanol-treated rats received a 4.5-g/kg body weight ethanol (Merck KGaA, Darmstadt, Germany) solution in normal saline (20% w/v) by oral gavage once per day from GD7 through PN-21 (Sadeghzadeh et al. 2018; Sadeghzadeh et al. 2019; Shirpoor et al. 2015). The control group was treated with vehicle only (tap water). Following their birth, litters were culled to 3 or 4 male pups/mother for preventing possible food deficiencies due to food competition among the litters. The male offspring from each group were anesthetized by ketamine 90 mg/kg and xylazine 10 mg/kg on PN-21 and PN-90 ($n = 8$ offspring from each group on PN-21 and PN-90). Then, the thoracic cavity was opened and the hearts were isolated and washed with cold saline. The apex of the hearts was fixed in buffered formalin and embedded in paraffin for histopathological purposes. The remaining part of each heart tissue was frozen in liquid nitrogen and stored at − 80 °C for later measurements.

Malondialdehyde and total antioxidant capacity assessment

Malondialdehyde (MDA) as the end product of lipid peroxidation was evaluated through a reaction with thiobarbituric acid (Sigma-Aldrich; St. Louis, MO, USA) in the heart samples according to manufacturer's protocol (Niehaus and Samuelsson 1968). In brief, 0.3–0.4 g of the heart tissue was homogenized in ice-cold KCl (150 mM) and then centrifuged at 3000×g for 10 min. Afterwards, 0.5 ml of this supernatant was combined with 3 ml phosphoric acid (1% v/v), and then following vortex mixing, 2 ml of 6.7 g l^{-1} TBA was subjoined to the samples. The specimens were heated at 100 °C for 45 min. After cooling down on ice, n-butanol (3 ml) was combined and the products were further centrifuged at 3000×g for another 10 min. Then, the absorbance of the products was considered at 532 nm through spectrophotometry. The absorbance rate was compared with the standard curve (Lowry et al. 1951). Total antioxidant capacity (TAC) was measured by a Randox (Crumlin, County Antrim, UK) total antioxidant status kit, in which 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfanate) (ABTS) is incubated with peroxidase and H_2O_2 resulting in the radical cation ABTS + production. This has a stable blue-green color, which is evaluated using a 600-nm automatic analyzer (Abbott model Alcyon 300; Abbott Laboratories, Abbott Park, IL, USA). Antioxidants in a sample cause the suppression of this color production to a degree that is related to their concentration (Shirpoor et al. 2014).

Western blotting

Hsp70, ERK1/2, and JNK (phosphorylated and total) in the myocardial tissue were determined by Western immunoblotting (Mohaddes et al. 2017). Briefly, the protein concentration of the supernatant was measured by the Bradford assay kit (Sigma-Aldrich, USA), and then, 20 μg of protein was loaded into each well after mixing with a 2× sample loading buffer. The proteins were separated in 10% SDS gels and then transferred to PVDF membranes in an hour for all the loaded samples. Subsequently, the blocking of the membranes was performed in a 5% skim milk buffer containing 0.1% Tween 20 for 1.5 h and then probed with primary antibodies against Hsp70, ERK1/2, JNK, and β-actin overnight at 4 °C in a shaker incubator. After 4× 5-min washing with a Tris-buffered saline solution containing 0.1% Tween 20, the HRP-conjugated secondary antibody (1:7000, Cell Signaling) was added to the membranes. After 1 h of incubation in the shaker, the membranes were bathed in wash buffer and washed at least 3× for 5 min. Then, the membranes were incubated with the enhanced chemiluminescence (ECL, Amersham) reagents in the dark room. This was followed by exposing the membrane to an X-ray film and visualizing the

Table 1 The antibodies used in Western blotting assays

Primary antibody	Company	Dilution	Catalog number
ERK1/2	Santa Cruz	1:500	sc-292838
p-ERK1/2	Santa Cruz	1:500	sc-16981-R
JNK	Santa Cruz	1:500	sc-7345
p-JNK	Santa Cruz	1:500	sc-6254
Hsp70	Santa Cruz	1:500	sc-66048
β-Actin	Santa Cruz	1:300	sc-130657

chemiluminescence of the binding by means of a visualizing machine. The intensity of the bands was determined using the ImageJ software (IJ 1.46r version, NIH, USA) and normalized to the bands of the internal control (beta-actin). The antibodies used in Western blotting assays (catalog numbers and companies) are shown in Table 1.

TUNEL assay

The TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling) evaluation of myonuclei positive for DNA strand breaks was characterized by a fluorescence detection kit (Roche Applied Science, Indianapolis, IN) and fluorescence microscopy. After dewaxing and rehydrating, paraffin tissue sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 8 min on ice. Terminal deoxynucleotidyl transferase (TdT) with fluorescein-dUTP as the TUNEL reaction mixture was added to the sections in 50-μl drops and incubated for 60 min at 37 °C in a humidified chamber in the dark. The slices were washed three times in PBS for 5 min each. Following embedding, the sections were visualized with an Olympus DP80 microscope equipped with an Olympus MaguaFire SP digital camera. Finally, the TUNEL-positive nuclei and TUNEL-negative cells were counted blindly to determine the percentage of apoptotic cells with the Image-Pro image analysis software (Media Cybernetics, Bethesda, MD) at the final magnification of × 400.

Hematoxylin–eosin staining

An amount of 5 μm of paraffinized ventricle was used for histopathological staining. The morphological changes were evaluated using Harris' hematoxylin and eosin (H&E) staining protocols under a light microscope (Olympus BH-2, Tokyo, Japan) in a blinded manner.

Statistical analysis

Normal distribution of data was examined with the Kolmogorov–Smirnov test. The data were statistically analyzed using the independent samples t test. The significant level was assessed at $p < 0.05$. The results were expressed as means ± SD.

Results

MDA and TAC assessment

MDA, as a lipid peroxidation marker, and TAC levels were taken as the indicators of oxidative stress. As shown in Figs. 1 and 2, a remarkable elevation in the MDA content accompanied by attenuation in TAC level ($p < 0.001$) was detected on PN-21 and PN-90 by maternal ethanol consumption during the pregnancy and lactation periods compared with the control groups.

Fig. 1 Effects of maternal ethanol consumption on the MDA level in myocardium tissue in different groups. The level of MDA increased in E-PN21 (14.03 ± 1.2) and E-PN90 (7.98 ± 0.49) compared with that in C-PN21 (1.75 ± 0.14) and C-PN90 (1.93 ± 0.12), respectively. ***$p < 0.001$ vs C-PN21; ###$p < 0.001$ vs C-PN90. All data are expressed as the means ± SD (n = 8)
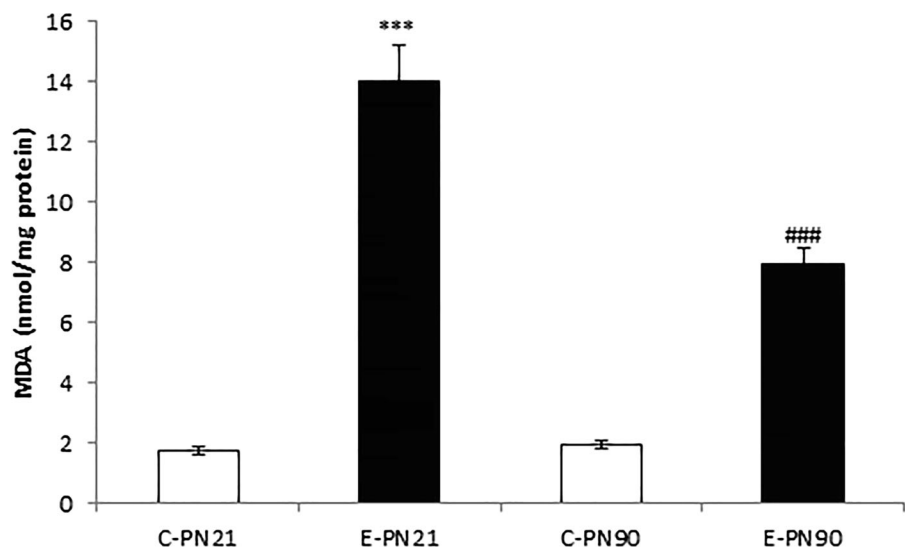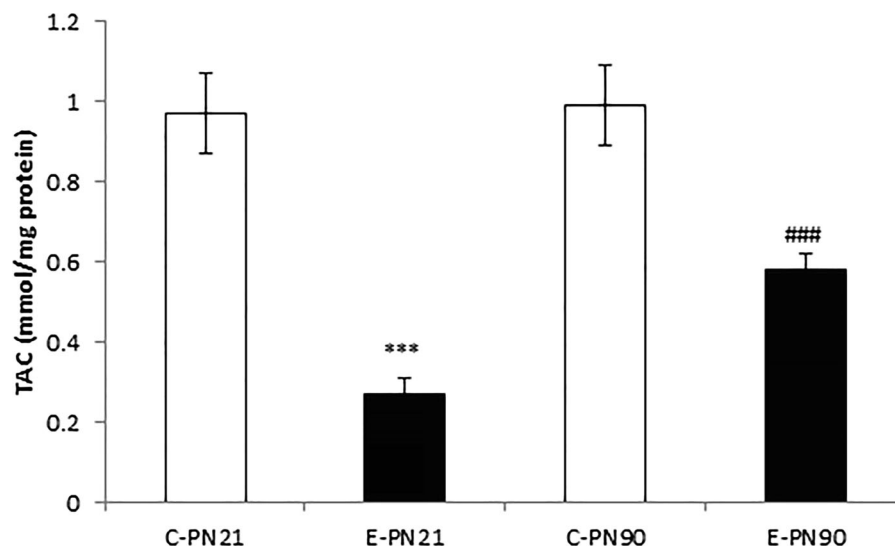
Fig. 2 Effects of maternal ethanol consumption on TAC in myocardium tissue in different groups. The level of TAC decreased in E-PN21 (0.27 ± 0.04) and E-PN90 (0.58 ± 0.04) compared with that in C-PN21 (0.97 ± 0.1) and C-PN90 (0.99 ± 0.1), respectively. ***$p < 0.001$ vs C-PN21; ###$p < 0.001$ C-PN90. All data are expressed as the means ± SD (n = 8)

ERK1/2, JNK, and Hsp70 protein expressions

JNK and ERK signaling pathways are highly involved with stress stimuli including oxidative stress which is associated with cell mortality and apoptosis. However, Hsp70, a highly conserved family of stress response proteins, was conferred to protect cells from stress insults. To investigate the effects of maternal ethanol consumption on Hsp70, JNK, and ERK signaling, the Western blotting assay was performed to examine the Hsp70 content and phosphorylated protein levels of JNK and ERK1/2 in the myocardium of the offspring exposed to ethanol consumption (Figs. 3 and 4). Based on the present Western blot results, maternal ethanol consumption in pregnancy and lactation significantly ($p < 0.001$) enhanced phosphorylation of ERK1/2 and JNK in the myocardium of the offspring at the end of lactation and 90 days after birth compared with the C-PN21 and C-PN90 groups, respectively. Furthermore, our results showed that ethanol consumption notably enhanced cardiac Hsp70 protein expression in litters 21 ($P < 0.01$) and 90 ($P < 0.05$) days after birth, exhibiting the protective role of this inducible protein in a stress situation.

TUNEL

To determine whether ethanol exposure induced apoptosis in cardiomyocytes, the TUNEL assay was performed. The results verified that ethanol exposure increased the number of TUNEL-positive cells in the E-PN21 ($p < 0.01$) and E-PN90 ($p < 0.001$) groups, as compared with that in the C-PN21 and C-PN90 groups, respectively (Fig. 5).
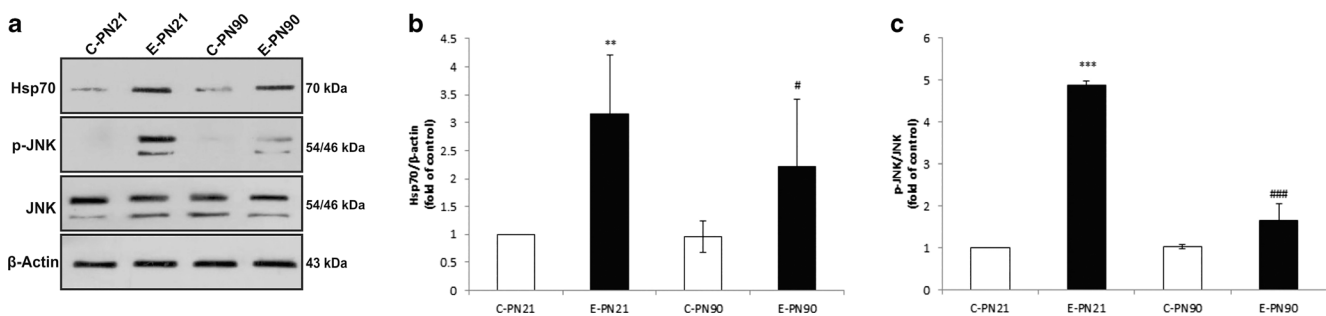

Fig. 3 The effect of maternal ethanol consumption on the phosphorylation of p-JNK and protein expression of Hsp70 in the myocardium of all experimental groups. The blotting images of p-JNK, JNK, and Hsp70 (**a**). The bar charts represent the quantitative analysis of the protein levels of p-JNK and Hsp70 (**b**, **c**) normalized against JNK and β-actin, respectively. The level of Hsp70 increased in E-PN21 (3.15 ± 1.06) and E-PN90 (2.22 ± 1.19) compared with that in C-PN21 (1 ± 0.0) and C-PN90 (0.96 ± 0.28), respectively. Similarly, the level of p-JNK/ JNK increased in E-PN21 (4.87 ± 0.1) and E-PN90 (1.65 ± 0.4) compared with that in C-PN21 (1 ± 0.0) and C-PN90 (1.04 ± 0.05), respectively. All data are expressed as the mean ± SD (n = 8). **$p < 0.01$ vs C-PN21; ***$p < 0.001$ vs C-PN21; #$p < 0.05$ vs C-PN90; ###$p < 0.001$ vs C-PN90
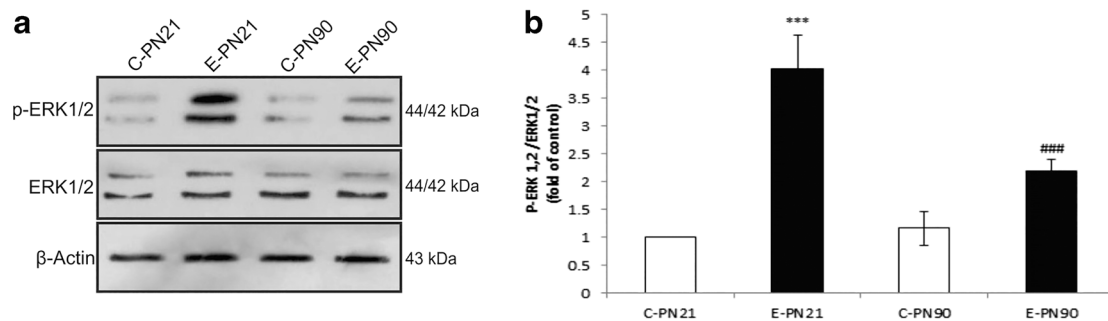
Fig. 4 The effect of maternal ethanol consumption on the phosphorylation of ERK1/2 protein in the myocardium of all experimental groups. The blotting images of p-ERK1/2 and ERK1/2 (**a**). The bar charts represent the quantitative analysis of p-ERK1/2 (**b**) normalized against ERK1/2. The level of p-ERK1/2/ERK1/2 increased in E-PN21 (4.03 ± 0.61) and E-PN90 (2.2 ± 0.2) compared with that in C-PN21 (1 ± 0.0) and C-PN90 (1.16 ± 0.31), respectively. All data are expressed as the mean $\pm$ SD (n = 8). ***$p < 0.001$ vs C-PN21; ###$p < 0.001$ vs C-PN90

Hematoxylin–eosin staining

In the heart slices of the C-PN21 and C-PN90 groups, a normal histological myocardial structure was observed. However, it was noted that severe histopathological changes such as cell vacuolization and cellular disarrangement were observed in all parts of the myocardial sections in the E-PN21 and E-PN90 groups in comparison with the control group animals. In addition, the increased cardiomyocyte transverse cross-section area was coupled with edema in cells, resulting in a significant reduction of interstitial space between fibers in the myocardial slices of the E-PN21 and E-PN90 groups (Fig. 6).

Discussion

In the present study, we demonstrated for the first time that maternal ethanol exposure had deleterious effects on myocardial tissues in offspring via oxidative stress, apoptosis, and regulation of Hsp70, ERK 1/2, and JNK signaling pathways.

To date, there have been few studies examining the adverse effects of maternal ethanol exposure on myocardial remodeling in pups (Denny et al. 2017; Jones and Smith 1973; Shirpoor et al. 2015; Tobiasz et al. 2018; Webster et al. 1984). Maternal ethanol intake induces alterations in some measures of morphological changes in the cardiovascular system including vascular contraction, blood pressure (Adickes et al. 1990; Daft et al. 1986), cell proliferation, capillary density, endothelial disarrangement, and PMN infiltrations in pups (Shirpoor et al. 2015). In corroboration with this issue, here in the present study, we extended the evidence to show ethanol treatment had adverse impact on the myocardial tissues of the pups. The increased myocardial transverse section area, cell vacuolization, and cellular disarrangement along with a reduction of interstitial space between fibers and also cell death or apoptosis were exhibited in the ethanol-exposed offspring on both PN-21 and PN-90. However, its exact

mechanism is not yet known, although several explanations were considered in the literature.

Oxidative stress, described as an excess production of reactive oxygen species (ROS), has been demonstrated to be involved in the pathophysiology of myocardial remodeling and dysfunction (Junior et al. 2019). Excessive ROS production triggers lipid peroxidation, persistent DNA strand breaks, and morphological and functional damage and can lead to irreversible damage or cell death (Mustroph et al. 2018).

According to previous reports, ethanol consumption destructs normal oxidative metabolism, leading to ROS accumulation, and thus creates oxidative stress. Oxidative stress, which has been strongly implicated in congenital heart defect, is a potent apoptotic inducer (Wu et al. 2016). In line with previous studies, as shown in the heart (Shirpoor et al. 2015) and other tissues (Cesconetto et al. 2016; Nogales et al. 2017; Shirpoor et al. 2014), we demonstrated that maternal ethanol exposure disrupted the oxidative balance in the myocardial tissue of the pups that was manifested by increasing the MDA and decreasing the TAC content.

Apoptosis is a common pathological process and plays an important role in the pathogenesis of ventricular remodeling (Gallo et al. 2015). Prenatal and late gestational ethanol exposure increases apoptosis and some apoptotic markers including caspase-3, caspase-8, and bcl-2, leading to myocardial dysfunction and remodeling in newborns (Goh et al. 2011; Ren et al. 2002; Yan et al. 2017). Our results suggested that maternal ethanol consumption–induced myocardial toxicity mediated through DNA fragmentation was shown by using the TUNEL assay. Through microscopic examination, TUNEL-positive cells were found on PN-21 and persistently expressed on PN-90. As mentioned above and according to our data, apoptosis caused myocardial damage after prenatal alcohol consumption, although they did not illustrate detailed mechanisms.

MAPKs are signaling pathways with an important role in complex cellular processes including proliferation, survival, and cell death, and are a ROS downstream gateway to
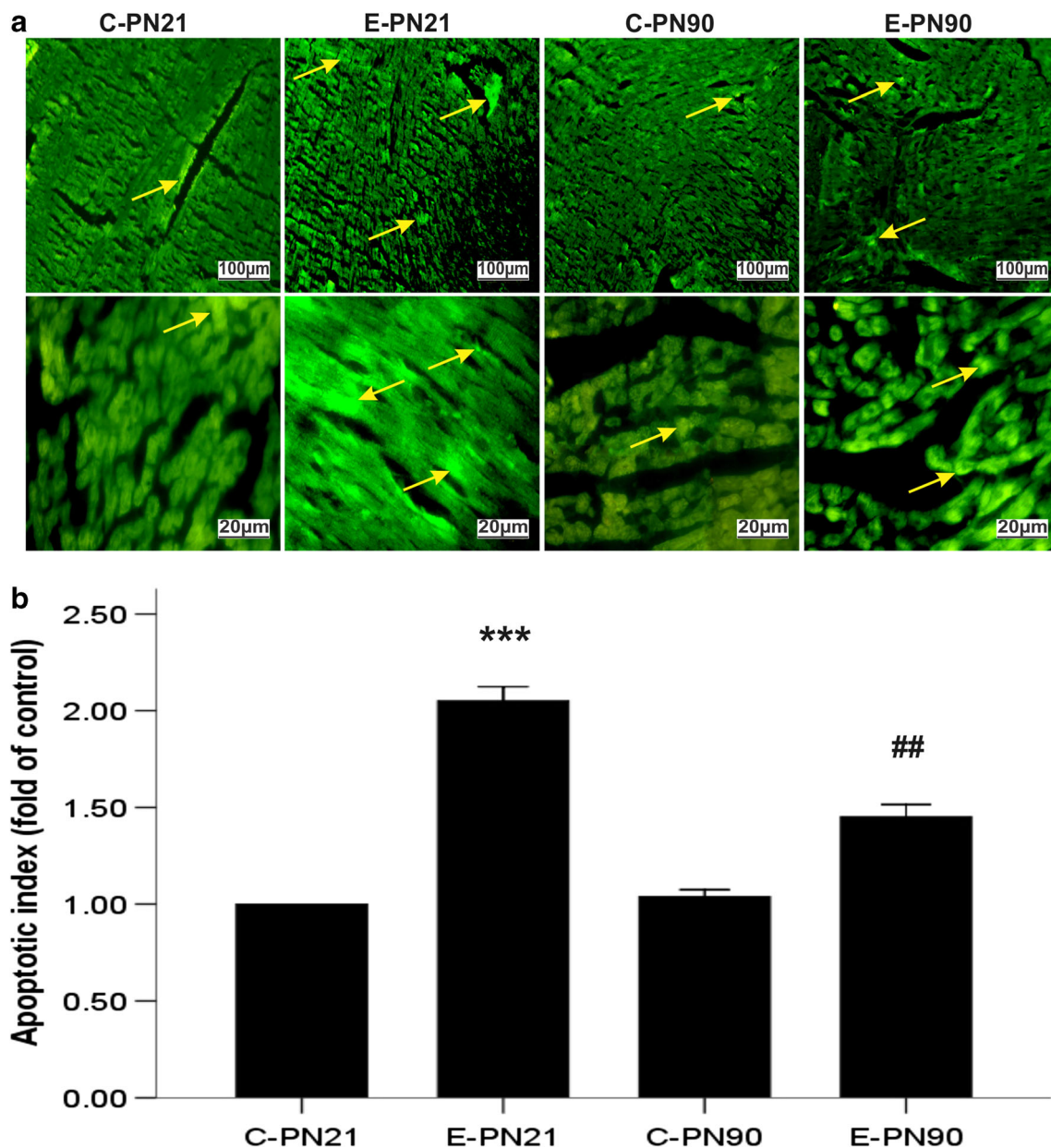
Fig. 5 Assessment of apoptosis in four different groups. **a** Images of apoptotic cells in the myocardium of pups from four groups with TUNEL staining. Ethanol exposure caused an increased number of apoptotic cells in the pups' myocardium (yellow arrow). Magnification × 400. **b** Quantitative analysis of the apoptotic index (percentage of TUNEL-positive nuclei, %). Apoptotic index increased in E-PN21 (1.87 ± 0.26) and E-PN90 (1.47 ± 0.26) compared with that in C-PN21 (1 ± 0.0) and C-PN90 (1.06 ± 0.08), respectively. All data are expressed as the mean $\pm$ SD ($n = 8$). ***$p < 0.001$ vs C-PN21; ##$p < 0.01$ vs C-PN90. Scale bars are as indicated

apoptosis (Gao et al. 2018). JNK activation has commonly been associated with cell inflammation and apoptosis, whereas the ERK pathway is implicated in cellular survival and growth (Sun et al. 2015b). The specificity of activation or inhibition of downstream molecules delineates the result of ERK1/2 phosphorylation on cell survival, which is anti-apoptotic, but in some cases, pro-apoptotic (Lu and Xu 2006; Wan et al. 2016). With that in mind, ERK activation was shown to be accompanied by cardioprotection in several studies (Portbury et al. 2012; Rose et al. 2010; Sun et al. 2006),

but, conversely, by myocardial apoptosis and dysfunction in some other studies (Wan et al. 2016). For instance, ERK1/2 activation in ethanol exposure leads to myocardial oxidative stress and disturbances (El-Mas and Abdel-Rahman 2015). Another report showed that cardiac apoptosis was induced by ischemia/reperfusion in H9c2 cells accompanied by ERK activation (Sun et al. 2015b).

JNK has been demonstrated to have a link to apoptosis mediated by ROS. Specifically, JNK inhibition prevents bcl-2 phosphorylation and dissociation of the Beclin 1–Bcl-2
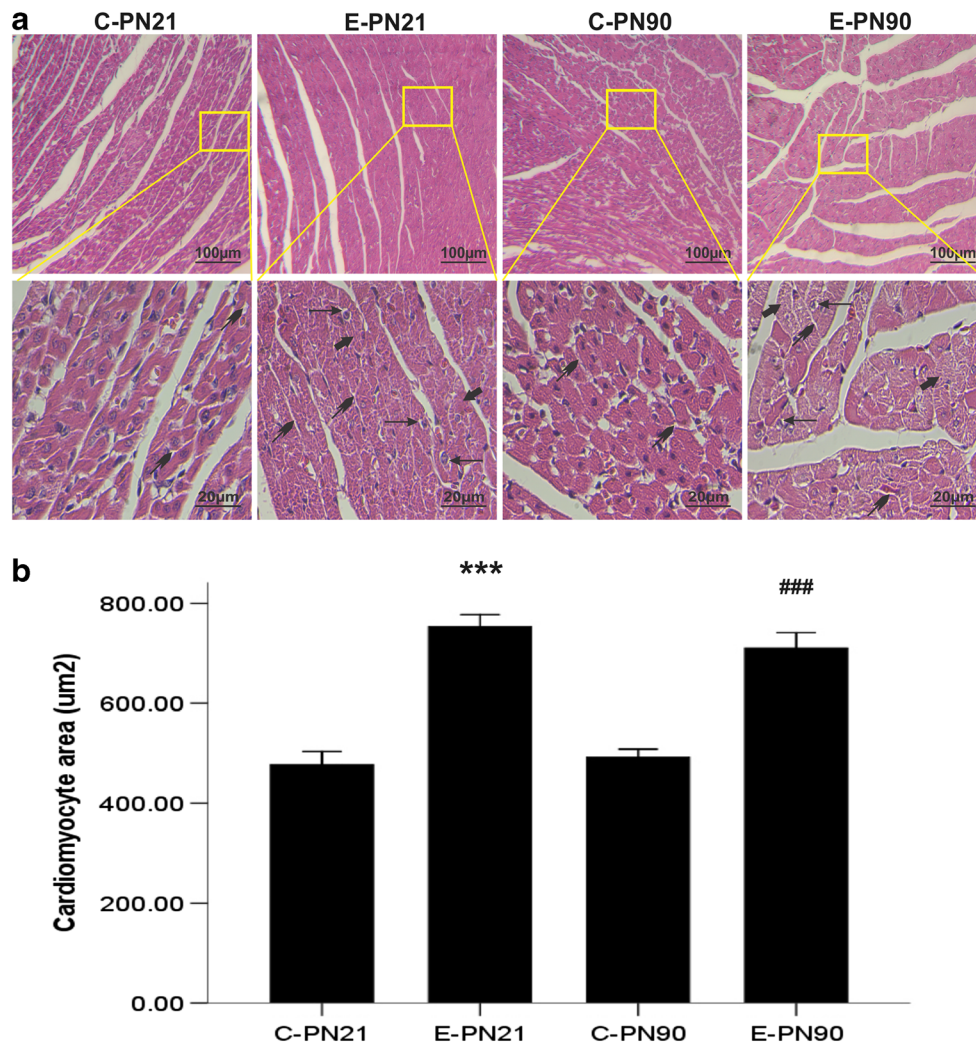
Fig. 6 Histological analysis of heart tissue in different groups. **a** Hematoxylin and eosin (H&E) staining micrographs of transverse sections of the myocardium (magnification × 400; scale bar 20 μm) and **b** quantitative analysis of cardiomyocyte cross-sectional area (measurements of 20 cardiomyocytes in AU). In both E-PN21 and E-PN90, severe histopathological changes such as cell vacuolization (◄—) and cellular disarrangement were observed. Also, the cardiomyocyte transverse section area (➡) was significantly increased which was accompanied with reduction of interstitial space between fibers (—➡) compared with the control groups. Cardiomyocyte area increased in E-PN21 (753 ± 156) and E-PN90 (710 ± 173) compared with that in C-PN21 (477 ± 118) and C-PN90 (492 ± 85), respectively. All data are expressed as the mean ± SD (n = 8). ***$p < 0.001$ vs C-PN21; ###$p < 0.001$ vs C-PN90. Scale bars are as indicated

complex to reduce markers of apoptosis (Steiner and Lang 2017). Accordingly, acute ethanol exposure induced autophagy associated with heart toxicity in part through the activation of JNK signaling pathway (Zhu et al. 2018).

As a preliminary finding, our analysis showed that maternal ethanol consumption during pregnancy and early postnatal days caused the activation of MAPKs by strengthening the phosphorylation of ERK1/2 and JNK proteins, which can be important in myocardial apoptosis.

Heat shock proteins are a family of molecular chaperones, involved in protein folding, and participate in the oxidant-caused lethal response, thereby playing a key role in recovery from stress insult (Sun et al. 2015b; Zhao et al. 2019). Moderate alcohol consumption improved cardiac preservation during oxidative insult by Hsp70 induction and distribution (Sato et al. 2002; Su et al. 1998). Consistent with this, embryonic rat heart–derived H9c2 myocytes were rescued from simulated ischemia via overproduction of Hsp70, as a biochemical stress indicator (Chong et al. 1998; Mestril et al. 1994). Likewise, Hsp70 is considered an effective inhibitor of apoptosis due to inhibition of anti-apoptotic proteins (Liu et al. 2016). Hence, relying on the upregulation of cardiac Hsp70 expression in the ethanol-treated group, cardiac cells attempt to retrieve homeostasis to hamper the long-term damage

caused by ethanol feeding. Therefore, in this study, based on the results, we reported that maternal ethanol treatment increased the expression of Hsp70 protein level, as the major stress-inducible protein in fetal heart, indicating the activation of the protective survival pathways against the detrimental effects of ethanol. Our results also revealed a drop in PN-90 points for the most markers studied. It may justify developing an adaptive response between interacting molecules during prolonged stress or reversible alterations after the withdrawal of alcohol consumption (El-Mas and Abdel-Rahman 2015; Singh et al. 1976).

One potential limitation of the present study was that we did not apply molecule inhibitors to accredit the signaling pathway mechanism. Another limitation of our study was that we did not study the effect of ethanol exposure on functional parameters including left ventricular developed pressure (LVDP), heart rate (HR), rate pressure product (RPP; LVDP $\times$ HR), and dp/dt in perfused heart according to the Langendorff method to further illuminate this phenomenon.

To the best of our knowledge, this was the first study of its kind to investigate the modification of ERK1/2, JNK, and Hsp70 proteins in the myocardium of pups after ethanol exposure which is related to oxidative stress, apoptosis, and cardiovascular abnormality along with heart tissue damage. The damage was demonstrated by a transverse section area, cell vacuolization, and cellular disarrangement along with a reduction of interstitial space between fibers.

Conclusion

Herein, we realized that maternal ethanol consumption during pregnancy and early postnatal days induced oxidative stress and apoptosis in part by modulating ERK1/2, JNK, and HSP70 protein expressions. These results provide further comprehension of the impact of alcohol abuse and molecular mechanisms on the pathogenesis of congenital heart disease. This surely will promote attractive opportunities for preventing congenital heart disease.

Compliance with ethical standards

All animal procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care Committee, the Urmia University of Medical Sciences.

Conflict of interest The authors declare that they have no conflict of interest.

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