



## Comparison of myocardial apoptosis in 21 and 90 days after birth in pups induced by maternal long term ethanol consumption

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### Abstract

**Background & Aims:** This study was designed to assess and the cardiac apoptosis after the prenatal and early postnatal ethanol treatment in pups.

**Materials and Methods:** Pregnant Wistar rats treated with ethanol 4.5g/kg BW once per day from the seventh day of gestation (GD7) throughout lactation. Apoptotic cells, body weight, heart weight and their association on postnatal day 21 (PN-21) and postnatal day 90 (PN-90) was evaluated in male pups.

**Results:** The results showed that maternal ethanol consumption increased apoptotic cells, decreased heart weight, body weight, and HW/BW after 21 and 90 days of birth compared with the controls. It has found more apoptotic cells in 90 days after births compared with 21 days of age.

**Conclusions:** our findings revealed that maternal ethanol intake in pups decreased HW/BW ratio in part through apoptosis, which is more predominant in 90 days of age.

**Keywords:** ethanol; offspring; apoptosis; heart

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### Introduction

Maternal ethanol consumption is a major cause of birth defects. It exerts teratogenic effects such as growth retardation, abnormal facial features, and several organ damages, collectively known as fetal alcohol syndrome (FASD) (1, 2). Several cardiovascular disorders emerge up to 50% of children diagnosed with FASD (3). Some reports have shown that prenatal ethanol exposure impaired cardiac maturity morphologically and functionally (3). However, its underlying mechanism

remains unknown. Apoptosis is considered as a possible key event during the development of cardiomyocytes (4). Loss of cardiomyocytes will result in heart dysfunction and may contribute to the development of heart failure (5, 6). Both in vivo and in vitro studies have reported that alcohol exposure can cause apoptosis of cardiomyocytes (7, 8). Consistently ethanol consumption during pregnancy and lactation led to increased apoptotic molecules in the harvested myocytes in pups. In this study, we used male Wistar

pups exposed to ethanol in utero as a model of FASD. Therefore, we assumed that fetal exposure to ethanol during pregnancy and lactation would induce myocardial apoptosis in 21 and 90 days after birth, which is manifested by decreasing heart weight to body weight ratio.

## Materials & Methods

### Animals and experimental design:

Our study was performed in accordance with the National Institutes of Health Guidelines for the Care and approved by the Urmia University of Medical Sciences, Animal Care Committee. Adult female Wistar rats (200–250 g) were kept on a 12:12 h light/dark cycle, with controlled temperature ( $22 \pm 1$  °C). Rats were given ad libitum access to food and water. The rats were bred overnight with males and were tested for fertility the next morning. The presence of a vaginal plug used as an indicator to confirm the mating event, which often leads to pregnancy. Day 0 of gestation (GD0) was assumed by the presence of a vaginal copulatory plug. On gestation day 7 (GD7), female rats were singly housed and randomly divided to 4 groups (n = 8): (1) control-PN21, (2) ethanol-PN21, (3) control-PN90, (4) ethanol-PN90.

Ethanol-treated rats received 4.5 g/kg body weight ethanol (Merck KGaA, Darmstadt, Germany) solution in normal saline (20% w/v) by a feeding tube once per day from GD7 through PN 21 (2). The control group was received 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 used for the study at 21 and 90 days of age. They were anesthetized by ketamine 90 mg/kg and xylazine 10mg/kg (n=8). Then the thoracic cavity was opened and heart tissue was isolated immediately. After weighting heart, a section of the ventricle was fixed in buffered formalin and was embedded in paraffin for further analysis.

### TUNEL assay:

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) evaluation of

myonuclei positive for DNA strand breaks was determined by fluorescence detection kit (Roche Applied Science, Indianapolis, IN) and fluorescence microscopy. After dewax and rehydrate, 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), fluorescein-dUTP as TUNEL reaction mixture was aggravated to the sections in 50- $\mu$ l drops and incubated for 60 min at 37 °C in a humidified chamber in the dark. The slices were washed 3 times in PBS for 5 min each. After embedding, the apoptosis index was evaluated using a light microscope. Results were presented as a percentage of TUNEL-positive cells to the total cell.

### Statistical analysis:

Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. The significant level was assessed at  $p < 0.05$ . Results are expressed as means  $\pm$  S.E.M.

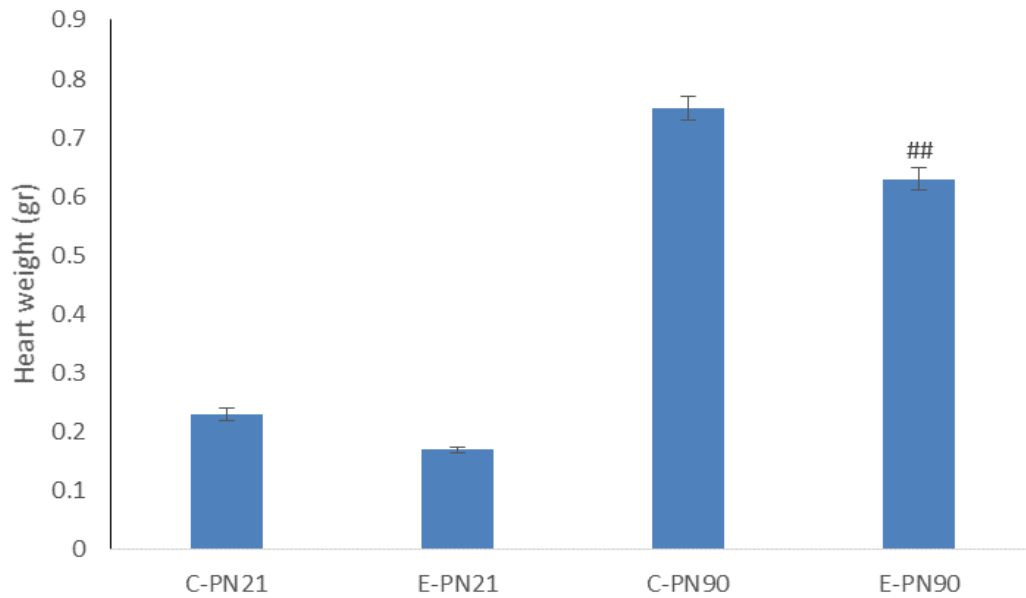
## Results

### Body weight, heart weight, heart weight/body weight ratio:

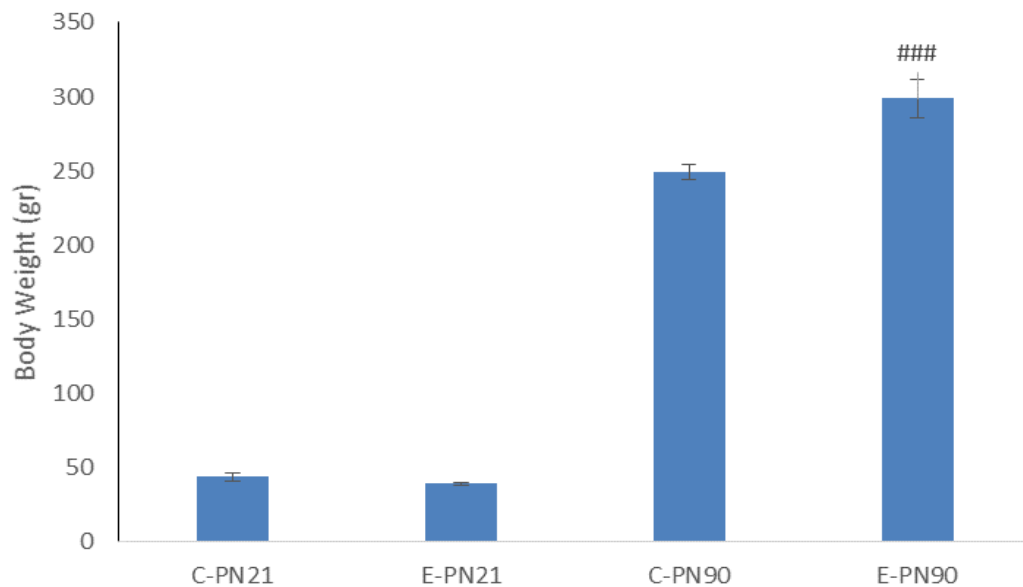
As shown in fig 1 heart weight of offspring in the E-PN21 and E-PN90 ( $p < 0.01$ ) group decreased compared to controls. However, it was not significant in the E-PN21 group. In addition body weight showed a significant increase in E-PN90 ( $p < 0.001$ ) compared to C-PN90 (fig 2). Also, we demonstrated that the HW/BW ratio decreased in E-PN21 ( $p < 0.05$ ) and E-PN90 ( $p < 0.01$ ) compared to control groups.

### TUNEL:

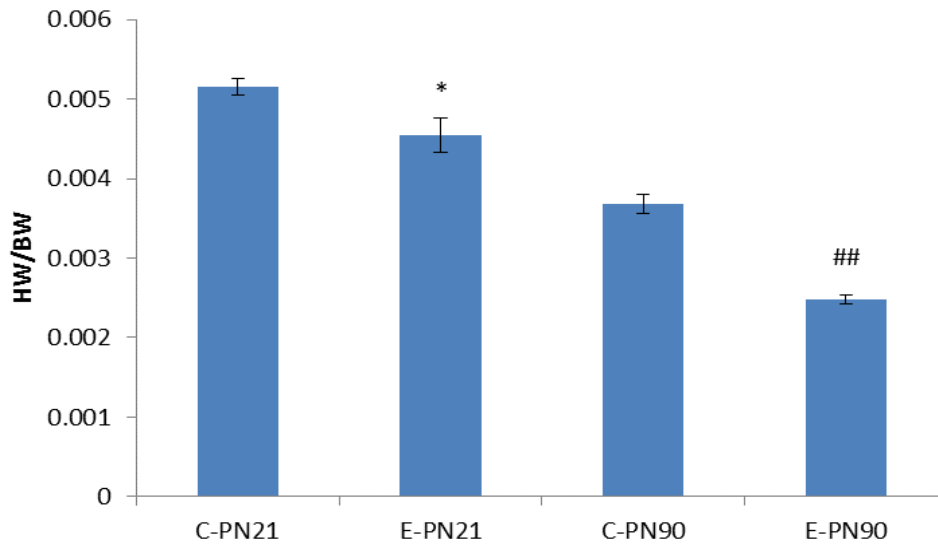
As shown in Fig 4, the cells in the control groups were negative for TUNEL staining. The number of apoptosis cells in myocardial tissue was appeared in ethanol groups compared to control groups. Quantitative analysis of the number of TUNEL (+) cells showed in table 1. These cells in myocardial tissue 90 days after births are significantly higher than 21 days of age ( $p < 0.001$ ).



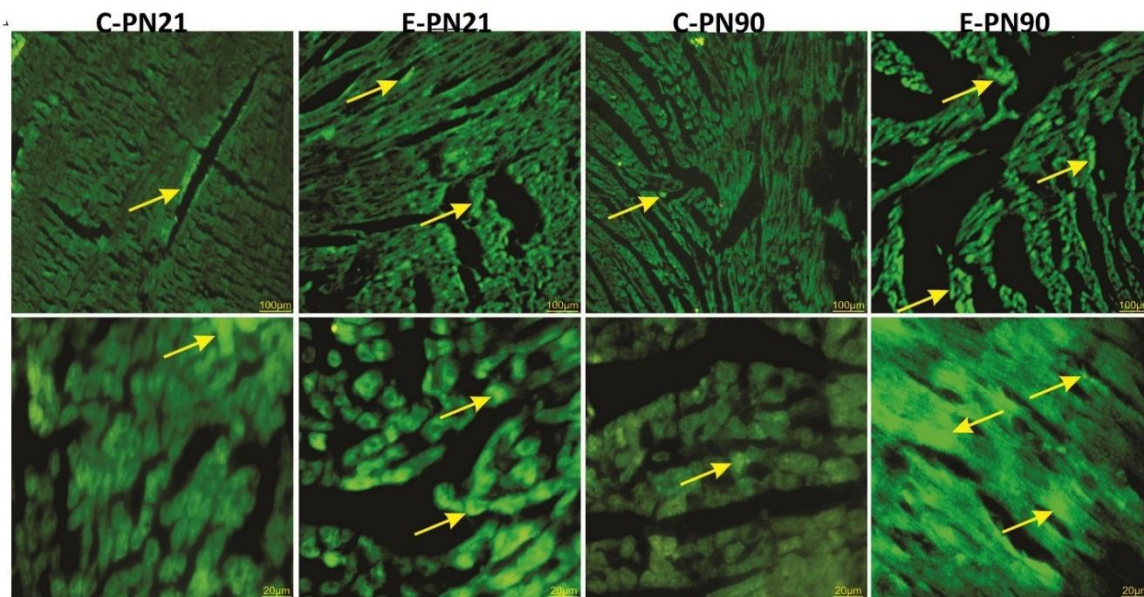
**Fig 1.** Effects of maternal ethanol consumption on heart weight in different groups. <sup>##</sup>  $P < 0.01$  vs C-PN90. All data are expressed as the means  $\pm$  SEM (n=8). C-PN21: control-PN21, E-PN21: ethanol-PN21, C-PN90: control-PN90, E-PN90: ethanol-PN90



**Fig 2.** Effects of maternal ethanol consumption on body weight in different groups. <sup>###</sup>  $P < 0.001$  vs C-PN90. All data are expressed as the means  $\pm$  SEM (n=8). C-PN21: control-PN21, E-PN21: ethanol-PN21, C-PN90: control-PN90, E-PN90: ethanol-PN90



**Fig 3.** Effects of maternal ethanol consumption on HW/BW (Heart weight/Body weight) in different groups. \* P<0.05 vs C-PN21, ## P<0.01 vs C-PN90.. All data are expressed as the means ± SEM (n=8). C-PN21: control-PN21, E-PN21: ethanol-PN21, C-PN90: control-PN90, E-PN90: ethanol-PN90



**Fig 4.** Expression of TUNEL (+) apoptosis cells in the heart tissue of pups exposed to ethanol in uterus. Arrows indicate TUNEL positive cells Scale bar= 20µm

C-PN21: control-PN21, E-PN21: ethanol-PN21, C-PN90: control-PN90, E-PN90: ethanol-PN90

**Table 1.** Quantitative analysis of the number of TUNEL (+) cells

Groups	C-PN21	E-PN21	C-PN90	E-PN90
TUNEL (+) cells	1±0.0	1.48±0.25**	1.05±0.15	2.06±0.27###\$\$\$

\*\* P<0.01vs C-PN21, ### P<0.001vs C-PN90, \$\$\$ P<0.001vs E-PN21 . All data are expressed as the means ± SEM (n=8). C-PN21: control-PN21, E-PN21: ethanol-PN21, C-PN90: control-PN90, E-PN90: ethanol-PN90

## Discussion

Several studies declared that ethanol is toxic to the heart and may cause cardiomyopathy (9). It is well known that maternal ethanol exposure causes teratogenic disorders in myocardial tissue in pups including abnormal heart development and histological damages (2, 10, 11). However, the underlying mechanisms are not clearly discovered. In this regard, oxidative stress, apoptosis, and inflammation were highlighted in organ malfunction (12).

Key evidence suggest that apoptosis of cardiomyocytes could have occurred in alcoholic cardiomyopathy (7). However, there is a little information in pups with prenatal ethanol exposure.

The major findings of the present study are decreased heart weight, body weight, HW/BW ratio and increased apoptosis in myocardial tissue of offspring in 21 and 90 days after birth following maternal ethanol usage. Furthermore, apoptotic positive cells were found in E-PN21 and E-PN90. However, there are higher apoptotic cells in E-PN90 compared to E-PN21.

Reduced heart weight to body weight is consistent with a teratogenic effect of ethanol and may contribute to altered myocardial function (8). It was known that ethanol reduces specific cellular contents of actin and myosin in cardiac myocytes (13). This may lead to morphological abnormalities and functional disorders which are related to decrease heart weight (14).

Apoptosis is a process led to programmed cell death with orderly activation of specific intracellular signaling pathways (15, 16). These signaling pathways finally lead to fragmentation of DNA (5). It was indicated that loss of cardiomyocytes through apoptosis contributes to developing failure of myocardial function and lead to cardiomyopathy (4).

In this study, we demonstrated that ethanol consumption by mothers in pregnancy-induced apoptosis in myocardial tissue of pups in 21 days of age and also in 90 days of ages. Our results are in agreement of previous studies which revealed that prenatal ethanol exposure promotes postnatal myocyte apoptosis in 10-12 weeks after birth at a dosage of 6 gr/kg/day (8).

Despite increasing apoptosis in 90 days (12 weeks) of age, in this study we found apoptotic cells in 21 days after birth. It shows that this process triggered cell death immediately in neonates. In addition, apoptotic features became more severe as the pups grew up in PN90.

Ren et al reported that apoptotic cells were found in 10-12 weeks after birth in pups from alcoholic mother (8). In addition, Prenatal alcohol exposure induced cardiac apoptosis in the embryonic hearts in cardiac progenitor cells (17). However, daily exposure to a moderate dose of ethanol in the late gestation accelerates the maturation of cardiomyocytes and increases left ventricular tissue volume and also a significant increases in relative fetal heart weight. They also declared that apoptotic gene expression increased in the ethanol-exposed hearts (18)

In all, our data is the first which declared that maternal ethanol consumption during pregnancy and early postnatal days caused myocardial apoptosis in 90 days of age more than 21 days of age.

## Conclusions

In conclusion, this study demonstrated that ethanol exposure in prenatal and postnatal decreased HW/BW ratio may be related to apoptosis which is more prominent in 90 days of birth than 21 days of age. However, further research is needed to describe detailed mechanisms.

## Acknowledgments

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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