

Influence of Maternal Ethanol Exposure on Systemic Hemodynamic Variables and Histopathological Changes in the Aorta Wall of Male Rat Offspring: A Three-month Follow-up

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What's Known

- In animal models, fetal alcohol exposure was found to cause various heart and vascular abnormalities, including heart cell proliferation, increased arterial stiffness, and impaired vascular vasodilation function.
- Furthermore, studies found that long-term ethanol exposure in pregnant rats resulted in an increase in mean arterial pressure in offspring at six months of age.

What's New

- Maternal ethanol exposure causes aorta wall remodeling on post-natal days 21 and 90.
- Ethanol causes an increase in inflammatory mediators in aorta tissue, as well as a change in hemodynamic variables, on post-natal days 21 and 90.

Abstract

Background: Alcohol consumption in pregnancy is associated with an increased risk of cardiovascular abnormalities, but the mechanisms are unknown. This study evaluated the impact of ethanol exposure on the offspring's aorta structural, functional, and molecular alterations on postnatal (PN) both on days 21 and 90.

Methods: This experimental study was conducted at Urmia University of Medical Sciences (Urmia, Iran) in 2019. Twenty Pregnant Wistar rats on the seventh day of Gestation Day (GD) were randomly divided into two groups: control and ethanol-treated groups (n=10 per group). From the seventh day of GD throughout lactation, rats in the ethanol group were fed binge alcohol (4.5 g/Kg body weight) once daily. Systemic hemodynamic variables in the offspring were analyzed using waveform contour analysis 90 days after birth. On postnatal days (PN) 21 and 90, aorta wall histological alterations and the level of inflammatory factors were assessed in the aorta of male offspring. The statistical differences were examined via an independent samples *t* test. $P < 0.05$ was considered to be statistically significant.

Results: The results revealed that offspring in the ethanol group had higher systolic, diastolic, mean arterial pressure, and diastolic pressure than the control group ($P < 0.001$). The level of aorta tissue tumor necrosis factor (TNF)- α , intercellular adhesion molecule (ICAM)-1, nuclear factor (NF)- κ , and endothelin-1 were significantly higher in the ethanol offspring group than in the control group ($P < 0.001$). Histopathological changes such as total aorta thickness, tunica media, tunica adventitia, elastin fiber thickness, fiber interval, and elastin/media ratio significantly increased in the aorta of the offspring of the ethanol group compared to the control group 21 and 90 days after birth.

Conclusion: Our findings suggest that prenatal and early postnatal ethanol exposure-induced cardiovascular abnormalities are, in part, due to predisposing the aorta to atherosclerosis, which was mediated through the aorta wall remodeling and inflammation process.

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Introduction

Alcohol consumption during pregnancy is associated with a number of fatal adverse effects, including premature birth, intrauterine growth restriction, craniofacial deficits, and central nervous system dysfunction, collectively named fetal alcohol spectrum disorders (FASD).^{1, 2} As the neurodevelopmental disorders induced by maternal alcohol consumption are visible and have obvious manifestations, early studies on FASD in terms of neurodevelopmental and craniofacial deficits have received much attention. Over the last two decades, researchers have focused on the impact of maternal ethanol consumption on cardiovascular system abnormalities, particularly in response to binge drinking.^{3, 4} Fetal alcohol exposure was found to induce some measures of heart and vascular abnormalities such as heart cell proliferation, increased arterial stiffening, and altered vascular vasodilation function.^{4, 5} Turcott and others also reported that long-term ethanol exposure in pregnant rats increased the mean arterial pressure and impaired the aortic endothelium-dependent relaxation in the offspring at six months of age.⁶ Cardiovascular malformation and vascular stiffness have also been reported in children exposed to ethanol during the gestation period.^{7, 8} Our laboratory recently found that ethanol exposure during pregnancy and lactation caused cardiac structural and biochemical changes, hippocampus defects, and testis abnormalities in the rats' offspring 21 and 90 days after birth.^{5, 9, 10} These studies provide evidence that chronic ethanol exposure during cardiovascular system development predisposes the adult offspring to a risk of cardiovascular dysfunction.

The purpose of this study was to explore the effect of maternal ethanol ingestion on the aorta of male rat offspring in terms of functional histological and molecular endpoints at days 21 and 90 after birth.

Materials and Methods

Experimental Procedure

This experimental study was conducted at Urmia University of Medical Sciences (Urmia, Iran) in 2019. The study protocol was approved by the Ethics Committee of Urmia University of Medical Sciences (code: IR.UMSU.RES.1398.129). The animal care and experimental procedures were performed according to the national guidelines, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Twenty pregnant date-mated rats were housed separately and assigned to two groups:

alcohol and control.⁵ From Gestation Day (GD) seven to postnatal day (PN) 21 (lactation period), the rats in the alcohol group were given ethanol at a dose of 4.5 g/Kg body weight (Merck KGaA, Darmstadt, Germany) diluted with distilled water (20% w/v) intragastrically by gavage once a day. Due to the impact of sex differences on response to ethanol, only male pups were used in this study. During the weaning period, the pups (control and ethanol-exposed) had free access to nipples. Ketamine (Alfasan, Holland; 10%, 80 mg/Kg B.W, IP) and xylazine (Alfasan, Holland; 2%, 10 mg/Kg B.W, IP) were used to anesthetize the offspring from each group on PN21 and PN90 (n=8 offspring from each group PN21 and PN90).

Blood Pressure and Hypertension

Systemic hemodynamic variables and heart rates were determined using a physiograph and a digital waveform contour analysis method directly recorded from the carotid artery (NARCO, Bio-system, USA). The detail of the protocol were outlined in our previous work.¹¹ Power Lab Software (ADInstruments, Australia) was used to analyze the data, i.e., the recorded digital volume pulse.

Tissue Sampling

After blood pressure measurement (only in PN90 pups), the thoracic cavity was opened, and the aorta was dissected from the root to the abdomen descending section. The harvested aorta was washed and cleansed to remove any adventitial tissues, fat, and blood clots.

For histological analysis, formalin was used to fix aorta samples. After the dehydration steps, the samples were embedded in paraffin and sectioned at 5 μ m for histological evaluation. In order to assess the histopathological alterations, eight slides per group and four non-overlapping fields of view per section from two to three sections per animal were analyzed.

For biochemical analysis, the remaining half of the aorta was rinsed with ice-cold normal saline and wiped with filter papers for biochemical analysis. After, mixing with an ice-cold extraction buffer (10% wt/Vol) including a 50 mM phosphate buffer (pH 7.4), Ultra Turrax (T10B, IKA, Germany) was used for homogenizing the samples. The products were then centrifuged at 10,000 \times g at 4 °C for 20 minutes, and the supernatant was frozen at -80 °C for further analysis.

Histopathological Assessment

Sections of the aorta were stained with Masson trichrome according to the

manufacturer's instruction (Asiapajohesh, Amol, Iran). The intensity of aorta fibrosis in different groups was assessed using a semi-quantitative method described by Ashcroft and others.¹² They applied criteria for scoring fibrosis intensity as 0=normal aorta tunica media, 1=minimal fibrosis thickening of the aorta tunica media, 2 and 3=moderate thickening of aorta tunica media without obvious damage to the structure of the aorta tissue, 4 and 5=increased fibrosis with definite damage to the architecture of the aorta and formation of fibrosis bands or small fibrosis masses, 6 and 7=severe distortion of the structure and large fibrosis areas, and 8=total fibrotic obliteration.

After preparing the tissue sections, Verhoeff's van Gieson and Periodic Acid-Schiff staining were used to detect elastin bands. To assess the atherosclerotic features, eight sections of the aorta from different experimental groups were used. Herein, the total aorta thickness, tunica media, tunica adventitia, smooth muscle cell layers' width, and elastic fiber thickness were measured using a linear scale-ocular micrometer with a 2.5 mm interval between divisions under x400 magnification with a digital camera equipped with a research microscope (Olympus, CX 31, Japan).

Biochemical Examinations

The levels of TNF- α , ICAM-1, NF- κ B, and endothelin-1 in the aorta tissue were analyzed using the quantitative sandwich enzyme immunoassay method, commercial rat TNF- α , ICAM-1, endothelin-1, and NF- κ B (Shanghaicrystal Day, Biotech, China) kits, according to the manufacturer's instruction.

Statistical Analysis

The normal distribution of the data within each group was verified through the Kolmogorov-Smirnov test using SPSS software (version 17.0; SPSS Inc, Chicago, IL, USA). In addition, the statistical differences between the control and ethanol groups were tested through an independent samples *t* test. The data obtained from each test were presented as mean \pm SEM, and $P < 0.05$ was considered to be statistically significant.

Results

Aorta Wall Cytokines and Chemokine Changes

The changes in inflammatory cytokines and chemokine levels in the offspring of the experimental groups are presented in table 1. Pre- and early postnatal animals exposed to ethanol showed significantly higher levels of pup aorta tissue, TNF- α , and NF- κ B on both PN21 and PN 90, when compared with control pups of the same age groups ($P < 0.001$). As compared to the control group, ICAM-1 level in the ethanol-treated groups showed a significant increase in the aorta wall of litters in both PN21 and PN90 ($P < 0.001$). On PN90, the Endothelin-1 level was significantly higher in the aorta tissue of pups from the ethanol group than in the control group ($P < 0.001$).

Aorta Wall Thickness Changes

The data regarding the aorta wall elastin layers' changes in litters is summarized in table 2 and figure 1. As compared to the control pups of the same age groups, the total aorta wall thickness in dams exposed to ethanol increased significantly on both PN21 and PN90 ($P < 0.001$). On PN21, tunica media and tunica adventitia thickness increased significantly in the aorta of the ethanol group compared to the control group ($P < 0.001$). On PN90, tunica media showed a significant increase compared to the control group ($P < 0.001$), but there were no significant differences regarding the tunica adventitia thickness changes between the ethanol and control groups ($P = 0.37$). The elastin fiber thickness and entire elastin thickness were significantly increased in the aorta of the ethanol-treated litters compared to the control group on both PN21 and PN90, $P = 0.044$, and $P = 0.015$, respectively. On PN90, the ethanol group had a significantly higher elastin fiber interval than the control group ($P = 0.014$). There was no significant difference between the ethanol and control groups in the elastin fiber interval ($P = 0.55$), on PN21. The smooth muscle cells thickness on PN90 was significantly greater than in the control group ($P < 0.001$). In the aorta of the ethanol-treated group, the elastin/media ratio was significantly higher than the control group on PN21 ($P = 0.011$).

Table 1: Ethanol exposure-induced alteration of tumor necrosis factor- α , intercellular adhesion molecule -1, nuclear factor- κ and Endothelin amounts in the aorta tissue of male offspring on postnatal days 21 and 90

Variable	Groups	Control 21 (n=8)	Ethanol 21 (n=8)	P value	Control 90 (n=8)	Ethanol 90 (n=8)	P value
TNF- α (ng/mg)		152.62 \pm 7.4	518.73 \pm 11.8*	<0.001	170.30 \pm 5	664.84 \pm 17.60*	<0.001
ICAM-1 (ng/mg)		0.75 \pm 0.02	0.86 \pm 0.04*	0.03	0.28 \pm 0.03	0.52 \pm 0.030*	<0.001
NF- κ B (ng/mg)		32.01 \pm 3.27	56.9 \pm 4.6*	0.005	22.57 \pm 2.20	49.80 \pm 2.10*	<0.001
Endothelin-1 (ng/mg)		-	-	-	48.40 \pm 3.30	79.33 \pm 2.85*	<0.001

The statistical differences between the control and ethanol groups were tested via an independent samples *t* test. Values expressed as mean \pm SEM. *Significant difference from the control group.

Table 2: effect of prenatal and early postnatal ethanol consumption on data related to aorta wall of control and ethanol groups in postnatal days 21 and 90

Groups	Control 21	Ethanol 21	P value	Control 90	Ethanol 90	P value
Total Aorta thickness (μm)	117 \pm 5.61	228 \pm 38.31*	0.014	167 \pm 5	260 \pm 14*	<0.001
Tunica Media (μm)	93 \pm 4.70	119 \pm 15.56*	0.05	83.50 \pm 4.3	188 \pm 8.1*	<0.001
Tunica Adventitia (μm)	22.90 \pm 1.10	56.8 \pm 6*	<0.001	83.50 \pm 7.3	70.40 \pm 11.9	0.37
Elastin Fiber Thickness (μm)	2.52 \pm 0.02	4 \pm 0.25*	0.044	3.62 \pm 0.12	6.25 \pm 0.79*	0.015
Fiber Interval	5.62 \pm 0.22	5.25 \pm 0.61	0.55	7 \pm 0.3	10.25 \pm 1*	0.014
Entire elastin thickness (μm)	26.63 \pm 0.82	37.50 \pm 3.4*	<0.001	32.62 \pm 1.98	65 \pm 9.21*	0.004
Elastin/Media	28.25 \pm 1.13	37.41 \pm 2.84*	0.011	39.23 \pm 1.66	44.09 \pm 4.6	0.315
Entire SMC thickness (μm)	52.77 \pm 1.86	51.25 \pm 4.14	0.73	46.75 \pm 4.1	90.93 \pm 1.86*	<0.001
Media/Aorta	80.04 \pm 1.38%	59.01 \pm 3.55*%	<0.001	50.57 \pm 2.7%	72.73 \pm 2.89*%	<0.001
SMC/EFT	1.95 \pm 0.12	1.38 \pm 0.16*	0.021	1.56 \pm 0.11	1.53 \pm 0.12	0.86
SMC/Media	51.40 \pm 2.8	54.98 \pm 7.01	0.64	57.95 \pm 4.32	53.58 \pm 2.58	0.43

The statistical differences between the control and ethanol groups were tested via an independent samples *t* test. Values expressed as mean \pm SEM. *Significant difference from the control group

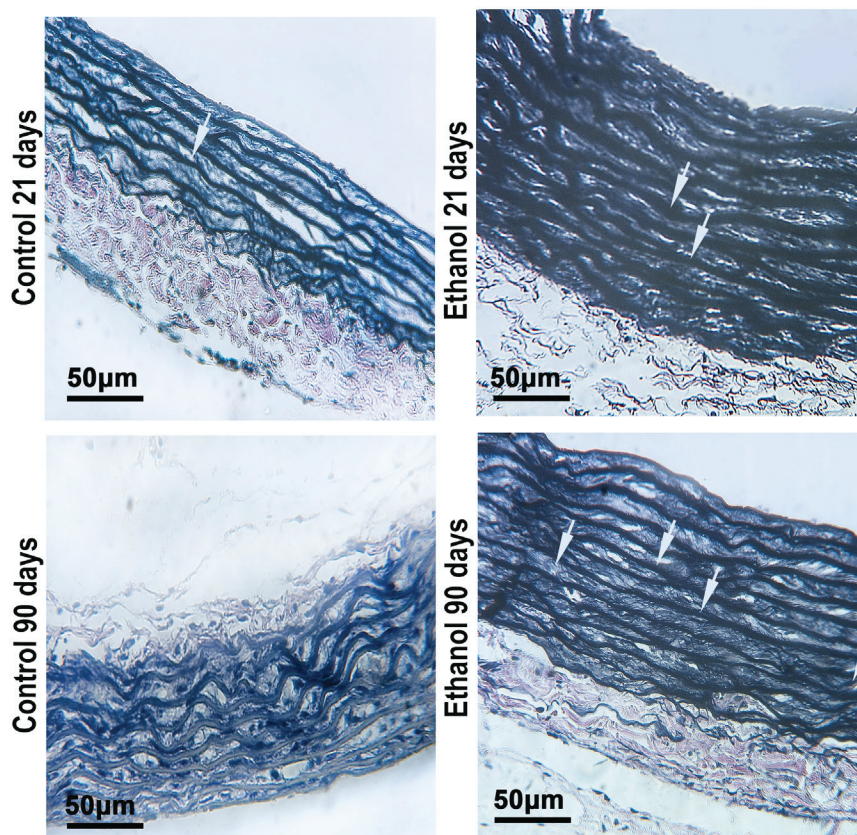


Figure 1: The ethanol-treated group showed an increase in total aorta thickness, tunica media, tunica adventitia, elastin fiber thickness, fiber interval, and elastin/media ratio compared to the controls on PN21 and PN90 (Verhoeff's van Gieson). Arrows show elastin layer thickness in different groups ($\times 400$).

On PN90, there were no significant differences in the elastin/media ratio between the ethanol and control groups ($P=0.31$). On PN21 and PN90, the media/aorta ratio in ethanol-exposed groups was significantly higher than that of the control groups ($P<0.001$).

Aorta Wall Collagen Changes

Masson's trichrome staining results revealed no lesion score in the aorta media of the control group on PN21 and PN90, as well as the

ethanol group on PN21 (grade=0). On PN90, the microscopic lesion scores in the tunica media of the aorta were 2 and 3, indicating moderate thickening of aorta tunica media without obvious damage to the aorta tissue structure. Tunica adventitia of the aorta was normally composed of collagen and fibrosis, which was visible in both the control and ethanol-treated groups. Ethanol-treated groups indicated a significant increase in fibrosis layer thickness on both PN21 and PN90, compared to the controls (figure 2).

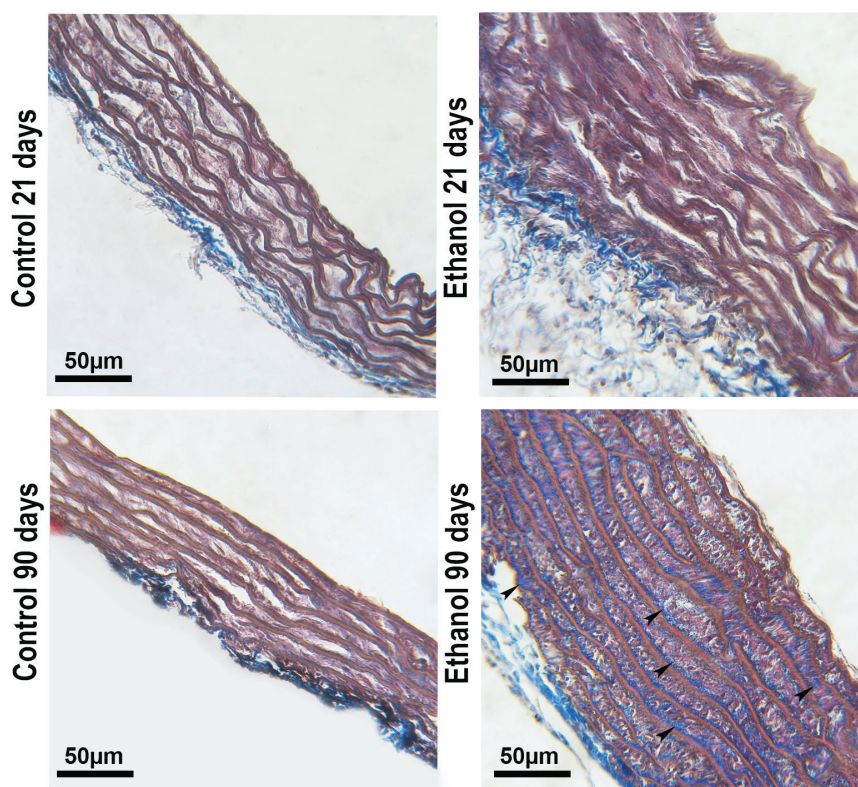


Figure 2: The arrows show the collagen accumulation in the intima region of the PN 90 ethanol treated rats, which appeared in blue color (Masson trichrome, ×400).

Table 3: effect of pre and early postnatal exposure of mothers on blood pressure variables in male offspring of rats

Variable	Groups	Control 90 (n=8) mean±SEM	Ethanol 90 (n=8) mean±SEM	P value
Heart rate (beat/min)		266.72±5.08	271.25±8	0.67
Psys (mmHg)		98.67±2.1	142.86±4.2*	<0.001
Pdias (mmHg)		87.60±2.6	114±5.2*	<0.001
M.A.P (mmHg)		91.42±2	121.20±4.7*	<0.001
Dicrotic. Pressure (mmHg)		94.37±1.7	127.15±4.7*	<0.001
Pulse. Pressure (mmHg)		12.18±1.29	29.74±6.1*	0.48

The statistical differences between the control and ethanol groups were tested via an independent samples *t* test. *Significant difference from the control group.

Blood Pressure

The digital waveform contour analysis method was used to analyze systemic hemodynamic variables and heart rates in PN90 pups, and the results are reported in table 3. Heart rates did not differ significantly between the ethanol-treated and control groups ($P=0.67$). On PN90, the ethanol-treated pups had significantly higher systolic, diastolic, and mean arterial blood pressures than the control pups ($P<0.001$). When compared to the control group, dicrotic notch pressure and pulse pressure were increased significantly in the ethanol group ($P=0.05$).

Discussion

The findings of the present study provide several lines of evidence such as increased aorta wall

thickness, high blood pressure, and increased inflammatory cytokines, including TNF- α , ICAM-1, NF- κ B, and endothelin levels in the aorta tissue of the offspring exposed to ethanol during pregnancy and early postnatal period 90 days after birth. All the measurements performed herein were known as possible risk factors for cardiovascular abnormalities, which also predispose the aorta wall to atherosclerosis. Previous studies on the effect of ethanol feeding during pregnancy on offspring blood pressure variables reported ambiguous results. Several studies found that mothers' ethanol exposure during the gestation period led to a significant increase in systolic, diastolic, and mean arterial pressure in their offspring, which is consistent with the findings of the current study.^{6, 13, 14} Other studies on animal models or on offspring of mothers who consumed

alcohol during pregnancy found that ethanol consumption during gestation had no effect on blood pressure variables.^{8, 15} The discrepancy between these studies and our findings could be attributed to technical differences in blood pressure measurement methods. Previous studies obtained blood pressure-related variables using tail-cuff methods in animal studies and brachial artery-cuff methods in human studies, while we employed contour analysis of hemodynamic variables using carotid arterial cannulation. Although this method is aggressive, the results are more informative and reliable than traditional methods such as tail-cuff methods.^{16, 17}

In our study, in addition to a significant rise in systolic, diastolic, and mean arterial pressure, there was a significant increase in diastolic or reflected pressure in pups obtained from the ethanol group 90 days after birth. The height of the dichroic notch indicates reduced nitric oxide (NO) production, which leads to aorta wall stiffness. It acts as a strong vasodilator contributing to vessel diameters, tone, resistance, and compliance of vasculature.¹⁸ Two reviews of pulse waves in human fingers and rabbit auricular arteries revealed that changes in the NO pathway were associated with variations in the relative height of the dichroic notch.^{18, 19}

In addition to an increase in diastolic pressure, the current study found a parallel significant increase in total aorta thickness, tunica media thickness, elastin fiber thickness, elastin fiber interval, and the media/aorta ratio in the ethanol group compared to the control animals. We also found significant collagen deposition (grade 2, 3) in the elastin fiber interval and a fibrosis score of grade 4-5 in the adventitia of the ethanol group, indicating severe adventitia thickening. It has been reported that elevated collagen deposition and elastin fraction in the artery wall increased arterial stiffness.²⁰

Recently, arterial stiffness has been proposed as an independent predictor of cardiovascular diseases, such as increased pulse pressure/hypertension, left ventricular dysfunction/hypertrophy, and myocardial ischemia/increased metabolic demands.²⁰⁻²²

It's worth noting that aortic media thickens is responsible for better tension distribution across the aortic wall, and thus its thickness is considered an index of generalized atherosclerosis.^{23, 24}

From a histological viewpoint, the media is formed by elastin fibers, and the fiber interval mainly includes smooth muscle cells and extracellular matrix proteins. In contrast to the control group, both of these components were significantly elevated in the ethanol group. Previously, ethanol consumption during

gestation-induced cardiovascular anomalies was shown to prevent normal cardiogenesis in FAS animal models through various molecular mechanisms, including retinoic acid signaling pathway, sonic hedgehog signaling, and Wnt/ β -catenin signaling pathway.²⁵⁻²⁷

While acknowledging the previous works, we proposed in this study that inflammatory cytokines, which are key molecular mediators in atherosclerosis, may contribute to ethanol-induced aorta abnormalities in pups. Hence, since atherosclerosis is now recognized as a chronic inflammatory condition, alcohol consumption is clearly associated with a higher risk of atherosclerosis. On both PN21 and PN90, the level of NF- κ B in the ethanol-treated groups was significantly higher than in the control groups. The transcriptional factor of the nuclear factor kappa B signaling pathway in the vascular endothelial cells is responsible for numerous gene expressions involved in inflammation, immune responses, cell growth, and adhesion molecules such as ICAM-1, vascular adhesion molecule (VCAM)-1, TNF- α , IL-6, and beta-selectin on the cell surface.^{28, 29} In addition, numerous genes expressed by the NF- κ B pathway may be related to atherosclerosis plaque formation.

TNF- α has also been shown to directly activate NF- κ B, inducing an inflammatory response.³⁰ Although TNF- α elevation is secondary to NF- κ B or vice versa, our study found a significant increase in TNF- α and ICAM-1 in the aorta tissue of litters. Numerous studies have shown that TNF- α promoted the generation of reactive oxygen species (ROS) in the endothelial cell through the activation of NADPH oxidase.³⁰⁻³²

ROS generation and the subsequent TNF-activation seem to act as a second messenger, regulating the expression of adhesive molecules such as VCAM-1 and ICAM-1.³² Moreover, TNF- α , as a putative inflammatory cytokine, may contribute to the development and progression of atherosclerosis by facilitating the transcytosis of low density lipoprotein (LDL) across endothelial cells and the retention of LDL in the vascular wall.³³ In the current study, we found that ethanol significantly increased endothelin-1 level in the aorta tissue of rats. Endothelin-1 is primarily secreted by endothelial cells and plays a major role in the maintenance of basal vascular tone and vessel constriction. In addition, a study reported that endothelin-1 had mitogenic properties on vascular smooth muscle cells and stimulated the proliferation of coronary vascular smooth muscle cells. This effect of endothelin-1 has been observed in atherosclerotic coronary plaques and may be associated with atherosclerosis.³⁴

The current research had several limitations. Although evidence of LV dysfunction, such as decreased ejection fraction, is fairly straightforward to detect in rodents by echocardiography, we didn't use echocardiography in this study. This study focused only on the phenotypic effects of maternal alcohol consumption on the aorta of the offspring, with little attention paid to the mechanisms that could explain the phenotype.

Conclusion

Taking these findings together, we concluded that daily maternal alcohol consumption during gestation and in the early postnatal period induced aorta wall remodeling in the offspring, which manifested itself as increased aorta wall stiffness and hypertension even 90 days after birth. In addition, the elevation of vascular biomarkers of inflammation and atherosclerosis revealed a more significant and stronger association between maternal ethanol consumption-induced aorta structure changes through inflammatory stress. These findings suggest that maladaptive alterations in the fetal aorta in response to ethanol exposure during gestation may persist postnatally and increase the risk of cardiovascular abnormalities in adulthood.

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Authors' Contribution

H.S and A.S: Contributed to conception, design; S.S, F.N.M, R.N, and Y.R: Contributed to data analysis; Y.R: Contributed to critically revising the work; H.S, A.S, S.S, F.N.M and R.N: Contributed to drafting the manuscript. All authors approved the final manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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