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Research Paper

Maternal high salt-diet increases offspring's blood pressure with dysfunction of NO/PKGI signaling pathway in heart tissue

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ABSTRACT

Background: High salt-diets have become significant threats to human health, resulting in hypertension and cardiovascular diseases. Hypertensive disorders during pregnancy are complicated, since the maternal cardiovascular system undergoes extensive physiological changes during pregnancy. High-salt diets during pregnancy can disturb the intrauterine environment and negatively affect fetal development. Therefore, we explored how high-salt diets during pregnancy could affect the offspring.

Methods: Rats were divided into three groups and fed with low, normal, and high salt diets. The offspring were separated into three groups after weaning based on dietary salt concentration. The blood pressure and urine protein content of both dams and offspring were measured. To evaluate cardiac function, we used Masson staining and immunodetection to confirm the fibrosis status. Finally, we extracted protein from cardiac tissue to test the expression levels of the Nitric Oxide (NO)/cGMP-dependent protein kinase I (PKGI) pathway and the angiotensin receptor.

Results: High-salt diets increased blood pressure, and offspring previously exposed to high-salt environments were predisposed to hypertension. High-salt diets were also found to induce cardiac fibrosis and exacerbate fibrosis in offspring and alter the epithelial-mesenchymal transition (EMT). Under these conditions, the NO/PKGI pathway was activated in cardiac tissue and the type-1angiotensin II receptor (AT1R) was upregulated, though the type-2 angiotensin II receptor (AT2R) had the opposite effect.

Conclusion: High-salt diets induce high blood pressure and increase predisposition to hypertension in offspring. They are accompanied by cardiac fibrosis, which could be caused by the activation of NO/PKGI and upregulation of AT1R.

1. Introduction

A high-salt diet is a well-established cause of morbidity and mortality worldwide. Noncommunicable disease rates are increasing globally, with notable increases in cardiovascular and circulatory diseases.[1] While dietary habits in China are changing, the average salt intake is high and

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hypertension rates are increasing. Epidemiological studies have revealed a link between high salt intake, hypertension, and cardiovascular diseases (CVDs).[2] Hypertensive disorder during pregnancy was strongly associated with severe maternal and perinatal morbidity and increased the incidence of CVDs in mothers and offspring.[3] Hypertension is caused by both environmental and genetic factors, and excessive sodium intake is a key determinant of hypertension.[4]

Perinatal maternal exposure can affect fetal development. Evidence has demonstrated that elevated blood pressure during pregnancy could result in adverse outcomes in offspring,[5] such as an increased risk of hypertension.[6] Intrauterine changes during critical periods of development can permanently influence the fetus, eventually leading to CVDs in adulthood.[7] There is an increasing amount of evidence supporting fetal origins of adult disease (FOAD).[8] However, the underlying molecular and cellular mechanisms remain unknown.

Maternal high-salt intake can affect multiple organs, including the heart, kidney, vasculature, and brain.[9–11] Animal models have demonstrated that high-salt diets can alter the activity of the renin-angiotensin system (RAS)[10,12] and nitric oxide (NO) signaling pathways.[13,14] Cardiac local RAS also regulates homeostasis, proliferation, adaptation, and myocardial remodeling. AT1R and AT2R, which are Angiotensin-II (AngII) receptors, also mediate cardiac fibrosis and hypertrophy. It is well-known that the RAS[15] and NO signaling pathways are associated with cardiovascular dysfunction caused by hypertensive disorder.[16] Animal experiments demonstrated that the downstream component of AT1R, Adenosine 5′-monophosphate(AMP)-activated protein kinase (AMPK), controls endothelial nitric oxide synthase (eNOS) phosphorylation and catalysis activity.[17] The AngII/AT1R pathway also activated protein phosphatase 2A (PP2A), which further decreased eNOS phosphorylation levels and NO content.[18] NO regulates many factors through the NO/cyclin guanosine monophosphate (cGMP) pathway and activates downstream effectors, including cGMP-dependent protein kinase I (PKGI).[19]

Previous studies have typically used high-salt diets containing 8% NaCl. When Ferreira et al. fed the rats with 8% NaCl, they found that their blood pressure increased significantly and that rats fed a high-salt diet developed cardiac hypertrophy.[20] In our study, we fed the high-salt diet rats with 8% NaCl to determine the relationship between a high-salt diet, blood pressure, and cardiac function.

We hypothesize that the RAS and NO/PKGI signaling pathways could be involved in regulating maternal high-salt-induced cardiac remodeling during pregnancy, and lead to hypertension disorders in their offspring.

2. Methods

2.1. Animal experiments

Three weeks of age male and female Sprague Dawley rats were purchased from the Medical Laboratory Animal Center of Guangdong province (Guangzhou, Guangdong, China). Female rats were randomly assigned to feed with purified standard chow with 0.5% NaCl (Normal salt-dieted group, N group), with 0.26% NaCl (Low salt-dieted group, L group), and with 8% NaCl (High salt-dieted group, H group). At 12 weeks of age, female rats were time-mated using an oestrous cycle monitor. Day 0 of pregnancy was determined by the presence of spermatozoa in a vaginal smear examination. At 4 weeks of age, weaned offspring of N group were continuously fed with normal salted dieted (NN group). However, weaned offspring of H group were respectively divided into three groups, which included continuously feeding with 8% Nacl (HH group), feeding with 0.5% NaCl (HL group), and with 0.26% NaCl (HN group). All animals had free access to deionized water and food. All the animals were housed in pathogen-free, temperature-controlled, and air -conditioned facilities under a 12-h light/dark cycle. All the animal care and procedures were performed according to the protocols approved by the Animal Experimental Committee of Guangzhou Medical University(approved No 2018-061).

2.2. Measurement of maternal and offspring blood pressure

From maternal 8-weeks old to postpartum 3 weeks and from offspring 8-weeks old to 12 weeks old, systolic blood pressure (SBP) and diastolic blood pressure(DBP) were weekly measured using blood pressure monitor (Softron BP-2010A, tail-cuff system, Tokyo, Japan). The measurements were repeated three times and each interval was 5 min. The average SBP was for statistics.

2.3. Assessment of 24h urine collection

Blood samples were collected from the orbital sinus of maternal rats at eighth week, twelfth week, gestational 18 days, and postpartum 3 days, and offspring rats at 8 weeks and 12 weeks. At the same days, urine of maternal and offspring rats was collected by metabolic cage. Maternal and offspring's urine protein content in 24 h was measured by Coomassie Blue G-205 staining method (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) in accordance with the instructions provided in the aforementioned assay kit.

2.4. Determination of cardiac fibrosis index

The chest of the rats was immediately opened after anesthesia. The heart was removed and fixed in 4% paraformaldehyde for 24h. Next, it was dehydrated with gradient ethanol and vitrified with xylene. Then it was embedded in paraffin and excised carefully in 4 mm for Masson staining. The slices were observed under optical microscope with DC750 digital camera system (Leica Microsystems, Wetzlar, Germany). The results were analyzed by the computer software Image-pro plus 6.0.

2.5. Immunodetection of protein expression

Soluble proteins were collected in a reducing lysis buffer and resolved using SDD-PAGE. After electro-blotting the proteins onto a charged membrane, the protein onto polyvinylidene difluoride membranes, the proteins were exposed to primary and secondary antibodies. The antigen-antibody complexes were detected with enhanced chemiluminescence and visualized with a charge-coupled device camera-gel imaging system (Bio-Rad, ChemiDoc XRS+). Anti-a-SMA, *anti*-vimentin and *anti*-GAPDH were purchased from Proteintech Inc (Chicago, USA). Anti-AT1R, Anti-AT2R, anti PKGI, *anti*-GC and *anti*-eNOS were purchased from Abcam Inc (Cambridge, UK). and data were analyzed with the computer software ImageJ. Quantitative bands densitometry was conducted in ImageJ software. Relative protein expression was determined by comparing target protein densities with the density of an internal reference protein (GAPDH).

2.6. Statistical analysis

All experiments were repeated at least three times. Representative images and summative data were shown. The results were analyzed by SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA), the quantitative data were reported as means ± standard deviation (SD), was subjected to one-way analyses of variance (ANOVAs). When indicated, a Tukey's range test was performed post hoc. For abnormal distribution the data, the method of Kruskal-Wallis was used. In all case, P values < 0.05 were considered significant.

3. Results

3.1. High-salt exposure elevated blood pressure and proteinuria in both maternal rats and offspring

As salt exposure increased over time, the SBP levels in maternal rats and offspring started to increase. The SBP of maternal H groups from 8-weeks old to 3 weeks postpartum was higher than that of N groups and L

groups (Fig. 1A). The SBP of the dams increased from 8 weeks until 10 weeks, when it peaked and slowly decreased. During pregnancy, blood pressure mildly decreased and was lowest at the time of delivery, after 19 days of gestation. After delivery, we monitored the SBP from 1 week postpartum to 3 weeks postpartum, when it started to increase again. Of the offspring, the SBP of HH group was the highest in all groups and that of the NN group was the lowest (Fig. 1C). We also found that, as the exposure time from the high-salt diet increased, their SBP also increased. The SBP of the offspring subjected to high-salt exposure in utero was close to that of the NN group at 8 weeks, while other groups showed no significant differences. However, when they were 12-weeks-old, the SBP of the HH group was much higher than that of the NN group. This differed from the dams experiencing pregnancy, since the offspring didn't exhibit a decreasing trend or a peak in the SBP curve.

The maternal protein levels in urine samples obtained from the H group were typically higher than those obtained from N group and L group (Fig. 1B). The data indicate that proteinuria was detected in the H group in the eighth and the twelfth week, and was worse after 18 days of gestational. Even though the urine protein content decreased after delivery when it was measured 3 days postpartum, the value in the H group was still higher than in the N group. Despite significant differences in the urine protein content of 8-week-old dams, we detected no significant changes in 8-week-old offspring (Fig. 1D). Proteinuria occurred in the HH group at 12 weeks as the high-salt treatments progressed. However, other intrauterine exposed groups, including the HL and HN groups, showed no significant changes in urine protein compared to the NN group.

High-salt exposure could promote endothelial mesenchymal transition by upregulating levels of αSMA and vimentin expression, increasing the cardiac fibrosis index.

We assessed changes in the cardiac fibrosis index to investigate the relationship between high-salt exposure and myocardial fibrosis. Data obtained from Masson staining indicated that rodents experienced more severe myocardial fibrosis when they were exposed to a high-salt environment. The maternal myocardial fibrosis index in the H group increased compared to the N group (Fig. 2A). Similarly, this index was higher among offspring in the HH group than in the NN group, both at 8 weeks and 12 weeks (Fig. 2B), and it was higher at 12 weeks than 8 weeks. We then performed Western blot analysis to test the expression levels of α-SMA and vimentin in cardiac tissue (Fig. 3A-C), which are specific biological markers of Endothelial mesenchymal transition (EndMT). Similar to the results of Masson staining, α-SMA and vimentin were both upregulated in the cardiac tissue of maternal rats fed with a high-salt diet compared to the normal-salt diet group. This indicates that the cardiac fibrosis index was higher as salt intake increased. The expression levels of the HH_8w group and HH_12w group exceeded that of the NN_8w group and NN_12w group in the offspring.

3.2. High salt levels stimulated the NO/PKGI pathway and activated RAAS in heart tissue

Next, we studied the role of the NO/cGMP/PKGI pathway in the myocardium via Western blot analysis. The expression levels of eNOS and PKGI were upregulated in cardiac tissue collected from maternal rats in the H group and in its offspring in the HH_8w group and the HH_12w group. Of these, the expression levels of guanylate cyclase (GC) showed no statistically significant difference (Fig. 4A-C). We also detected AT1R and AT2R expression levels in the myocardium of maternal rats and their offspring to identify whether the RAS system participated in functional cardiac variation, while higher levels of AT1R and lower levels of AT2R were detected in rats that were administered high-salt diets. AT1R was upregulated in the H group, HH_8w group, and HH_12w group, while AT2R was downregulated in the N group, NN_8w group, and HH_12w group (Fig. 4D-F).

4. Discussion

In this study, we found that maternal high-salt intake elevated blood pressure and increased urine protein content in both maternal rats and

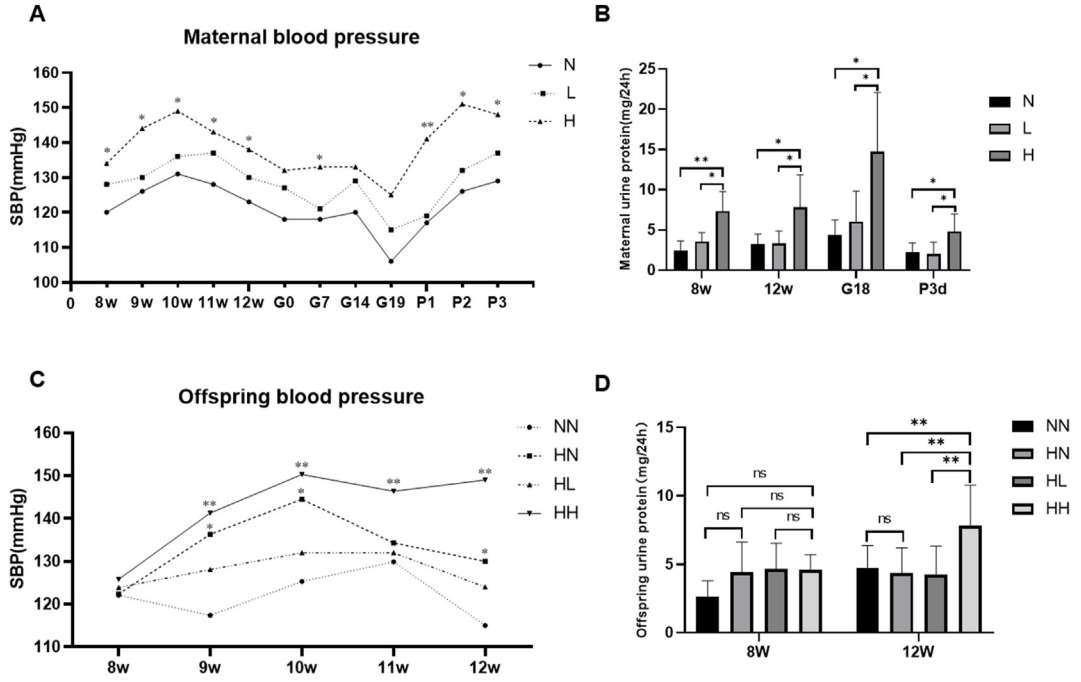

Fig. 1. High salt-diet increased systolic blood pressure and the level of urine protein in maternal rats and exerted a profound influence in offspring. A: The systolic blood pressure in dams of high salt-diet (H), low salt-diet (L) and normal salt-diet (N) group on 8 weeks to postpartum 3 weeks. B: The level of 24h urine protein in dams of H, L and N groups on 8 weeks to postpartum 3 weeks. Data represented as mean ± SEM, n = 8. *P ≤ 0.05, **P ≤ 0.01, vs N group. C: The systolic blood pressure in offspring of NN, HH, HL, and HN group on 8 weeks–12 weeks. D: The level of 24h urine protein in dams of NN, HH, HL, and HN group on 8 weeks–12 weeks. Data represented as mean ± SEM, n = 8. *P ≤ 0.05 , **P ≤ 0.01, vs NN group.

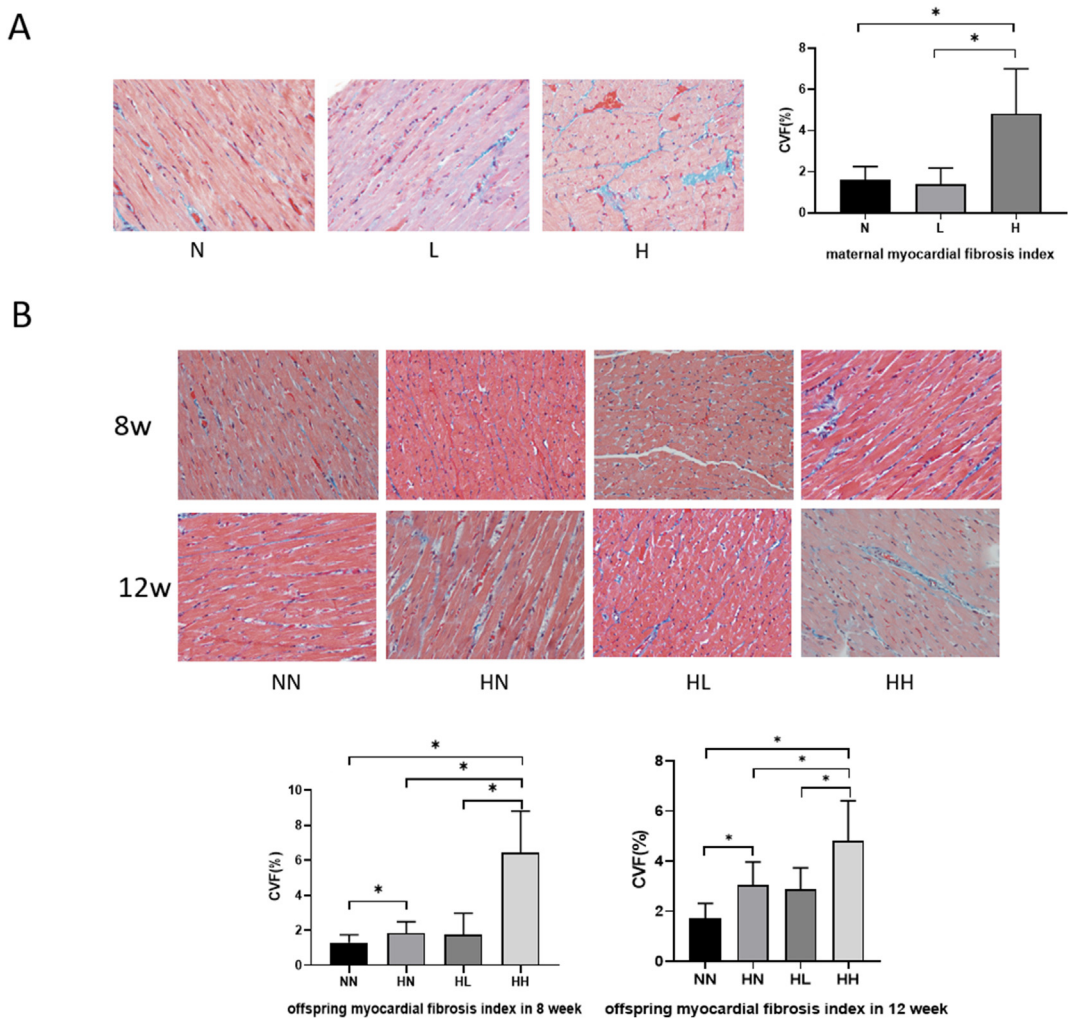

Fig. 2. The fibrosis changes in heart tissue and myocardial fibrosis index measured by Masson staining in N, L and H groups (dams) (n = 4,3,3) and NN, HN, HL and HH groups (offspring) at eighth (n = 3,4,4,3) and twelfth week (n = 4,4,4,4). Western blotting was performed on the heart tissue. Data were quantified using the ImageJ software, and expressed as the fold change in maternal group and offspring groups at eighth and twelfth week. Data represented as mean ± SEM. *p ≤ 0.05.

their offspring. We fed the rats with an 8% NaCl high-salt diet and monitored changes in SBP and proteinuria levels. Before pregnancy, the SBP baseline of dams in the H group was higher than in the N group, as was the 24h urine protein as early as the eighth week. During pregnancy, the vasculature undergoes physiological changes and ensures a blood supply to placenta and fetus, so maternal SBP levels remained relatively low compared to non-pregnancy. SBP again increased after delivery. However, when treated with 8% NaCl, the offspring from the maternal H group were not significantly different from each other and did not differ from the NN group in terms of SBP or urine protein at the eighth week. As the exposure time grew longer, SBP and urine protein significantly increased in the HH group at the twelfth week.

The heart is affected by hypertension, and cardiac dysfunction can trigger hypertensive disorders. High salt intake could activate vascular remodeling in a fetal programming manner,[21,22] increasing the risk of hypertension and cardiovascular disease in adulthood. While the myocardial structure also changes in response to a chronic high-salt intake,[23] the reason is unclear. Therefore, this study aims to determine the long-term effects of a high-salt diet on heart tissue. This shows that a high-salt diet aggravates myocardial fibrosis and perinatal high salt exposure also contributes to fibrosis during the offspring's cardiac development.

During myocardial fibrosis, fibroblasts proliferate and transform into myofibroblast phenotypes, synthesizing excessive cardiac extracellular matrix, while degradation of the cardiac extracellular matrix decreases. Fibroblasts can then express specific smooth muscle markers, such as (α-SMA).[24] The positive expression of α-SMA and other endothelial markers after myocardial fibrosis confirms the existence of endothelial mesenchymal transition (EMT).[25] Endothelial mesenchymal transition (EndMT) is a form of EMT, and is characterized by the upregulation of α-SMA and vimentin, which are class-III intermediate filaments found in mesenchymal cells, through the decomposition, phenotypic change, and migration of endothelial cells.[26] EndMT plays an important role in cardiac development, which generally occurs during the embryonic stage of cardiac remodeling. Previous studies have demonstrated that EndMT does exist in myocardial fibrosis.[27] Other animal studies have provided evidence that high salt intake induced EndMT, which is considered a vital process in fibrogenesis.[28–30] We found that the expression of α-SMA and vimentin were both upregulated in the cardiac tissue of female rats and their offspring after long-term high-salt exposure, indicating that EndMT was involved in myocardial fibrosis induced by long-term high-salt exposure.

Previous research has reported that high salt intake stimulated cardiomyocyte hypertrophy and interstitial fibrosis via local AT1R activation,[31] while the angiotensin receptor blocker attenuated the hypertrophy and fibrosis in cardiac cells.[32] In addition, high salt intake can exacerbate tubulointerstitial fibrosis of the kidney associated with

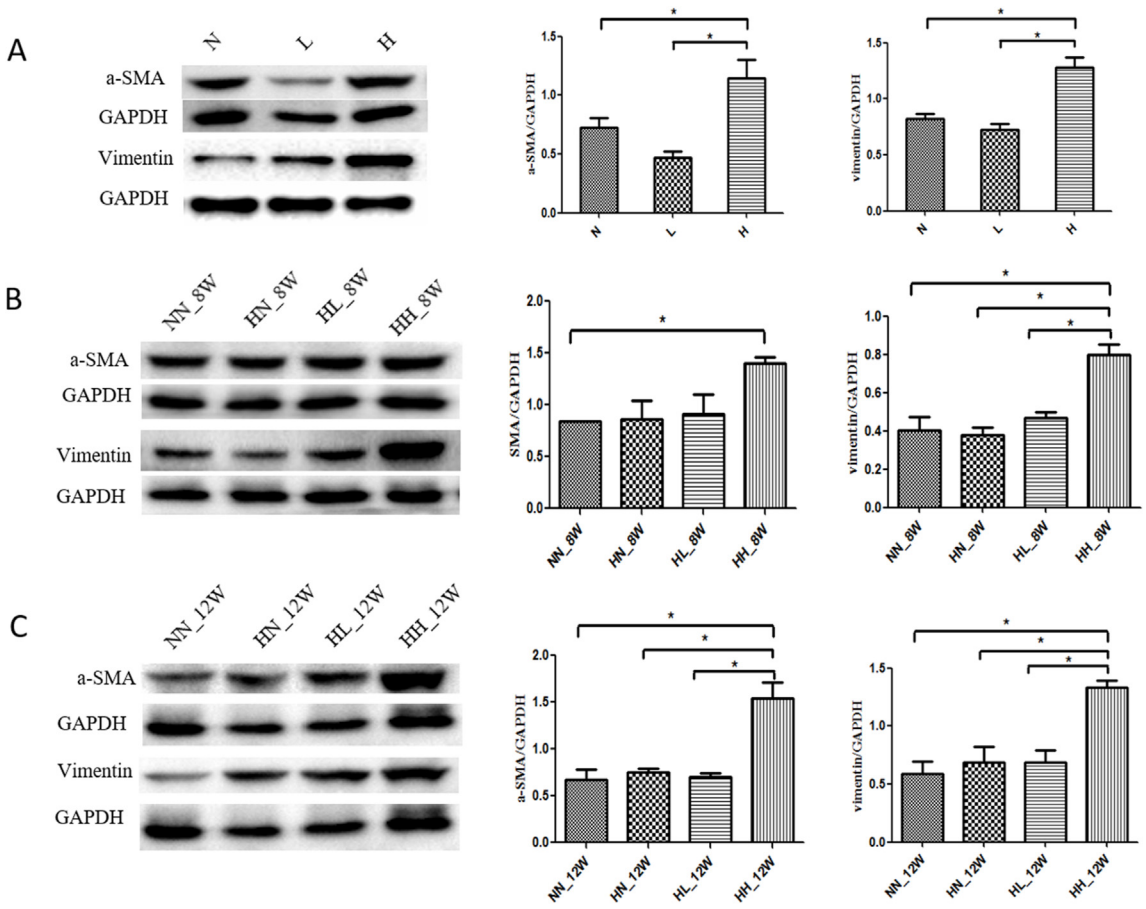

Fig. 3. Western blotting was performed on the heart tissue. Proteins levels were quantified using the ImageJ software, and data are expressed as the fold change in N, L and H groups (dams) (n = 3) and NN, HN, HL and HH groups (offspring) at eighth (n = 3) and twelfth week (n = 3). Data represented as mean ± SEM. *P ≤ 0.05.

AT1R overexpression,[30] and excess perinatal salt disturbed renal RAS function in adult rats.[33] Moreover, maternal high-salt intake affects the vascular RAS function in offspring, promoting endothelial dysfunction in rats.[34] However, little is known about the cardiac effect in offspring following high in utero salt exposure.

Our findings indicate that the local angiotensin II receptor could play a crucial role in cardiac fibrosis due to long-term high-salt exposure in female rats and their offspring. AT1R overexpression and AT2R downregulation were observed in cardiomyocytes as myocardial fibrosis was aggravated. This indicates that high salt exposure in maternal rats during pregnancy can affect the cardiac tissue of both female rats and their offspring, where RAS may participate in programming cardiac remodeling.

We also demonstrated that the expression levels of eNOS and PKGI in the cardiac tissue of rats in the high-salt diet group were upregulated, but there was no significant difference in GC expression in any group. Previous research has found that the NO/GC/PKGI pathway is involved in hypertension induced by high-salt diets. Decreases in PKGI were detected in rodent aortas in the high-salt diet group.[35] The downregulation of the soluble-GC (sGC)-related pathway of renal vasculature was observed in rat offspring after exposure to high salt-diet.[36] However, because our study focused on cardiac dysfunction and heart tissue has low levels of GC expression, it was difficult to determine significant differences in GC. High in utero perinatal salt exposure can alter the NO/PKGI pathway in the cardiac tissue of the offspring. Additionally, activating the NO/PKGI pathway was associated with inositol 1,4,5-trisphosphate (IP3),[37] and AT1R stimulation was also involved with the activation of IP3 signaling.[38] Further experiments should investigate the role of IP3 signaling in high salt-induced hypertension.

Our study indicated that this effect could negatively affect offspring through fetal programming. Similarly, a previous study found that the offspring of mice exposed to adverse environments during pregnancy developed hypertension related to aberrant DNA methylation of the gene encoding AT1R.[39] Therefore, we must elucidate how this mechanism functions and how this effect passes to the next generation.

Our study demonstrated that high-salt diets during pregnancy can induce hypertension and is related to elevated blood pressure in their offspring. Offspring from pregnancies complicated by hypertension were more likely to develop high blood pressure in adulthood. Furthermore, our data indicated that a maternal high-salt diet can cause cardiac fibrosis, which may exist in utero and involve the AT1R mediated NO/PKG1 signaling pathway. This indicates that it can disturb the EMT process and lead to cardiac fibrosis.

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “Maternal high salt-diet increases offspring's blood pressure with dysfunction of NO/PKGI signaling pathway in heart tissue”

Conflict of interest statement

The authors declare no conflict of interest.

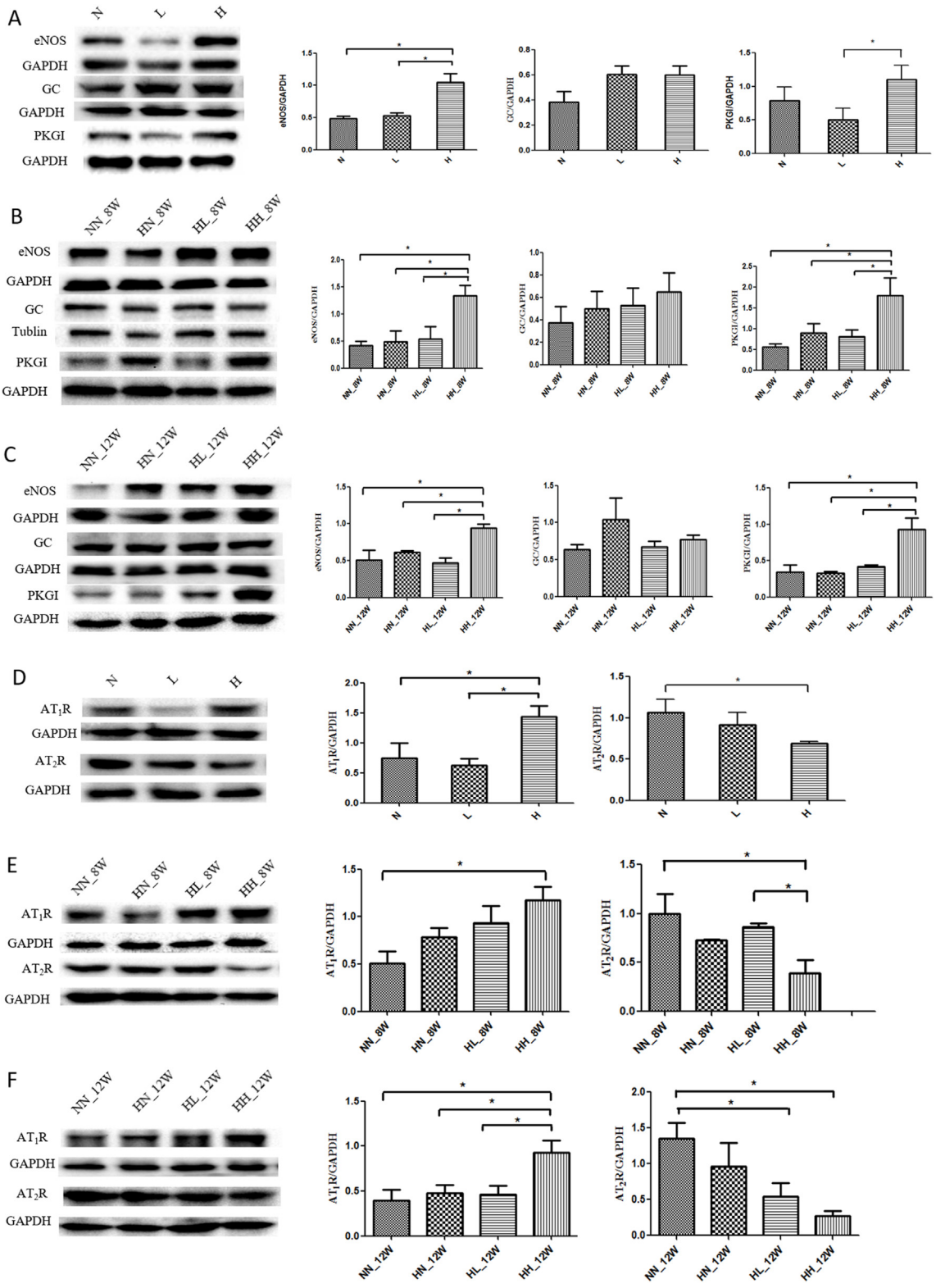

Fig. 4. Western blotting was performed on the heart tissue. Proteins levels were quantified using the ImageJ software, and data are expressed as the fold change in N, L and H groups (dams) (n = 3) and NN, HN, HL and HH groups (offspring) at eighth (n = 3) and twelfth week (n = 3). Data represented as mean ± SEM. *P ≤ 0.05.

Contributions

CD and CJ designed the experiments. HM, LX and RL performed the experiments. LX,HL and DL collected data. HM, LX,RL,PJ and YJ performed the statistical analysis. HM and CJ wrote and edited the manuscript. CJ and CD supervised the work.

Consent for publication

The Authors agree to publication in the Gynecology and Obstetrics Clinical Medicine.

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