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High salt-induced excess reactive oxygen species production resulted in heart tube malformation during gastrulation

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Abstract

An association has been proved between high salt consumption and cardiovascular mortality. In vertebrates, the heart is the first functional organ to be formed. However, it is not clear whether high-salt exposure has an adverse impact on cardiogenesis. Here we report high-salt exposure inhibited basement membrane breakdown by affecting RhoA, thus disturbing the expression of Slug/E-cadherin/N-cadherin/Laminin and interfering with mesoderm formation during the epithelial-mesenchymal transition（EMT）. Furthermore, the DiI^{+} cell migration trajectory *in vivo* and scratch wound assays *in vitro* indicated that high-salt exposure restricted cell migration of cardiac progenitors, which was caused by the weaker cytoskeleton structure and unaltered corresponding adhesion junctions at HH7. Besides, down-regulation of GATA4/5/6, Nkx2.5, TBX5 and Mef2c and up-regulation of Wnt3a/β-catenin caused aberrant cardiomyocyte differentiation at HH7 and HH10. High-salt exposure also inhibited cell proliferation and promoted apoptosis. Most importantly, our study revealed that excessive reactive oxygen species（ROS）generated by high salt disturbed the expression of cardiac-related genes, detrimentally affecting the above process including EMT, cell migration, differentiation, cell proliferation and apoptosis, which is the major cause of malformation of heart tubes.

Keywords: High salt; chick embryo; heart tube; reactive oxygen species; cardiac progenitor migration and differentiation

Introduction

The human body requires a small amount of salt to work properly, while too much of it can be detrimental to its health. Excess salt (sodium chloride) consumption has been associated with many adverse health effects, including hypertension and cardiovascular mortality in epidemiological and clinical studies (World Health Organization) [1, 2]. Sodium and chloride ions can transfer across the placental barrier mainly by passive diffusion [3, 4]. Having high-salt diet during pregnancy may affect the development of the heart in the fetus [5].

The heart is the first organ that becomes functional in the vertebrate embryo. Its development mainly consists of cardiac progenitors of the splanchnic mesoderm (primary and secondary heart field), cardiac neural crest, and the pro-epicardium (PE). Organisms need sodium chloride to maintain a normal and stable osmolarity of the tissue extracellular environment and balanced homeostasis. Osmolarity determined by solute concentration is defined as the number of osmoles (Osm) of solute per litre (L) of solution, such as sodium chloride, which is dissociated into Na^+ and Cl^-. While our body requires a certain amount of salt to work properly, too much of it is bad for health. A report summarizing 13 studies from eight countries concluded that a high salt diet is closely related to cardiovascular events [6]. According to the Centers for Disease Control and Prevention (CDC), most Americans consume too much sodium in their daily diet, with a current average of 3.4g/d. However, the World Health Organization (WHO) recommends a sodium intake of <2g/d (equivalent to 5 g/d of salt) for adults, with lower amounts for children [7]. Changes in offspring blood pressure and cardiovascular structures have also been reported [8]. In a previous study, we demonstrated that exposure to high salt dramatically increased chick mortality, which

mainly resulted from defects in cardiovascular development. Salt can enhance the risk of abnormal heart tube looping and blood congestion in the heart chamber in 4.5-Day chick embryos, which is linked with oxidative stress and ROS production [9]. Uetake et al. indicated that the high salt might induce ROS production and exacerbate ROS-associated cellular macromolecules damage and tissue damage [10]. However, it is unclear to what extents a maternal high-salt intake may affect cardiac function in offspring during gastrula stage. The myocardium of the heart is composed of multiple highly specialized physiological processes. Each of them is a highly ordered processes involving multiple signaling pathways and their intersection with transcriptional regulatory networks [11]. Mesp1 and Mesp2, as the basic helix–loop–helix (bHLH) transcription factors, are required in migration of the precardiac mesoderm cells at the primitive streak [12]. Nkx2.5, GATA factors, myocardin, and Tbx20 are cardiogenic transcription factors, which play a pivotal role in cardiomyocyte differentiation [11]. The inhibition of canonical Wnt/β-catenin signaling can either enhance or inhibit cardiogenic differentiation [13]. In this context, any disruption of these genes expression may result in congenital heart malformations.

Chick embryo, as a classic development model, has the major advantage of the accessibility for surgical observation and functional interference approaches [14]. Thus, investigations in chick embryos on whether high-salt exposure could affect offspring's cardiogenesis during gastrula stage can provide valuable data and facilitate a detailed understanding of the underlying cellular and molecular mechanisms.

Materials and methods

Chick manipulations

Fertilized chick eggs were obtained from the Avian Farm of the South China Agriculture University. EC (early chick) culture [15] was employed to culture these gastrula chick embryos. For high-salt exposure at the early embryonic stage, Hamburger-Hamilton (HH) [16] stage 0, 4, or 7 chick embryos from fertilized eggs were incubated with 240 mOsm/L (control), 260 mOsm/L, 280 mOsm/L, or 300 mOsm/L (high-salt) in early chick (EC) culture medium in a humidified incubator (Yiheng Instruments, Shanghai, China) at 38°C and 70% humidity until the required developmental stage. For the Vitamin C (VC) -treated embryos, the HH0, HH4, or HH7 chick embryos in EC culture were incubated with 2.86mM VC (Beijing Ding Guo Chang Sheng, China) according to experimental requirements. Chick embryo treatment of VC is 0.5mg/egg [9, 17]. In EC-culture, it is 1ml/embryo, so the final concentration is 2.86mM (=0.5 mg/ml) in culture medium. Briefly, high salt or VC was directly applied to either EC culture or in vitro culture medium to reach the final concentrations 300 mOsm/L high salt and 2.86mM respectively. Alternatively, 300 mOsm/L high salt was directly applied to one side of the gastrula-stage embryos, with the other side being exposed to 240 mOsm/L salt as a control.

Cell trace with DiI

Cell trace with DiI (Carbocyanine dye 1,1V-dioctadecyl-3,3,3V,3V-tetramethyl indocarbocyanine perchlorate, Molecular Probes, Inc.) was performed according to a standard protocol as described previously [18].

Explant, primary and cell culture

Explant culture: The anterior primitive streak (cardiac progenitor cells) was cultured *in vitro* for 4.5-Day as previously described [19]. Each treatment was performed in triplicate. In the control group, approximately, 95% of the cells in the explant culture were determined to be cardiomyocytes which showed the presence of myosin heavy chain using MF20 immunofluorescent staining as previously reported [20], and cell proliferation using pHIS3 immunofluorescent staining.

Primary culture: Primary cardiomyocyte cultures were established from day 14 chick embryo hearts.

Cell culture: H9c2 cells were purchased from Guangzhou Jennio Biotech Co., Ltd, China and cultured in culture medium (DMEM-F12 GIBCO).

Cell counting kit-8 assay

Cell viability was assessed through a modified cell counting kit-8 (CCK8; Dojindo Molecular Technologies, Japan) assay of H9c2 cells and performed as previously reported [21]. Cell viability was indirectly established by the ratio of the absorbance value of 300mOsm/L high salt, high salt+114μM (=20μg/ml) VC or high salt+228μM (=40μg/ml) VC-treated cells relative to the control (240mOsm/L) [22, 23]. The final results were determined from analysing five independent experiments.

Scratch wound migration assay

A "scratch wound" was created by scraping a monolayer culture of H9c2 cells using a sterile 10 μL pipet tip and the length of the wound gap was measured using Image pro-Plus

software as described previously [24, 25]. The assays were performed three times using triplicate culture wells.

Immunofluorescent staining and Hochest/PI staining

Chick embryos were harvested and fixed in 4% paraformaldehyde solution overnight at 4°C. The fixed embryos were implemented according to a standard protocol [25]. Whole-mount embryos were performed using the following primary antibodies: MF20 (1:500, DSHB, USA), E-cadherin (1:50, BD Transduction Laboratories, USA), N-cadherin (1:50, DSHB, USA), Laminin (1:100, DSHB, USA); pHIS3 (p-histone H3, 1:400, Santa Cruz Biotechnology, USA) and C-caspase3 (Cleaved Capase-3, 1:200, Cell Signaling, USA). Then embryos were incubated with labeled secondary antibodies at room temperature for 2 hours. Nuclei were stained after incubation with DAPI (1:1000, Invitrogen, USA) for 1 hour. For Hochest/PI staining, the cells were cultured and washed twice with cold PBS, and then incubated with Hochest/PI for 45 min at 37°C in the dark. The photography and cryostat microtome were performed as previous reported [18, 25].

In situ hybridization

Whole-mount in situ hybridization of chick embryos was performed according to a standard in situ hybridization protocol [26]. Briefly, digoxigenin-labeled probes were synthesized for VMHC[17], GATA5[27], BMP2 and Nkx2.5 (supplied by Dr. Thomas M. Schultheiss).

Western blot

HH4, HH7 and HH10 chick embryos were collected and performed as pervious reported [21]. Antibodies: E-cadherin (BD Transduction Laboratories, USA), N-cadherin (DSHB, USA), Slug (DSHB, USA), Mef2c (Abcam, USA); GATA4 (Abcam, USA); TBX5 (Abcam, USA); GATA6 (Abcam, USA); β-catenin (Abcam, USA); PCNA (Santa Cruz Biotechnology, USA); C-caspase3 (Cell Signaling, USA); HRP-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG (Cell Signaling Technology, USA).

RNA isolation and quantitative PCR

Total RNA was isolated from HH4, HH7and HH10 chick embryos using a Trizol kit (Invitrogen, USA) according to the manufacturer's instructions and performed as described previously [28, 29].

Measurement of ROS

To assess the extent of oxidative stress caused by high salt, samples from HH7 and HH10 chick embryos or primary cardiomyocytes were homogenized and analyzed for SOD (superoxide dismutase), GSH-Px (glutathione peroxidase) and MDA (malondialdehyde) [30]. The protein content of the chick embryos or primary cardiomyocytes was performed as pervious reported [31].

Quantitation of apoptotic cells

Annexin V-FITC (BD Bioscience, USA) and Propidium Iodide (PI) double staining were used to identify and quantify apoptotic chick myocardial cells [31].

Data analysis

Statistical analysis for all the experimental data generated was performed using a SPSS 13.0 statistical package program for Windows as pervious reported [21, 23, 31].

Results

High salt disrupted the heart tube morphology in gastrula chick embryos.

There are three crucial phases from fertilized egg to gastrulation: primitive streak (HH4), primitive heart field (HH7) and primitive heart tube (HH10) (Fig. S1A). To investigate the effects of high-salt exposure on heart tube formation in chick embryos, we cultured the embryos with 240 mOsm/L (control), 260 mOsm/L, 280 mOsm/L, and 300 mOsm/L as shown in Fig. 1A to HH10. Compared with the control groups, the rate of growth of embryos diminished and their mortality increased with increasing concentrations of salt (Figs. S1B-E).

Some atypical C-looping of the heart tubes were evident in the embryos treated with high salt (Fig. S1F). According to phenotype, they were divided into four classifications: normal (Figs. 1A-A2), mild (Figs. 1B-B2), intermediate (Figs. 1C-C2) and severe (Figs. 1D-D2), and all were stained with MF20 antibody. Furthermore, there were some delay in the formation of heart tubes in 280 and 300 mOsm/L salt groups (Figs. 1E-E2), which had not formed any C-loops when they died. In the 240 mOsm/L salt-treated embryonic heart, the heart tubes are fully C-looped. In contrast, some heart tubes treated with high-salt presented in varying degrees of deformities（Fig. 1F）, of which the 300 mOsm/L high-salt exposure had the most serious impact on formation of the heart tube. Time-lapse

microscopic recording of the process of heart tube formation from HH7 to HH10 treated with 240 and 300 mOsm/L salt (Supplemental movie).

High-salt exposure caused heart tube malformation by interfering with EMT during chick gastrulation.

To detect the effects of high salt exposure on the first phrase, primitive streak in HH4 stage, chick embryos were cultured as shown in Fig. S2A. Results showed that 300 mOsm/L high-salt exposure retarded the chicken embryos development and caused heart tube malformation (Figs. S2B-F).

Cardiogenic cells undergo EMT, which are located in the bilateral heart fields in the anterior lateral plate mesoderm (Fig. 2A). Here, E-Cadherin in control group was mainly expressed on ectoderm and mesoderm (Figs. 2B-B1’’). In contrast, E-Cadherin in high-salt induced embryos was just expressed on the apical side of ectoderm (Figs. 2C-C1’’) and the expression of N-Cadherin was stronger in the endoderm in high-salt induced embryos (Figs. 2D-D1’’) than that in control (Figs. 2E-E1’’). During chick gastrulation, the earliest sign of EMT is the breakdown of BM at the midline [32]. Compared to the midline distance of the embryos in control group (Figs. 2F-F1’’), the high-salt one was shortened ($P < 0.001$; Figs. 2G-G1’’, H). The quantitative PCR data (Fig. 2I) showed the expression of RhoA is up-regulated. Besides, high-salt treatment up regulated Mesp1, Mesp2, Wnt3a, BMP2, and FGF8. Western-blotting indicated that high-salt treatment suppressed the expression of E-Cadherin, while enhancing N-Cadherin, slug and β-catenin (Figs. 2J-J’), which implied that cardiac precursor cells specification was disturbed.

Furthermore, EMT underlies the alterations of adheren junctions (AJs), tight junctions (TJs), gap junctions (GJs) and Desmosomes [33] (Fig. 2K). The quantitative PCR data showed that 300 mOsm/L high-salt treatment reduced the expression of E-cadherin, versican, Occludin, and α-catenin; increased the expression of N-cadherin, Vinculin, Par3, β-catenin, Plakoglobin, Desmoplakin, ZO-2, Claudin-1, Claudin-5, Claudin-12, α-actin and Cx43, but had no effect on expression of ZO-1 (Figs. 2L-M). RhoA, CDC42 and Rac1, all small GTPase of the Rho family, are the basis of cell-cell adhesion and migration [34]. Additionally, the cell cycle is a vital process by which a single-celled fertilized egg develops into a mature organism. In high-salt group, the expression of Rac1 was down-regulated; CDC42, P21 and CyclinD1 were up-regulated analyzed by quantitative PCR (Fig. 2N).

High-salt treatment repressed cardiomyocyte migration during gastrula chick embryo development.

To verify the effects of high salt exposure on the formation of primitive heart field, the chick embryos were exposed to high salt from HH4 to HH7 (Fig. S3A). The results show high-salt suppressed the growth of the embryos and led to the abnormal heart tube formation at HH10 (Figs. S3B-G).

Cardiac progenitor cells in the lateral plate mesoderm migrate symmetrically and form the cardiac crescent [35]. Embryos were developed to about HH4, then cultured with either 240 mOsm/L (control) on two sides (upper images in Fig. 3A) or with 300 mOsm/L high-salt on one side (lower images in Fig. 3A). Then, DiI dye was injected into the anterior primitive streaks of these embryos at HH4 to record the migration trajectory of cardiac progenitor cells. Images were taken after 8- and 18-hour incubations. The results show that

the Dil^+ mesoderm cells in the control group migrated symmetrically at bilateral sides of embryos ($P > 0.05$; Fig. 3B), while significantly fewer Dil^+ mesoderm cells were observed after 8- and 18-hour incubations in the sides with high-salt treatment compared to the controls ($P < 0.001$; Figs. 3C-D). To investigate the nature of cell migration further, we employed chicken cardiac muscle cells cultured *in vitro* in the presence of high-salt [36]. The images show that the cardiac muscle cells exposed to high-salt partly lost their migratory capability, and were accompanied by a loss of cell polarization (Fig. 3E). The scratch-wound assay showed that high-salt exposure inhibited chicken cardiac muscle cell migration, as reflected in the extent of "wound" closure. The dotted line shows the wounded area ($P < 0.001$; Figs. 3F-G). Furthermore, anterior primitive streak explants were cultured and immunofluorescently stained with MF20 antibodies 4.5-Day after culture ($p < 0.001$; Figs. S4B-C). The result of migration length at 4.5-Day determined that high-salt treatment significantly inhibited cardiomyocyte migration.

To further investigate the effect of high salt on cytoskeletal reorganization, the ability of cardiac muscle cells to form stress fibers was tested, since these provide the contractile force required for cell-like motility [37]. The shape and the stress fibers of cardiac muscle cells stained for F-actin are shown in Fig.3H. The majority of control cells formed prominent stress fibers. However, in cells cultured with high-salt, actin filaments remained diffusely distributed in the cytoplasm ($P < 0.001$; Figs. 3I-J). Elongation of cells treated with high salt was significantly less than that of control cells ($P < 0.001$; Fig. 3K).

Cells migration properties are related to cellular cytoskeleton modulation or to relevant adherence factors, including AJs, TJs and GJs etc. [38, 39]. Quantitative PCR data (Fig.3L) revealed that high-salt treatment reduced the expressions of α-catenin, β-catenin, α-actin,

E-Cadherin, N-Cadherin and CX43, but increased the expression of Vinculin. In addition, Rho GTPases and SRF-Myocardin are also important in actin cytoskeleton and contractile processes. The quantitative PCR data (Fig.3M) revealed that high-salt treatment reduced the expressions of RhoA, Rac1, CDC42, SRF and Myocardin. The difference in Dil^+ cardiac progenitor cell migration *in vivo* and the inhibition of the migration, polarization, and protrusion formation of cardiac cells *in vitro* clearly suggests that high-salt exposure restrained the cell migration, polarization and protrusion formation of cardiac precursors towards the site of heart tube formation.

High salt influenced the differentiation of cardiac precursors during gastrula chick embryo development.

Figure 4A reveals the principal signaling pathways and key transcription factors related to the regulation of cardiomyocyte differentiation. To explore whether high-salt exposure affects these crucial gene expressions of cardiomyocyte formation, we firstly exposed high salt to one side of the embryos, using the other side as control. The GATA5 and Nkx2.5 whole-mount *in situ* hybridization showed that both of GATA5 and Nkx2.5 expressions in the high-salt treated sides of embryos were dramatically reduced in comparison to control side (Figs. 4B1-B2, B1'-B2'), which could be clearly seen in their transverse sections (Figs. 4B1''-B2''). The expression of VMHC, ventricular myosin heavy chain is also suppressed with high-salt exposure as well (Figs. 4B3-B3''). Using MF20 immunofluorescent staining, we could see that there are less $MF20^+$ cells in the monolayer cells crawled out from the *in vitro* cultured explant of anterior primitive streak in high-salt treated group (Fig. S4B). Moreover, quantitative PCR data showed more gene expression alterations induced by

high-salt exposure, which is down-regulated GATA4/5/6, Nkx2.5, VMHC. ISL1, TAL1, etc., up-regulated Wnt3a and β-catenin in HH7 chick embryos exposed to high-salt (Figs. 4C-D). The stage of HH7 to HH10 is the major phase of cardiac precursors' differentiation. To further investigate the effects of high-salt exposure on differentiation, the embryos were cultured as shown in Fig. S5A. The results illustrated that high-salt exposure disturbed the differentiation of cardiac precursors and resulted in abnormal heart formation (Figs. S5B-F). To address the possible mechanism about high-salt exposure-induced phenotypes, we further detected the expression of genes related to heart tube formation using quantitative PCR. We displayed that the expression of cardiomyocyte differentiation-related genes (GATA4/5/6, BMP2/4, Fgf8/10, Nkx2.5, and Tbx20) was significantly down-regulated in high-salt treated HH10 chick embryos compared to control (Figs. 4E-F). All data imply that high-salt exposure certainly caused the alteration of crucial gene expressions for heart tube formation during early embryo development, which in turn effected the differentiation of cardiac progenitor cells.

High salt exposure inhibited cell proliferation and promoted apoptosis.

Cardiac cell proliferation and apoptosis during embryonic and fetal development are associated with congenital heart malformations and myocardial defects [40, 41]. To investigate the involvement of cell proliferation and apoptosis in the malformation of heart tube exposed to high salt, we performed double immunofluorescent staining for MF20 and pHIS3 or MF20 and c-Caspase3 co-expression in embryos. The results indicated that the number of $pHIS3^{+}$ cells on heart tube in high salt-treated embryos was significantly lower than in control embryos ($p < 0.001$; Figs. 5A-A2''; B-B2'', C). Likewise, number of

c-Caspase3^{+} cells was significantly smaller in high-salt treated embryos ($p < 0.001$; Figs. 5D-D2''; E-E2'', F).

High-salt exposure inhibited cell proliferation and promoted apoptosis through excess ROS.

In our previous, we have found that excess ROS affected cell proliferation, as well as apoptosis and resulted in embryonic cardiovascular dysplasia [9]. To further explore whether the influence of excess ROS on proliferation and apoptosis, we employed the H9c2 cells cultured *in vitro* in presence of high salt. First, we used H9c2 cells to establish whether VC, an antioxidant compound, could be used to rescue the cells from the effects of high-salt exposure. In the presence of high salt, H9c2 cells viability rate was significantly reduced at 36-hour but could be rescued by different doses of VC (Fig. 6A). Next, we exposed the chicken cardiac muscle cells with high salt and found that the ROS generation was significantly increased after 36-hour. To study this correlation, VC was added together with high salt and the ROS generation was reduced to some extent (Fig. 6B). In our previous study, we found that 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH, a free radicals generator) induced excess ROS [17, 42] and AAPH-treated embryos developed cardia bifida with two cavities [20]. Then we performed immunofluorescent staining for pHIS3 and PI/Hochest with cells exposed to 300 mOsm/L high salt. The result revealed compared with control group, the pHIS3^{+} cells cultured with high salt and AAPH were fewer (Figs. 6C-D). And the PI^{+} cells, labeled with dead cells were much more (Figs. 6E-F). In addition, by using flow cytometry, we found that apoptosis of chick cardiac muscle cells dramatically enhanced with high-salt exposure, while combinational application of VC and high salt

distinctly suppressed the increase of high-salt induced cell apoptosis in the cultured chick cardiac muscle cells (Figs. 6G, H-H''). The results of western blotting analysis of HH10 chick embryos revealed that PCNA expression was down-regulated by high-salt treatment and could be rescued by the addition of VC. And c-Caspase3 expression was also correspondingly altered in the presence of high salt and addition of VC (Figs. 6I-I'). Anterior primitive streak explants culturing stained with pHIS3 antibody suggested that high salt suppressed cell proliferation as well ($P < 0.001$; Figs. S4B, D).

High salt through excess ROS production disturbed the specification, migration, differentiation and fusion of the heart tube.

Excess ROS generation in tissues and cells is always cytotoxic [43]. To further explore the influence of excessive ROS production on embryonic cardiovascular dysplasia, we employed HH7 and HH10 chick embryos, treated with 240, 300 mOsm/L salt, AAPH and 300 mOsm/L salt with the addition of VC. SOD, GSH and MDA activity assay kits are used to determine the activities of SOD, GSH and MDA in HH7 chick embryos. GSH is a reducing agent oxidized with ROS to GSSG. The results show that both activities of GSH and SOD reduced in presence of high salt; MDA activity in presence of high salt increased and fell back after addition of VC (Fig. 7A). The results of quantitative PCR data show that high-salt exposure increased the expression of ROS-related genes, including SOD1, SOD2, GPx and GLR, VC had rescued effect on the HH7 chick embryos, which was the further evidence that ROS was increased by high-salt exposure (Fig. S6A). The results of western blotting showed that the high-salt exposure dramatically inhibited GATA4, GATA6, TBX5 and Mef2c expression at HH7, while AAPH just inhibited GATA4 and TBX5 at protein

level. The decrease induced by high salt could be rescued by addition of VC (Figs. 7B-B'). Moreover, quantitative PCR data showed more gene expressions' alterations induced by high-salt exposure. High-salt and AAPH exposure reduced the expression of Zic3, GATA4/5/6, TBX20, Nkx2.5/2.3/2.6, Myocardin, VMHC, Hopx, Fgf8/10, SRF, ISL1 and TAL1, while it induced the expression of BMP2/4, Wnt3a and β-catenin. The abnormal expression of genes after the addition of VC reflects a return to some level of normalcy (Figs. S6B-C). Next, we also examined the ROS production of HH10 chick embryos at protein (Fig. 7C) and mRNA levels (Fig. S6D). The date showed that the ROS was activated with high-salt treatment at the period of cell differentiation and heart tube fusion. We detected some genes related to the fusion of endocardial cushions by quantitative PCR. The high-salt and AAPH exposure suppressed the expression of Epha1/3, Tmem2 and CCN1, but high salt promoted the expression MMP2, which was different from the effect of AAPH on MMP2 (Fig. S6E). The results of western blotting and quantitative PCR illustrated that high-salt treatment inhibited the expression of Zic3, GATA4/5/6, TBX5/20, Mef2c, Nkx2.5/2.3/2.6, Myocardin, VMHC, BMP2/4, Hopx, Fgf8/10, SRF, Wnt3a and β-catenin and ISL1, and VC had rescued effect on the genes expression, except Mef2c, Hopx and Wnt3a. AAPH exposure had similar influence on these genes' expression except β-catenin (Figs. 7D-D', F-G).

Since VC was valid in rescuing the genes expression related to the formation of heart tube, we cultured the embryos to investigate whether excess ROS could also inhibit the growth of embryos and induce cardia bifida (Figs. 7E). From the result, we could found that the development of embryos and embryonic mortality was clearly improved after VC addition compared with high-salt treatment, but it was slightly severer than that of control

groups (Figs. 7G-J). Importantly, the morphological observation of heart tube at HH10 also showed that addition of VC significantly rescued the malformation of heart tubes, 80% of the high-salt treated heart tubes could be fused with the addition of VC (Fig. 7K).

Discussion：

High-salt exposure in pregnant rats has been shown to cause a long-term negative impact on vascular development in their offspring [44]. The offspring of high-salt diet-fed dams had lower blood pressure and heart rate, indications of both left ventricular systolic and diastolic function, and a decreased aortic vasodilation response to NO, which indicated organic damage to the heart [45]. During embryogenesis, the heart is the first organ to be developed. Severe developmental defects and malformation in the heart could cause the death of embryo. It is not clear whether the morphology and molecular biology of the heart tube are affected by high-salt exposure during the gastrula period.

Cardiac precursor cells derive from epiblast cells after undergoing EMT process. In chicken embryos, RhoA controls tissue polarity and cell movement of cardiogenic progenitors [46]. Besides, other Rho GTPases, CDC42, Rac1, are central to dynamic actin cytoskeletal assembly and rearrangement and are the basis of cell-cell adhesion and migration [47, 48]. High-salt treatment enhanced the expression of RhoA and laminin (Figs. 2F-I), which inhibited the initiation of the EMT process. Additionally, high-salt destroyed the cytoskeleton and enhanced cell junctions (Figs. 2L-N), inhibited cell migration and specification. Thus, we found the excessive ectopic expression of E-cadherin and N-cadherin, which indicated abnormal mechanism during the EMT process of heart tube formation [25] (Fig. S2).

The myocardial precursor cells initiated at the anterior primitive streak of gastrula embryos migrate symmetrically and differentiate to form the crescent heart. The DiI^{+} migration *in vivo*, scratch wound assays and anterior primitive streak explants *in vitro*, showed that the migration of cardiac cells were suppressed by high-salt treatment (Figs. 2A-G, Figs.S4B-C). Actin cytoskeletal structures that are characteristic of cell migration were replaced by a diffuse distribution of actin with high-salt treatment (Figs. 2H-I) [49]. Cells cultured with high-salt lost the elongated shape associated with motile cardiac cells and adopted short shuttle-like or round-like, unpolarized shapes. Importantly, the acquisition of a rounded cell shape was accompanied by a loss of the actin structures associated with cell polarization and perturbed stress fiber formation. Many of these processes are mediated by cellular cytoskeleton modulation, relevant cell-cell adherence factors and Rho GTPases [38, 49], which play vital roles in the uniform contraction of the heart musclem [50, 51]. In addition, the activation of the RhoA signaling pathway can induce the formation of stress fibers and single-cell motility *in vivo* [39, 48]. These results determined that high-salt treatment through suppressing polarization and protrusion formation of cardiac precursors inhabited the cell migration towards the site of heart tube formation.

During embryo development, morphogenesis of heart tube depends on a number of precisely coordinated cardiac-associated genes. Mutation of Mesp1, the first indicator of cardiogenic mesoderm, leads to cardia bifida, and cells that are doubly mutant for Mesp1 and Mesp2 are selectively unable to contribute to heart development [12]. The earliest and later cardiac progenitor cells to express Mesp1 form the first heart field (FHF) and second heart field (SHF). A summary of the key transcription factor interactions that regulate FHF and SHF differentiation is shown in Fig. 4A. The activated expression of cardiogenic

transcription factors play the vital role as cardiac differentiation progresses during addition of SHF cells to the linear heart tube. Whole-mount *in situ* hybridization showed that high-salt exposure dramatically restricted the expression of GATA5, Nkx2.5 and VMHC at cardiac crescent. Furthermore, early expression of Islet1 is dependent on canonical Wnt/β-catenin pathway and on Fgf8 signaling, both of which are required for proliferation of early cardiac progenitors [52]. FGF is able to substitute for pharyngeal endoderm and probably cooperates with BMP to induce cardiac mesoderm and then drives mesodermal cells into myocardial differentiation [53]. In addition, it is notable that the Wnt/β-catenin pathway, concerting with BMP and FGF, is initially required for the induction of mesoderm, while sustained activation inhibites heart tube formation in HH7 to HH10 [54, 55]. Epha1/3, Tmem2, MMP2 and CCN1 regulate the coordination of myocardial and endocardial morphogenesis, which are relevant to the fusion of the heart tube [56].

Congenital heart malformations and myocardial defects are related to cardiac cell proliferation and apoptosis. Various studies have illustrated that excessive ROS could induce inordinate apoptosis. In this study, high-salt treatment inhibited cell proliferation and promoted apoptosis, the results of cardiomyocytes (Figs. 6C, E) and chick embryos' heart malformation are similar to that of AAPH (an inducer of free radicals) [20], which demonstrated that high-salt might through generating excess ROS production to induce heart deformity. Furthermore, this study found that excess ROS is responsible for a significant disturbance to gene expression by exposing chick embryos to AAPH in a similar manner to embryos treated with high-salt [20], both of which could suppress the expression of key cardiogenic transcription factors, especially GATA4/5/6, Nkx2.5, Tbx5 and Mef2c, and combination of Vitamin C application with high-salt could rescued high salt-induced

reduction to some extent (Figs. 4 and 7). The results confirm that high-salt exposure could inhibit cardiac progenitor cell differentiation. Most importantly, adding Vitamin C to high-salt treatment from HH0 to HH10, dramatically improved the development of embryos and of heart tube, reduced the generation of deformities (Fig. 7E), which further supports the conclusion that high-salt exposure generated excessive ROS production and led to discorded cardiac-related gene expression, which detrimentally effected EMT, cell migration, cell differentiation, fusion, cell proliferation and apoptosis, which eventually induces heart tube malformation (Fig.8).

To eliminate the osmotic stress effects, mannitol was used as an osmotic pressure-matched control for high-salt treatment [18, 40, 41, 43]. From the result we found that except the effect of osmotic pressure, high-salt exposure had even bigger impact on the growth of chick embryos and caused more serious heart tube malformation (Fig. S7). Culturing embryos during HH4-HH7 with high-salt had serious negative effects on heart tube morphogenesis (Fig. S3), notably, on the embryo mortality classification of the heart tube at HH10. Perhaps the stage of HH4 to HH7 is the vital one of cardiac precursor cell migration and differentiation, which plays an irreplaceable role in the embryonic development. As for culturing form HH0 to HH4 with high-salt, the disturbed EMT process can be saved to a certain extent in the following process; as for culturing form HH7 to HH10 with high salt, it mainly affects the cell differentiation. Thus, the proper and synchronized migration and differentiation of the cardiac progenitor cells in mesoderm-derived primary heart field is the key to normal heart tube development. Figure 8 summarizes schematically how high salt might induce these changes. Further experiments, such as whole genome sequencing or further experiments on animals, are required to

explore the precise molecular mechanisms by which high-salt affects cardiogenesis. It can help us to improve the understanding of high salt metabolism in the body.

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Competing Financial Interest

The authors have declared that no competing interests exist.

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Figure legends：

Figure 1. The classification of progressive high-salt exposure-induced heart malformations in gastrula chick embryos.

(A) Normal **(B)** Mild **(C)** Intermediate **(D)** Severe **(E)** Delayed. Representative appearances of phenotypes classification of chicken embryos and their heart tubes in gastrulating chick embryos. **(A1-E1)** Bright-field images of whole HH10 embryos. **(A2-E2)** Anti-mouse MF20 antibody immunofluorescent-staining of whole heart tubes. Anti-mouse MF20 antibody and DAPI costaining of heart tubes transverse sections at the levels indicated by dotted white line of whole-mount immunostaining of heart tube. The distribution of heart tube phenotype classification ('+' means active result; '-' means negative result). **(F)** Bar chart shows the rate of heart phenotype classification (%) in 240, 260, 280 and 300 mOsm/L-treated groups. Scale bars = 500 μm (A1-E1); 300 μm (A2-E2); 200μm (A2'-F2'; A2''-E2'')

Figure 2. High-salt exposure interfered with the EMT process during chick gastrulation.

(A) Representation of the cardiac progenitor cells specification during EMT process regulated by the combination of BMP2, FGF8 and canonical Wnt signaling. (**B-G**) Bright-field images of whole HH4 embryos. (**B1-G1**) Anti-mouse E-Cadherin (B1-C1''), N-Cadherin (D1-E1''), Laminin (F1-G1'') antibody immunofluorescent-staining of primitive streak. **(H)** Bar chart shows the gap distance of laminin (μm) with 240 (control) and 300 mOsm/L-treated HH4 chick embryos. **(I)** Analysis of RhoA etc. gene expression in HH4 chick embryos treated with 240 and 300 mOsm/L salt by quantitative PCR. **(J)** Western blotting showing the expressions of E-Cadherin, N-Cadherin, slug, and β-catenin at

protein level in HH4 chick embryos. **(K)** Illustration shows the cell junction structure among cells, including adherens, tight and gap junctions. **(L-N)** Analysis of genes of adherens junction, E-Cadherin etc.; tight junction, ZO-1 etc.; and gap junction, CX43 expression in HH4 chick embryos treated with 240 and 300 mOsm/L salt by quantitative PCR. Scale bars = 500 μm (B-G; B1-G1); 200 μm (B1'-G1'; B1''-G1'')

Figure 3. High-salt exposure restricted cardiac progenitor cell migration.

(A) Bright-field and fluorescence images were taken at 0-, 8- and 18-hrs. The DiI-labeled cardiac progenitor cell of embryo migration following 240 mOsm/L salt-treatment on both sides of culture medium (upper images). The DiI-labeled cardiac progenitor cell of embryos migration following 300 mOsm/L high-salt treatment at the left side and 240 mOsm/L at right side of culture medium (lower images). **(B)** Bar chart shows the number of cardiac precursor cells migration based on upper images of A. **(C)** Bar chart shows the number of cardiac precursor cells migration based on lower images of A. **(D)** Bar chart shows the number of embryo incidence of symmetrical migration or asymmetric migration in 240 mOsm/L and 300 mOsm/L salt groups. **(E)** The sketch illustrates migration of chicken cardiac muscle cells as detected by the wound-healing assay. **(F)** The representative images of H9c2 cells scratch test at 0-, 12-, 24-hour incubation from 240 mOsm/L-treated and 300 mOsm/L-treated groups respectively. **(G)** The bar chart shows the percentage of wound closure (%) at 12-, 24-hour. **(H)** The sketch illustrates the stress fibers in chicken cardiac muscle cells. **(I)** Representative image of actin filaments in 240 mOsm/L-treated and 300 mOsm/L-treated chicken cardiac muscle cells were visualized by staining with F-actin (red), and cell nuclei were stained with DAPI (blue). White dotted lines show the long and short

axes of cells. (The boxed region shows stress fiber assay in chicken cardiac muscle cells). **(J)** Bar chart shows cells containing stress fibers (%). **(K)** Bar chart shows the ratio of long axis to short axis. **(L-M)** Analysis of genes of α-catenin etc. expression in HH7 chick embryos treated with 240 and 300 mOsm/L salt by quantitative PCR. Scale bars = 300 μm (A); 400 μm (F); 100 μm (Left images in I); 50μm (middle and right images in I).

Figure 4. High-salt exposure repressed the differentiation of cardiac progenitor cells.

(A) The diagram shown is a brief overview of a subset of all known transcription factor interactions and signaling pathways that drive the differentiation of first heart field (FHF; orange) and second heart field (SHF; blue) cardiac progenitor cells during development. Factors colored by both orange and blue represent regulators of both FHF and SHF. **(B)** The embryos were incubated with 300 mOsm/L (left) and 240 mOsm/L salt (right) at either side until HH7 and processed for in situ hybridization for GATA5 (B1), Nkx2.5 (B2), VMHC (B3). **(C-D)** Analysis of genes of Zic3 etc. and BMP2 etc. shown in A by quantitative PCR of HH7 chick embryos. **(E-F)** Analysis of genes of Zic3 etc. and BMP2 etc. shown in A by quantitative PCR of HH10 chick embryos.

Figure 5. High-salt exposure inhibited cell proliferation and promoted apoptosis

(A-B) Bright-field images of whole HH10 embryos treated with 240 and 300 mOsm/L salt. (A1-B1) Anti-mouse MF20 antibody immunofluorescent-staining of heart tube. (A2-B2) Anti-rabbit pHIS3 antibody immunofluorescent-staining of whole embryos. **(C)** Bar chart shows the number of $pHIS3^{+}$ cells on heart tube. **(D-E)** Bright-field images of whole HH10 embryos treated with 240 and 300 mOsm/L salt. (D1-E1) Anti-mouse MF20 antibody

immunofluorescent-staining of heart tube. (D2-E2) Anti-rabbit c-Caspase3 antibody immunofluorescent-staining of whole embryos. **(F)** Bar chart shows the number of c-Caspase3^{+} cells on heart tube. Scale bars = 500 μm (A-E); 300 μm (A1-E1; A2-E2); 200 μm (A2'-E2'; A2''-E2'')

Figure 6. High-salt exposure through generating excess ROS inhibited cell proliferation and promoted apoptosis.

(A) Cell viability rate (H9c2 cells) treated with 240, 300 mOsm/L salt, 300 mOsm/L salt in addition of 144μM or 288μM VC. **(B)** The quantitative analysis for intracellular ROS levels of cardiac progenitor cells in 240, 300 mOsm/L salt and 300 mOsm/L salt with the addition of 144μM VC. **(C)** Anti-mouse MF20 and anti-rabbit pHIS3 antibodies immunofluorescent-staining of H9c2 cells treated with 240, 300 mOsm/L salt, AAPH and 300 mOsm/L salt in addition of 144μM VC. **(D)** Bar chart shows the number of pHIS3^{+} cells of H9c2 cells. **(E)** PI (stained with red) and Hochest (stained with blue) dyes immunofluorescent-staining of H9c2 cells treated with 240, 300 mOsm/L salt, AAPH and 300 mOsm/L salt in addition of 144μM VC. **(F)** Bar chart shows the number of PI^{+} cells of H9c2 cells. **(G)** The flow cytometry was implemented in 36-hour cultured chick cardiac muscle cells of control, high salt-treated and high salt+VC-treated group. **(H)** The bar chart showing the comparison of the values of Q2 (late apoptosis) +Q4 (early apoptosis) in the cultured chick cardiac muscle cells among control, high salt and high salt+VC group. **(I)** Western blotting showing the expressions of PCNA and c-Caspase3 at protein level in HH10 chick embryos. Scale bars = 100 μm (C); 200 μm (E)

Figure 7. High salt through excess ROS production disturbed the genes' expression during heart tube formation.

We employed (A-B) HH7 chick embryos and (C-D) HH10 chick embryos, which were treated with to 240, 300 mOsm/L salt, AAPH and 300 mOsm/L salt with the addition of VC. **(A)** The activities of GSH, SOD, and MDA in HH7 chick embryos were assessed in presence/absence of high-salt and VC with the determination kits. **(B)** Western blotting showing the expressions of GATA4 etc. at protein level. **(C)** The activities of GSH, SOD, and MDA in HH10 chick embryos were assessed in presence/absence of high-salt and VC with the determination kits. **(D)** Western blotting showing the expressions of GATA4 etc. at protein level. **(E)** The strategy for applying 240, 300 mOsm/L simple salt and 300 mOsm/L salt addition with VC in chick embryos of EC-culture. **(F)** Representative appearance of 240 mOsm/L salt treated chick embryos for 0-, 18-, 34- and 38-hour. Representative appearance of 300 mOsm/L salt treated chick embryos for 0-, 18-, 34-, 38-, 41-, and 47-hour. Representative images of 300 mOsm/L salt in addition of VC treated chick embryos for 0-, 18-, 34-, 38-, and 41-hour. **(G)** Graph shows the mortality rate of three groups. **(H)** Bar chart shows the length of embryos following treatment at 18-, 38-hour. **(I)** Bar chart shows the length of embryos at HH10. **(J)** Bar chart shows the pair numbers of somites at 38-hrs. **(K)** Bar chart shows the rate of heart phenotype classification (%) with culturing in 240, 300 mOsm/L salt and 300mOsm/L salt in addition with VC from HH0 to HH10. Scale bars = 2000 μm (the images of 0h in F); 1000 μm (the images of 18-, 34-, 38-, 41-, 47-hrs in F).

Figure 8. Model depicting how high-salt exposure induced heart tube malformation during chick cardiogenesis.