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Original Article

Embryonic protective role of folate in arsenic-induced cardiac malformations in rats

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Abstract: Background: To investigate impacts of sodium arsenic ($NaAsO_2$) on embryonic cardiac development in rats and evaluate the protective role of folate in $NaAsO_2$ exposure rats. Methods: We divided 90 female rats randomly into 9 groups. Group A was the control; group B-F were the animals fed with $NaAsO_2$ in a series of increased doses, corresponding to 9.4 mg/L, 18.8 mg/L, 37.5 mg/L, 75 mg/L and 150 mg/L, respectively; group G-I were fed with 75 mg/L of $NaAsO_2$, in addition of folate with doses of 0.53 mg/kg, 5.3 mg/kg, and 10.6 mg/kg, respectively. Their fetus' general development and cardiovascular systems were examined. Nkx2.5, GATA4, TBX5 gene and protein expression were measured. Results: Relatively to group A, arsenic treated group C-F rats generated significantly lower weight of fetus and placenta ($P<0.05$), whereas the folate-treated groups H and I were significantly heavier than the arsenic-treated group E ($P<0.05$). We observed that incidences of cardiac malformations were significantly greater in arsenic-treated group E and F than group A ($P<0.05$). We found that the Nkx2.5 and GATA4 protein expression in the fetal hearts were downregulated in group B-F compared to group A. But the expression of them was significantly upregulated in group H-I relatively to group E ($P<0.05$). Moreover, the TBX5 gene expression was increased in both group D-F and G-I when they were compared to group A or group E, respectively ($P<0.05$). Conclusion: $NaAsO_2$ induce embryonic cardiac defection and folate supplement alleviate this impairment through modulation of the Nkx2.5, GATA4 and TBX5 gene expression.

Keywords: Arsenic, folate, congenital heart disease, Nkx2.5, GATA-4, TBX5

Introduction

Congenital heart diseases (CHD) manifest cardiovascular malformations during embryonic development. They are commonly diagnosed in children, in a frequency of 4 to 10 children per 1000 live births [1]. The genetic and environmental factors are the key determinants contributing to the multifactorial etiology of CHD. Currently, Environmental pollution is generally identified to be a major factor significantly correlating with occurrences of high incidences of CHD. Therefore, investigation of some key risk factors contributing to high incidences of CHD and their underlying molecular mechanisms might provide of proof-of-principle for early protective intervention.

Arsenic is a highly toxic metal, existing widely in our nature; it is a component mainly compounding pesticides, herbicides, alloy material, and medicine. It is absorbed by the respiratory and intestinal tracts and even the skin by a direct contact, and leads to multiple organ dysfunctions. During pregnancy, accumulated arsenic in the body will penetrate the placental barrier [2, 3] and directly cause embryonic dysplasia, manifesting a systemic fetal development malformations, with growth retardation, short limbs [4], low viability [5] and multiple organ malformations commonly including neural tube defects [6], urogenital abnormalities [7], limb bud and body hypoplasia [8]. Importantly, maternal arsenic exposure has been shown strongly correlating with the occurrence of CHD in their offspring. However, these retrospective studies are inevitable memory deviation [9-11] and the direct evidence of arsenic-induced CHD remains elusive.

The Nkx2.5, GATA-4 and TBX5 genes are the cardiac-specific transcription factors. They con-

trol many downstream genes, possibly involving cardiovascular development. Aberrant Nkx-2.5, GATA-4 and TBX5 expression (by genetically modification of animals by gene knockout) are associated with mammal heart malformations and CHD [12]. We hypothesized that arsenic exposure during pregestation and pregnancy could cause the fetal CHD, potentially mediated by downregulation of the cardiac-specific transcription factors-Nkx2.5, GATA-4 and TBX5 genes.

Supplementation of folate during pregestation and pregnancy has been shown an effective decrease in incidence of the fetal CHD [13, 14]. Folate breaks down the plasma homocystenine exerting a protective function [15-17]. However, whether folate supplementation during the pregnancy could protect cardiovascular development from arsenic toxicity remains largely unexplored; the associated protective activity involving changes of the expression of the cardiac-specific transcription factors-Nkx2.5, GATA-4 and TBX5 remains to be examined.

Our present study now shows that $NaAsO_2$ uptaken by SD rats increased high incidences of CHD. Conversely, folate supplementation significantly compromised the $NaAsO_2$-induced CHD, whereby sustaining expression of several cardiac-specific transcription factors in rat embryonic hearts.

Materials and methods

Animals

Animals were 30-40 days old female SD rats purchased from the Animal Center of Fujian Medical University, Fuzhou, China. Animal care and experimental proceedings were according to guidelines established by Fujian Medical University Animal Care Commission and following approval of the ethical committee. Animals were kept at 22±2 degrees centigrade, relative humidity of 55%, in the light for 12±1 hours and in the dark for another 12±1 hours. Fed water was changed twice a week.

Groups and toxicity exposure

A total of 90 female rats were divided randomly into 9 groups after one week adaptively fed. Group A was the control; group B-F were the animals fed with $NaAsO_2$ in a series of increased doses, corresponding to 9.4 mg/L, 18.8 mg/L, 37.5 mg/L, 75 mg/L and 150 mg/L, respectively, in drinking water; group G-I were fed with 75 mg/L of $NaAsO_2$, in addition of folate with doses of 0.53 mg/kg, 5.3 mg/kg, and 10.6 mg/kg, respectively.

The female and adult SD rats were caged overnight at 2:1 ratio after 6 weeks feeding. On the next morning, the male rats were removed from cages; vaginal smears of the female rats were obtained. In the event that smears were observed, the rat was accepted as being at day 0 of the gestational period. There were 10 mice per group; they were sacrificed after 6 weeks treatment. Caesarean were proceed on the day 16 following previous feeding. It was recorded miscarriage if embryo could not be seen in the maternal uterus on that day.

General and pathological observation

Caesarean were proceeded on day 16 under general anesthesia using 10% chloral hydrate. Numbers of miscarriage rats were recorded, and the live fetus and placenta were weighted. We randomly pick 3 fetuses from each live birth and fixed in 10% formalin. After fixture for 24 h, heart and bilateral lung (set as control) were separated, dehydrated, paraffin embedded and continuously dissected at a 7 um thickness. The sections were dyed with HE staining. Heart histological examination was observed by light microscope.

RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA of fetal heart tissue was extracted according to Trizol reagent's protocol (Invitrogen, USA). Amplification and detection of mRNA and synthesis of cDNA were accomplished by using iScript RT Supermix (Bio-Rad, USA) in accordance with the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Primers of Nkx2.5: 5'-ACCCTCGGGCGGATAAGA-3' and 5'-TCCTGCCGCTGTCGCTTAC-3'; GATA-4: 5'-AAACGGAAGCCCAAGAATC-3' and 5'-CACTGGATGGATGGAGGAC-3'; TBX5: 5'-CTCCACCCAACCCATACCCACT-3' and 5'-GCTGTGCCGACTCTGTCCTGTA-3'; GAPDH: 5'-TGATTCTACCCACGGCAAGT-3' and 5'-AGCATCACCCCATTTGATGT-3'. The relative expression of Nkx2.5, GATA-4, TBX5 mRNA were measured by the comparative cycle threshold (Ct) method. All experiments were analyzed in triplicate.

Table 1. Toxicity of $NaAsO_2$ on embryonic heart and preventive effect of folate

Groups	Pregnancy female rats	Miscarriage female rats	Weight of fetus/g ($\bar{x}\pm s$)	Weight of placenta/g ($\bar{x}\pm s$)
A	9	0	0.62±0.05	0.36±0.05
B	8	0	0.61±0.07▲ (P=0.270)	0.36±0.05▲ (P=0.853)
C	10	0	0.58±0.08* (P=0.000)	0.33±0.04* (P=0.000)
D	9	0	0.56±0.06* (P=0.000)	0.33±0.05* (P=0.002)
E	8	1	0.52±0.06* (P=0.000)	0.30±0.03* (P=0.000)
F	9	2	0.50±0.05* (P=0.000)	0.27±0.04* (P=0.000)
G	8	0	0.51±0.07# (P=0.240)	0.30±0.07# (P=0.472)
H	9	0	0.56±0.08Δ (P=0.001)	0.33±0.06Δ (P=0.000)
I	9	0	0.56±0.09Δ (P=0.000)	0.32±0.09Δ (P=0.004)

Annotation: ▲Compared with group A, P>0.05; *Compared with group A, P<0.05; #Compared with group E, P>0.05; ΔCompared with group E, P<0.05.

Table 2. Heart malformation effect of $NaAsO_2$ exposure and folate protective function

Group	Number of hearts	VSD	ASD	Pulmonary stenosis	Amount (abnormality rate)
A	27	0	0	0	0 (0%)
B	24	0	0	0	0 (0%)
C	30	0	0	0	0 (0%)
D	27	2	0	0	2 (7.4%)▲ (P=0.491)
E	21	3	1	1	5 (23.8%)* (P=0.012)
F	21	5	1	2	8 (38.1%)* (P=0.001)
G	24	2	2	0	4 (16.7%)# (P=0.713)
H	27	2	1	0	3 (11.1%)# (P=0.272)
I	27	1	2	0	3 (11.1%)# (P=0.272)

Annotation: ▲Compared with group A, P>0.05; *Compared with group A, P<0.05; #Compared with group E, P>0.05.

Western blot (WB)

Approximately 50 mg of heart tissues were completely homogenated by using a plastic grinding rod. Total proteins were extracted and quantified by BCA assay. WB was performed using total proteins. Denatured SDS protein was loaded onto a 10% SDS-polyacrylamide gel and blotted onto polyvinylidene fluoride (PVDF) membrane. Then PVDF membranes were incubated with blocking buffer at room temperature for 90 min. Membrane incubated with polyclonal first antibody (Nkx2.5 was purchased from Biobyt, British; GATA-4 from Santa Cruz, USA; TBX5 from Thermo, USA and Lamin B1 was purchased from Boster, China) overnight at 4 degrees centigrade, washed and incubated with Anti-rabbit IgG (HRP-linked Antibody, Merck, German). Membranes were incubated in reacting lipid of chemiluminescence for 2 min and exposure to X-ray. Protein staining was recorded by film scanning and analyzed by using Quantity One to calculate gray scale ratio of Nkx2.5/Lamin B1, GATA-4/Lamin B1, TBX5/Lamin B1 in each group.

Statistical analysis

Statistical analysis was performed by SPSS 19.0 version. The quantitative data ($\bar{x}\pm s$) were analyzed using Students' t-test, which in accordance with normal distribution and the variance was homogeneous. The Chi-square test or Fisher exact probability method was implied to identify count data. P<0.05 was accepted to be significant.

Results

General observation

In our study, the pregnancy rats were 8, 8, 10, 9, 8, 9, 8, 9, 9 in group A-I, respectively. Numbers of miscarriage rats in group A-I were 0, 0, 0, 0, 1, 2, 0, 0 and 0. Relatively to group A, arsenic treated group C-F rats generated significantly lower weight of fetus and placenta (P<0.05), whereas the folate-treated groups H and I were significantly heavier than the arsenic-treated group E (P<0.05). As showed in **Table 1**.

Arsenic-induced embryonic cardiac malformations in rats and protective role of folate

We eventually collected 27 fetal hearts in group A, none of them showed malformation by using light microscope. We observed normal four cav-

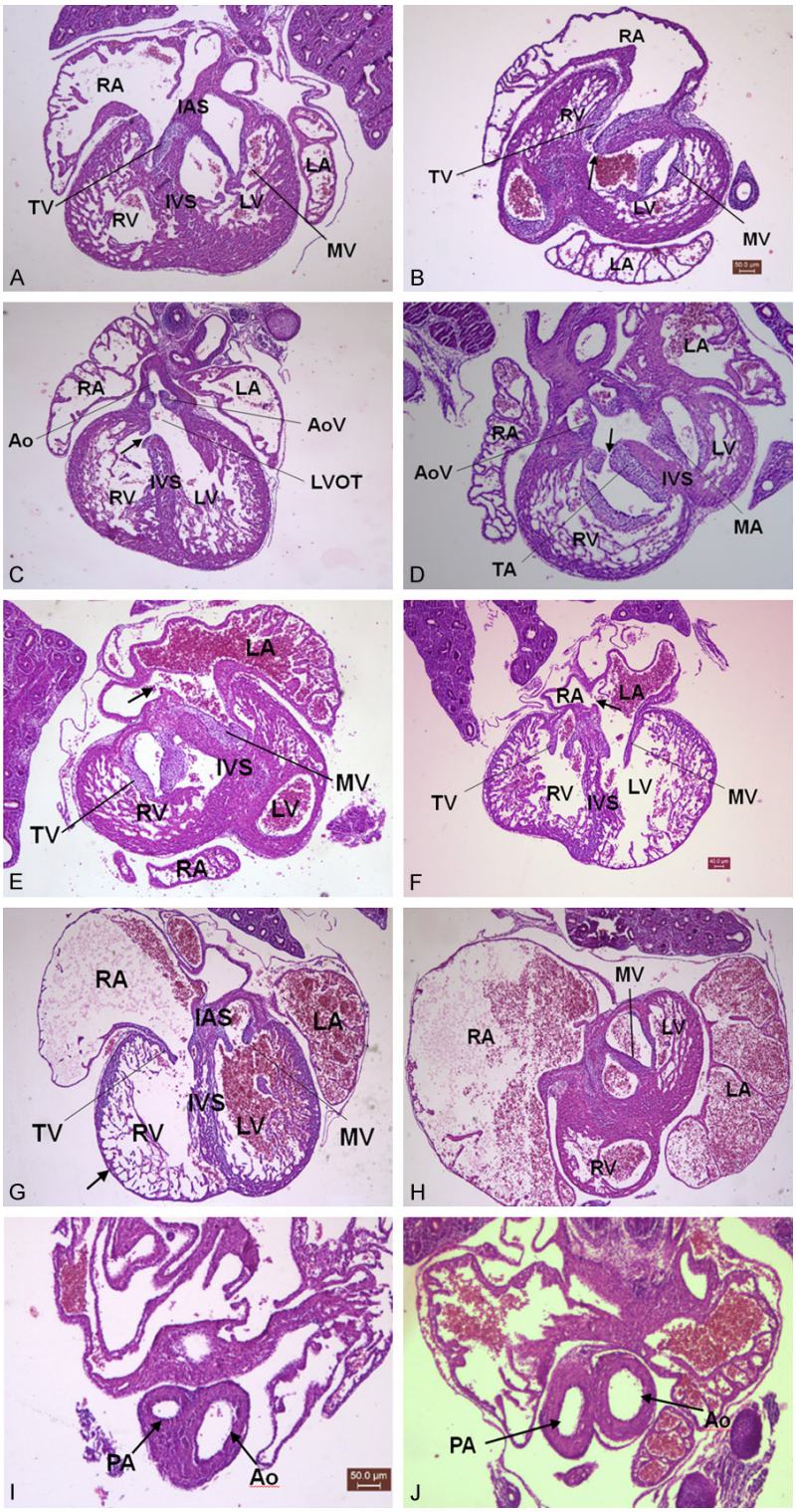
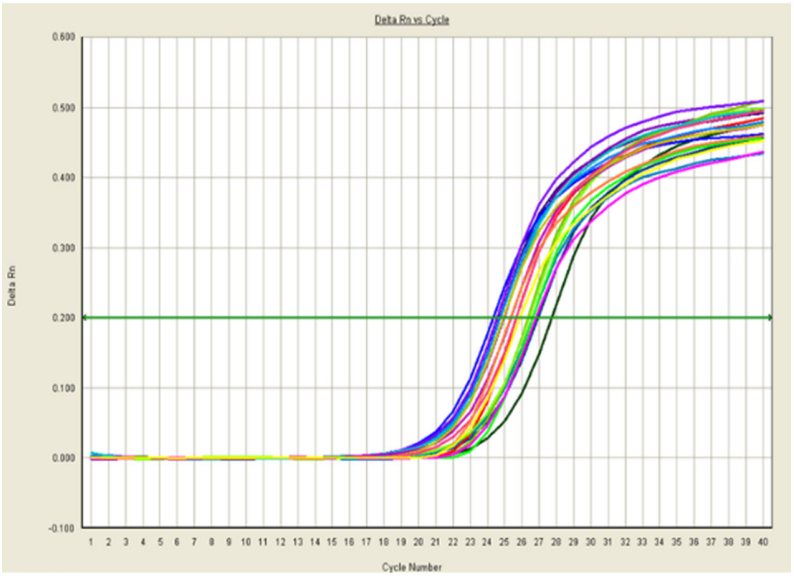
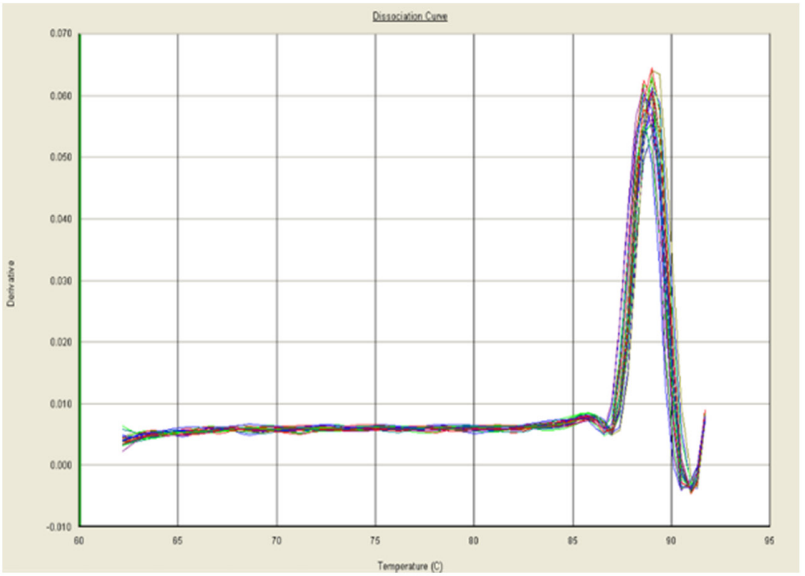


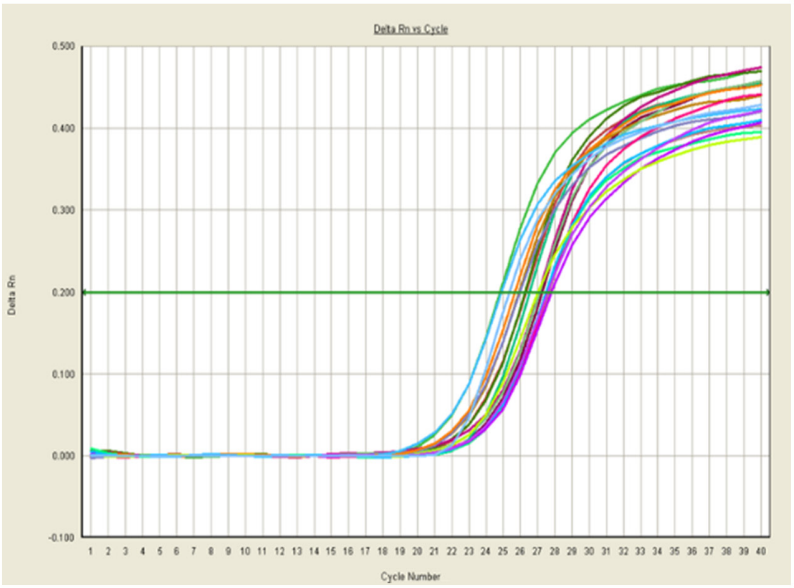
Figure 1. Effects of $NaAsO_2$ exposure on embryonic heart development during Pregestation/gestation. ×40. A: Normal heart; B-D: ↑VSD; E: ↑ASD; F: ↑ASD; G: ↑Thin ventricular wall; H: Large atrial cavity; I, J: ↑Pulmonary stenosis. LV: left ventricular; RV: right ventricular; LA: left atrium; RA: right atrium; IVS: interventricular septum; IAS: interatrial septum; MV: mitral valve; TV: tricuspid valve; Ao: aorta; AoV: aortic valve; LVOT: left ventricular outflow tract; PA: pulmonary arterial; ↑: anomalous structure.

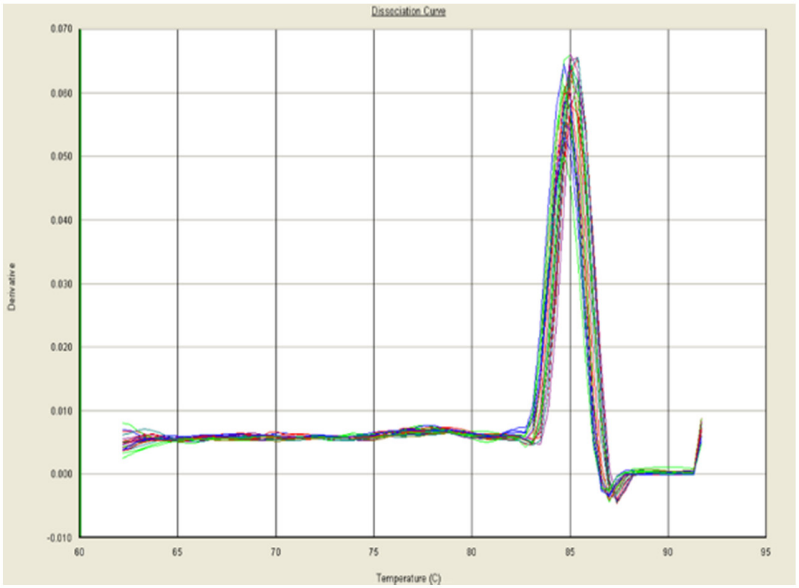
ity structure; well-developed atrial wall, ventricular wall, interventricular septum and large vessels; and maturity of atrioventricular valve and large artery membrane (slim valve). We also found none disorders in 24 and 30 fetal hearts derived from group B and C, respectively. However, 2 were ventricular septal defect (VSD) in 27 fetal hearts derived from group D; 3 were atrial septal defect (ASD), 1 had pulmonary stenosis in 21 fetal hearts derived from group E. We gathered 24 fetal hearts from group F, among them 5 VSD, 1 ASD and 2 pulmonary stenosis were found. Totally 24, 27 and 27 fetal hearts were collected from folate-treated group G to I, respectively. We observed 2 VSD and 2 ASD in group G, 2 VSD and 1 ASD in group H, and 1 VSD and 2 ASD in group I. The malformation rate of fetal heart in group A-I were: 0%, 0%, 0%, 7.4%, 23.8%, 38.1%, 16.7%, 11.1% and 11.1%, respectively. We observed that incidences of cardiac malformations were significantly greater in arsenic-treated group E and F than group A ($P<0.05$), (**Table 2**). There was no significantly difference of cardiac malformations in group E in comparison with groups G-I ($P>0.05$) (**Table 2**). Additionally, retardation of atrioventricular valve and large artery of fetal heart were common manifestations in groups with high-dose arsenic (≥37.5 mg/L $NaAsO_2$) and partly manifest thin ventricular wall, large atrial cavity and other disorders in fetal hearts (**Figure 1**).


Delta Rn vs Cycle
Delta Rn
Cycle Number

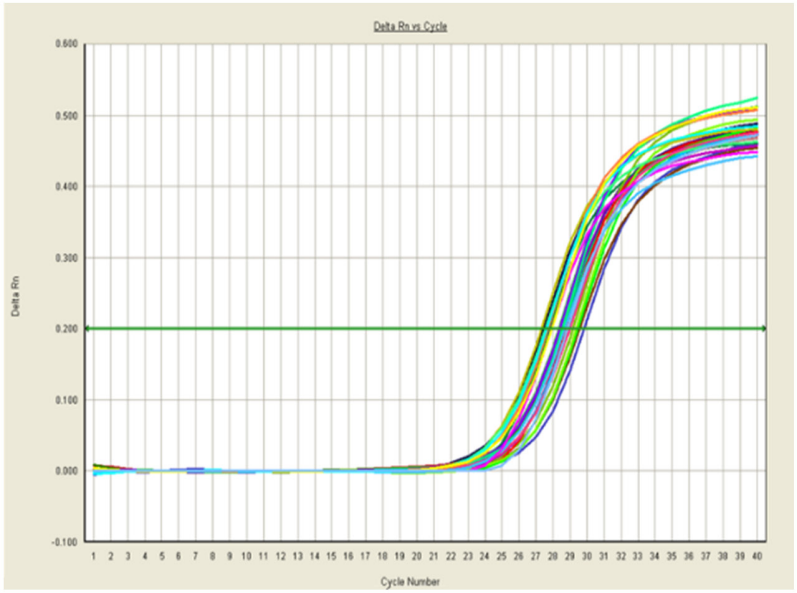

Dissociation Curve
Derivative
Temperature (C)

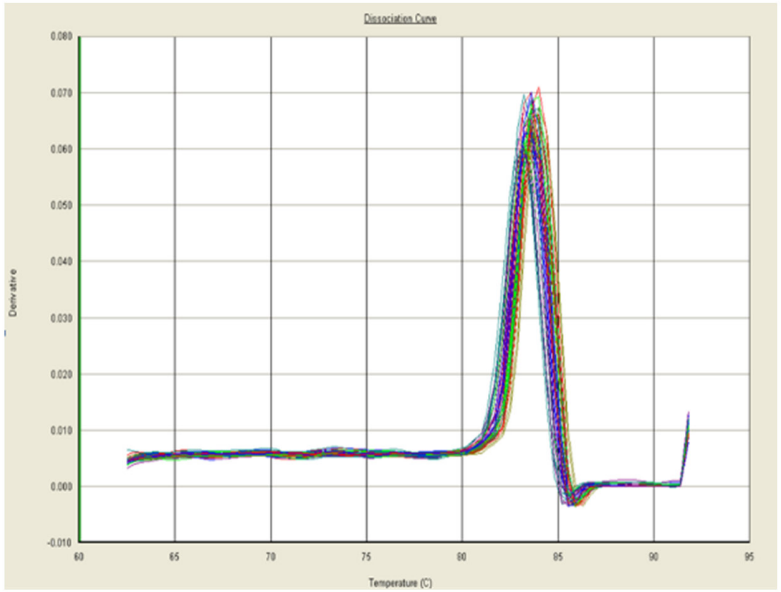
Nkx2.5


Delta Rn vs Cycle
Delta Rn
Cycle Number

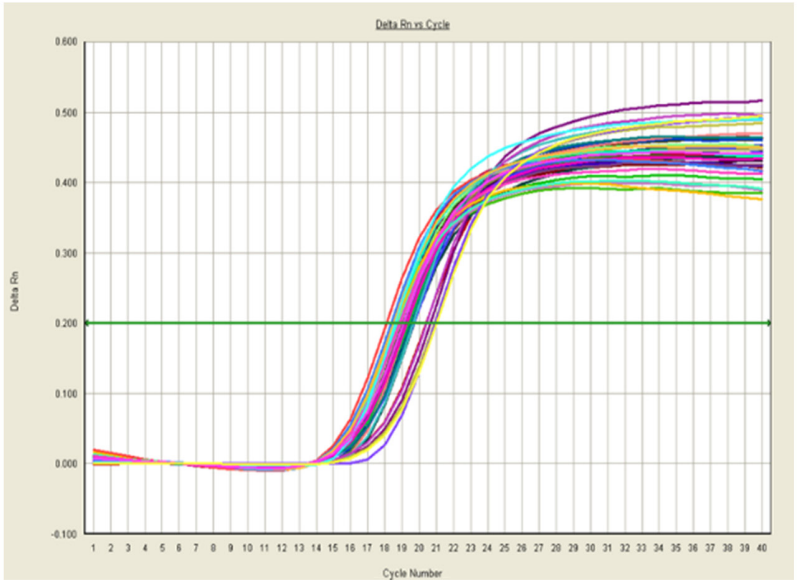

Dissociation Curve
Derivative
Temperature (C)

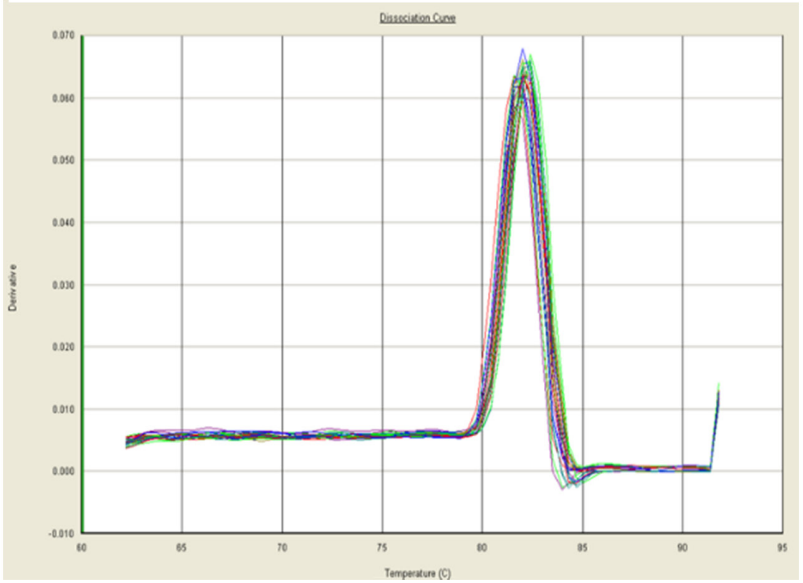
GATA4





TBX5





GAPDH

Figure 2. PCR amplification curve (left) and dissolution curve (right).

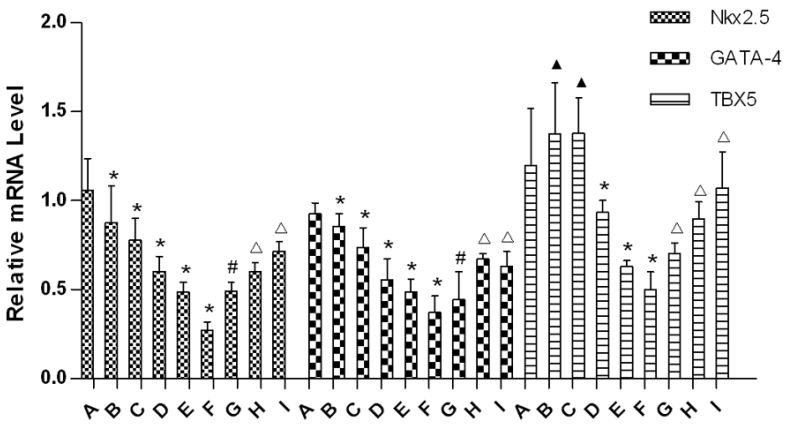


Figure 3. Relative mRNA levels of Nkx2.5, GATA4, TBX5 in each group's embryonic heart. (▲Compared with group A, P>0.05; *Compared with group A, P<0.05; #Compared with group E, P>0.05; △Compared with group E, P<0.05).

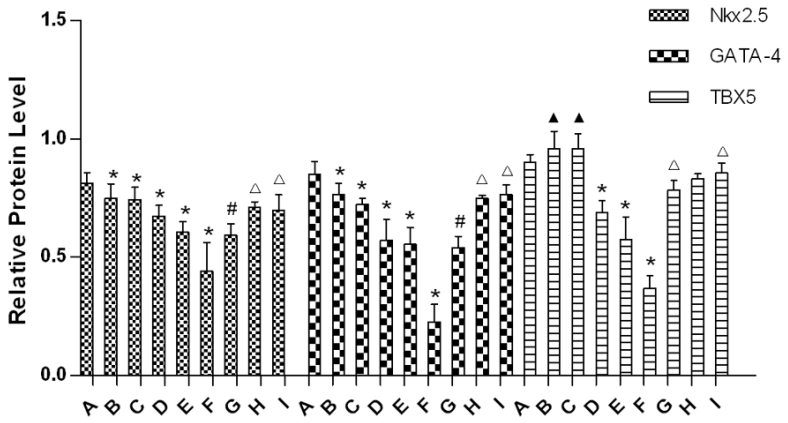


Figure 4. Relative protein levels of Nkx2.5, GATA4 and TBX5 in embryonic heart of each group. (▲Compared with group A, P>0.05; *Compared with group A, P<0.05; #Compared with group E, P>0.05; △Compared with group E, P<0.05).

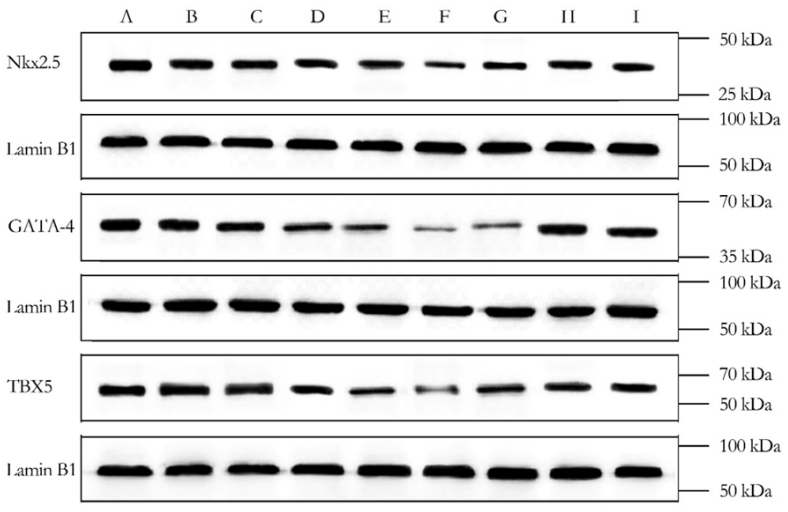


Figure 5. Results of Western Blot.

Possible mechanism of folate against arsenic toxicity on embryonic heart

Results of real-time PCR amplification curve and dissolution curve showed in **Figure 2**. We observed single sharp peak in dissolution curve in which confirmed results were specific products. Results of PCR showed that expression of Nkx2.5 and GATA4 mRNA in embryonic hearts were significantly downregulated in group B-F compared to group A (P<0.05). But the expression of them were significantly increased in group I, H relatively to group E (P<0.05). Moreover, expressions of TBX5 mRNA were significantly increased in both group D-F and G-I when they were compared to group A or group E, respectively (P<0.05), **Figure 3**.

WB assay revealed that Nkx2.5 and GATA4 protein expression in fetal heart were significantly decreased in group B-F compared to group A. However, the protein expression of them was significantly increased in group G-I relatively to group E (P<0.05), **Figures 4**, **5**.

Discussion

Most environmental risk factors often pose threat to normal fetal development; they penetrate the placental blood barrier, and directly damage embryonic cells, in association with impaired cellular differentiation, proliferation and mobilization [18]. Arsenic and associated metabolites are such

factors impair embryonic development at the teratogenic phase. Folate metabolism-essentially influences fetal development, regulating DNA synthesis, RNA transcription and tRNA methylation for protein expression and physiological organelle functions.

The loss of folate is essentially associated with dysmaturity of cellular DNA synthesis and cell proliferation. Folate supplementation at tetratogenesis for female rat decreased incidence of CHD in by 16.5% [19]. In line with these findings, our present study demonstrated the embryonic protective effects by folate; it decreased the incidences of heart malformation in rat embryo when they were exposed to arsenic.

Our present study showed that exposure of pregnant rat to arsenic led to a decrease in fetal and placental weights corresponding to a series increased arsenic-fed. We speculate that the embryonic cytotoxic $NaAsO_2$ might restrain embryonic development and cell differentiation and proliferation [20]; consequently leading to hypoplasia and inhibit placental angiogenesis [21]. Attenuation of fetal angiogenesis in turn induces chronic intrauterine hypoxia and hence fetal growth retardation.

In this report, we showed that the beneficial effects of folate supplementation; it compromises toxicity of $NaAsO_2$ and significantly increased the weights of rat fetus and placenta. Interestingly, protective effect of folate at 5.3 mg/kg and 10.6 mg/kg remained just the same. Based on that, intake proper dose of folate but not as much as possible is suitable to against toxicity of $NaAsO_2$. Our present study showed that the higher doses of arsenic were uptaken by pregnant rat the more incidences of CHD observed in the embryos. The most CHD happened to be VSD, ASD, pulmonary stenosis and other cardiac malformation The exposure to high doses of arsenic often occurred in parallel with retardation of atrioventricular valve and large vessels, thin ventricular wall and large atrial cavity. The developmental stages of heart were highly sensitive to external factors. These essential multiple stages including formation of atrioventricular, valve, large vessel of fetal heart and atrioventricular separation [22]. As an external toxicity, $NaAsO_2$ could interfere in any developmental stages of heart and lead to multi-structure abnormalities.

Conversely, we found that supplementation of folate during pregestation and gestation counteracted $NaAsO_2$-induced CHD, with a decrease in incidences of cardiovascular malformations.

Several previous studies have shown that mammal heart development is regulated by cardiac-specific transcription factors. The Nkx2.5 gene is a subtype of the NK type of homologous nuclear gene family of Nkx2, controlling the differentiation of heart cells, formation and looping of cardiovascular, separation of atrioventricular, formation of atrioventricular valve and conductive in atrioventricular. The Nkx2.5 mutations caused abnormal downstream expression of genes: ANF, BMP, MLC2V, N-myc, MEF-2C, dHAND, Msx2. They are involved in heart malformations [23].

The GATA family of zinc-finger transcription factors, GATA4 is proposed to regulate genes involved in myocardial differentiation and function. Since GATA4 contributes to 30%-40% of normal threshold values for regular development of heart. Homozygous GATA4 deletion is embryonic lethal due to defects of cardiac formations. Additionally, early alterations in GATA4 gene expression may result in cardiac dysplasia, endocardial cushion defects [24], ASD, pulmonary valve thickening, cardiac valve agenesia and other several cardiac malformation types [25].

The TBX5 gene is a member of a phylogenetically conserved T-box family of genes, which has recently been found to play a unique role in heart development. TXB5 combines downstream target genes with specific T-box domain, and regulates heart development throughout. It is necessary for initiation of atrioventricular cavity, separation of ventricular and cardiac [26]. The TBX5 deficiency is thus responsible for severe agenesia of atria and ventriculus sinister. Nkx2.5, GATA-4, TBX5 are the upstream transcriptional factors; they target several key genes and regulate cardiac development. They act in a coordinated fashion and decide each process of cardiac formation. Our present study now displays for the first time that the arsenic cardiac cytotoxic effect is associated with downregulation them at particularly in the phases of pregestation and gestation.

Conclusion

In conclusion, addition of folate had involved in multiple pathways to decrease the incidence of CHD before or during gestation. We present that folate could prevent $NaAsO_2$-induced CHD

through sustaining expression of several cardiac-specific transcription factors. Moreover, this article highlighted a new thought to explore etiology and preventative therapy of CHD.

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Disclosure of conflict of interest

None.

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