Ectopic Expression of Retinoic Acid Receptors and Change of Myocardial Structure in the Offspring Heart with Vitamin A Deficiency

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Summary Vitamin A is a key micronutrient required during crucial stages of embryonic development and vitamin A deficiency (VAD) results in embryonic heart malformation. The pleiotropic functions of vitamin A are mediated by specific nuclear receptors: the retinoic acid receptors (RAR α , - β , and - γ) and the retinoic X receptors (RXR α , - β , and - γ). The action of nuclear receptors has been implicated in controlling of cell proliferation, differentiation and apoptosis, and the expressions of these receptor genes are regulated by retinoic acid levels during the early stages of embryonic development. GATA-4 is one of the earliest transcription factors expressed in developing cardiac cells. However, the functional links of specific nuclear receptors to heart development in VAD embryos are not clearly understood. In our study, weaning female Sprague-Dawley rats were fed a modified diet containing different concentrations of vitamin A according to the American Institute of Nutrition 93 Growth Purified Diet. After 10-wk feeding, the female rats were mated with normal male rats, and a portion of them were transferred to a diet with enough added vitamin A for the pregnancy cycle. The embryo hearts were dissected out at embryonic day 13.5 (E13.5) to study the expression of RARs, RXRs and GATA-4. The embryo hearts from E18.5 were for observation of ultrastructural changes. In comparison to vitamin A supplemented groups, the embryo hearts from vitamin A insufficient groups exhibited ultrastructural changes and significantly lower expression of GATA-4, RAR α , and - γ , and higher expression of RXR α and - β . Our findings suggest that the down-regulation of RARs and the up-regulation of RXRs resulted from VAD affected GATA-4 gene expression, which resulted in ultrastructural changes in embryo hearts due to maternal insufficiency of vitamin A during pregnancy.

Key Words vitamin A deficiency, RARs, RXRs, GATA-4 gene, cardiogenesis

Vitamin A (VA) and its biological derivatives (the retinoids) play diverse roles in physiology, ranging from incorporation into vision pigments to controlling transcription of a host of important genes, as evidenced by the myriad of disorders that accompany deficiency or excess states. Vitamin A deficiency (VAD) is a public health problem in more than half of all countries, especially in developing countries, hitting hardest young children and pregnant women. According to the data from WHO, approximately 15% of pregnant women are estimated to be VAD all over the world (1). Maternal insufficiency of VA during pregnancy results in fetal death or severe abnormalities in the offspring, including aberrant heart development (2–4).

The VA active form, retinoic acid (RA), which acts as a ligand for the nuclear receptors the retinoic acid receptors (RARs) (RAR α , - β , and - γ) and the retinoic X receptors (RXRs) (RXR α , - β , and - γ), distinctly regulates embryonic development. It is an accepted fact that RARs and RXRs are indispensable for embryonic patterning and organogenesis, including cardiogenesis (5–8). Some studies show that the RAR γ gene is at very high levels in the endocardial cushions of the midgestation embryo (9). Data from Mangelsdorf et al. (10) show that the expression of RXR α and RXR β are highest in the whole embryo at midgestation (embryonic days 11.5–12.5), while RXR γ expression peaks slightly later (embryonic days 13.5–14.5). Kastner et al. indicate that at least four distinct retinoid receptors (RXR α , RXR β , RAR α and RAR β) are likely involved in controlling the ventricular myocardium development (11).

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The conserved GATA zinc-finger transcription factor family consists of six proteins (GATA-1–6). GATA-4 is one of the earliest transcription factors expressed in developing murine cardiac cells (12), although it is also found in the gut, gonads, liver, and endoderm (13, 14). Research work demonstrates that GATA-4 is expressed in the atrial and ventricular myocardium, endocardium, endocardial cushions, and outflow tract during mouse cardiogenesis (15). Deletion of GATA-4 in mice results in fetal death between embryonic days 7.0 and 9.5, caused by malformations in cardiac morphogenesis (16). In vitro experiments using embryonic stem cells showed that GATA-4 is indispensable for cardioblast survival and for terminal cardiomyocyte differentiation (17, 18).

There has been much research work focusing on the relationship between the retinoids and the expression of GATA-4 in embryonic heart development (19). The expression of GATA-4 in the VAD embryo is diminished in the cardiac inflow tract-forming area, endocardium and myocardium (20, 21). The heart abnormality arising from knockout RAR or RXR embryos has a similar expression. RAR α -null mutants all display an agenesis of the compact layer of the ventricular myocardium (11), while RAR γ has a distinct role in cardiac asymmetry and looping information (22). However, there has been less research work on the expression of RARs and RXRs at the same time in VAD embryo heart development with the change of GATA-4 gene expression.

Here, we establish a VAD, a marginally VAD and a VA-supplementary rat model to examine the expression of RAR and RXR genes at the early stage of embryo heart development, and to detect the response of GATA-4 genes in embryo hearts at different stages and different maternal VA statuses. Finally, we observe the ultrastructural characters of embryo hearts in order to study the development of embryo hearts affected by maternal VA statuses.

MATERIALS AND METHODS

Animals and diets. The study was performed on 80 weanling female Sprague-Dawley rats, weighing 50 to 65 g, obtained from Slac Laboratory Animal, Shanghai, China. They were housed in stainless steel cages under standard conditions: temperature of $22 \pm 3^\circ\text{C}$, humidity of 55–65% and 12-h light-dark periods.

The diets were formulated to the American Institute of Nutrition 93 Growth Purified Diet (23). The VA completely deficient (VAD) diet had no VA (Table 1). The VA marginally deficient (VAM) diet had a concentration of 0.132 mg VA/kg diet and the VA supplement (VAS) diet contained 3.3 mg VA/kg diet based on the VAD diet. The control diet (VAN) was supplemented with 1.32 mg VA/kg diet.

After a 1-wk acclimatization, all female rats were divided into 3 groups randomly. The control group ($n=16$) received the VAN diet. The VAD group ($n=32$) and the VAM group ($n=32$) were fed with the VAD diet and VAM diet, separately. Both diet and water were available ad libitum. After 10 wk, females were mated

Table 1. The component of vitamin A deficiency fodder.

Component	Content (%)
Cornstarch	52.95
Casein(without vitamin A)	20.00
Glucose	10.00
Soybean oil	7.00
Fiber	5.00
AIN-93G vitamins ¹ (without vitamin A)	3.50
AIN-93G minerals ² (without vitamin A)	1.00
Methionine	0.30
Choline bitartrate	0.25

¹ AIN-93G vitamins (g/kg or IU/kg): nicotinic acid 3.000, calcium pantothenate 1.600, vitamin B₆ 0.700, vitamin B₁ 0.600, vitamin B₂ 0.600, folic acid 0.200, biotin 0.020, vitamin B₁₂ 2.500, vitamin E 7,500 IU, vitamin D₃ 100,000 IU, vitamin K 0.075, glucose 974.655.

² AIN-93G minerals (g/kg): KH₂PO₄ 196.00, K₃C₆H₅O₇·H₂O 70.78, NaCl 74.00, K₂SO₄ 46.60, MgO 24.00, FeC₆H₅O₇ 6.06, ZnCO₃ 1.65, MnCO₃ 0.63, CuCO₃ 0.30, KIO₃ 0.01, Na₂SeO₄ 0.01025, (NH₄)₂Mo₇O₂₄·4H₂O 0.00795, Na₂SiO₃·9H₂O 1.45, CrK(SO₄)₂·12H₂O 0.275, LiCl 0.0174, H₃BO₃ 0.0815, NaF 0.0635, NiCO₃ 0.0318, NH₄VO₃ 0.0066, glucose 578.026.

overnight (18:00–06:00) with a normal male, and the morning with a vaginal plug was defined as embryonic day 0.5 (E0.5).

Both the VAD group and the VAM group were again randomly subdivided into two groups. The diet of one subgroup in the VAD group changed from the VAD diet to the VAS diet at E0.5 (VADS group, $n=16$), and the other subgroup in the VAD group continued to be fed with the VAD diet (VAD group, $n=16$). There were also two subgroups in the VAM group: one subgroup was fed with the VAS diet at E0.5 (VAMS group, $n=16$), the other subgroup continued to be fed with the VAM diet (VAM group, $n=16$). Five maternal rats were randomly selected in each group at E13.5 and were anaesthetized by phenobarbitone. The embryo hearts were rapidly dissected out and every heart was split in two pieces under a stereomicroscope, and immediately frozen in liquid nitrogen and then stored at -80°C until analysis for GATA-4, RAR and RXR gene mRNA and protein expression. Another five maternal rats in each group were anaesthetized at E18.5 and two embryos were randomly taken out from every litter. Under a stereomicroscope, the two embryo hearts were rapidly dissected out and two pieces of $1 \times 1 \text{ mm}^3$ myocardium were cut out of the left ventricle. One piece of tissue was put into 4% glutaraldehyde for the ultrastructural character observation, and the other tissue was put into 10% formalin for study of GATA-4 gene immunohistochemistry expression. The remaining embryo hearts of every litter were rapidly dissected out and frozen in liquid nitrogen, and then stored at -80°C for analyzing GATA-4 gene expression. The rest of the maternal rats in each group were prepared in case of misdiagnosis of pregnancy. Maternal blood was collected through the

tail before copulation (70th day) and through the heart after taking out embryos. The experimental procedures used in this study were approved by the Animal Care Committee of Shanghai Jiao Tong University, School of Medicine, Shanghai, China, and animals were cared for according to the Guiding Principles in the Care and Use of Animals.

Serum retinol analysis. Serum was separated by centrifugation at 3,000 rpm for 10 min at 4°C and frozen at -80°C until analysis. Serum retinol was extracted with hexane and dried under a nitrogen stream and dissolved into methanol. The retinol was subjected to high-performance liquid chromatography (Agilent 1200LC, USA) with a reverse phase C18 column (5 mm particle size; Beckman Instruments, Inc.) and a mobile phase of methanol : water (95 : 5) at the flow rate of 1.0 mL/min. The ultraviolet detection was at 325 nm.

Assays of RAR and RXR gene expression at E13.5.

1. Real-time PCR assays of mRNA level: Ten embryo hearts in every group were tested for mRNA (two embryo hearts per litter, five litters per group). Total RNA of the embryo hearts was extracted using Trizol (Invitrogen) following by the manufacturer's instructions, and then stored at -80°C until use. The reaction mixture contained 5 µg total RNA diluted in diethylpyrocarbonate-treated water, 50 µmol/L oligo (dT) primer, 4 µL reaction buffer (5×, 250 mmol/L Tris-HCl, pH 8.3, at 25°C, 250 mmol/L KCl, 20 mmol/L MgCl₂, 50 mmol/L dithiothreitol), 1 µL 10 mmol/L dNTP mix, 40 U RNase Inhibitor (Invitrogen), and 200 U Super-Scrip III Reverse Transcriptase (Invitrogen). The final reaction volume was 20 µL. The reaction mixture was incubated at 50°C for 50 min, and the reverse transcription was terminated by heating the mixture at 85°C for 5 min. The cDNA reaction mixture was diluted in diethylpyrocarbonate-treated water (Invitrogen), 0.5 µL Power SYBR® Green PCR Master Mix (20×) (Invitrogen) and 2 µL gene-specific primers (final concentration: 50 nmol/L each), in a final reaction volume of 25 µL. Thermocycler conditions comprised an initial activation step at 95°C (2 min), followed by a 3-step PCR program of 95°C (10 s), 60°C (30 s) for 40 cycles and 72°C (30 s) for primer annealing and extension. mRNA expression was quantified by the 2^{-ΔΔCt} method. Each real-time PCR was performed three times to evaluate data reproducibility. The primers for the genes are showed in Table 2. β-Actin was used as a control.

2. Western blot assay of protein level: The other part of the ten samples in every group was tested for RAR and RXR gene protein expression by using western blot. The embryo heart tissues were firstly homogenized, and the total protein in homogenates was quantified with the bicinchoninic acid (BCA) protein assay kit (Shanghai KangChen Bio-tech Inc., Shanghai, China). 20 µg of the total protein was resolved in SDS-PAGE, and protein was transferred onto PVDF membranes (Milipore, Temecula, CA, USA) electrophoretically at 200 mA for 2 h at 4°C. The membranes were blocked in 5% bovine serum albumin (BSA) for 1 h at room temperature. After 3 washes with tris-buffered saline and tween (TBST), the mem-

Table 2. PCR primers used in this study.

Gene		Sequence (5' to 3')
GATA-4	Forward	GCAGCAGCAGCAGCAGTGAA
	Reverse	TCTGAGTGACAGGAGATGGATAGCC
RARα	Forward	GGCTTCTTCCGACGCAGTAT
	Reverse	TGCGCACCTTCTCGATGA
RARβ	Forward	GGCTTCGGTCTCTGACTGA
	Reverse	AGCGGCTCTTGGAGCTTGT
RARγ	Forward	CACGAACTCCAGCGCAGAT
	Reverse	AGGCGAAGACGAGGTCTGTAA
RXRα	Forward	CAAGCACATCTGCGCTATCTG
	Reverse	CCGCTGTCTCTTGTCTGATCA
RXRβ	Forward	CGAAGCTCAGGCAAGCACTA
	Reverse	CGCTTGCTCCACTGTGCAGTC
RXRγ	Forward	CACGAACTCCAGCGCAGAT
	Reverse	AGGCGAAGACGAGGTCTGTAA
Rat-actin β	Forward	TGCCTCTGGTCTGTAACCTG
	Reverse	GGCAACATAGCACAGCTTCTCT

Table 3. Serum vitamin A concentration of maternal rats (µmol/L).¹

Group	Before copulation (70th day)	After taking out embryos (E13.5)	After taking out embryos (E18.5)
VAN	1.65±0.10 (16)	1.62±0.10 (8)	1.53±0.10 (8)
VAD	0.05±0.01 (32)*	0.03±0.01 (5)*	0.02±0.01 (6)*
VADS	Same as above	1.29±0.07 (6)*	1.21±0.09 (5)*
VAM	0.70±0.08 (32)*	0.57±0.08 (7)*	0.57±0.07 (9)*
VAMS	Same as above	1.56±0.09 (8)	1.57±0.06 (8)

¹ Values are mean±SD.

The number of maternal rats is given in parenthesis.

**p*<0.01, compared with VAN group.

branes were incubated with a HRP-conjugated antibody (Shanghai KangChen Bio-tech Inc.) at a 1 : 10,000 dilution for 1 h at room temperature.

Bands on the membranes were imaged by X-ray with chemoluminescence reagents (Shanghai KangChen Bio-tech Inc.). Finally, the bands were scanned and quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). The blots were incubated with mouse monoclonal antibodies to RARα (1 : 1,000), RARβ (1 : 1,000), RARγ (1 : 1,000), RXRα (1 : 1,000), RXRβ (1 : 1,000), RXRγ (1 : 1,000), and GAPDH (1 : 10,000, as a control). All the antibodies were purchased from Santa Cruz Biotechnology except antibody RXRβ from Upstate Company.

Real-time PCR assays of GATA-4 mRNA level at E13.5 and E18.5. The method was the same as above. The samples from E13.5 were the same tissue for analyzing RAR and RXR gene expression. Ten embryo hearts at E18.5 in every group were determined (two embryo hearts per litter, five litters per group) for GATA-4 gene mRNA expression. Table 2 shows the primers for the GATA-4 gene. β-Actin was used as a control.

Table 4. The expression levels of RAR and RXR mRNA in E13.5 embryo hearts.¹

	RARs ($\times 10^3$)			RXRs ($\times 10^3$)		
	RAR α	RAR β	RAR γ	RXR α	RXR β	RXR γ
VAN	6.10 $\pm$ 0.54	2.01 $\pm$ 0.54	1.21 $\pm$ 0.08	2.58 $\pm$ 0.45	0.68 $\pm$ 0.18	0.99 $\pm$ 0.38
VAD	3.93 $\pm$ 0.77**	1.79 $\pm$ 0.10	0.36 $\pm$ 0.12*	16.61 $\pm$ 4.88*	15.45 $\pm$ 5.77*	2.44 $\pm$ 1.57
VADS	5.71 $\pm$ 0.92	4.05 $\pm$ 0.62**	1.50 $\pm$ 0.73	6.01 $\pm$ 1.70*	6.97 $\pm$ 1.82*	0.90 $\pm$ 0.01
VAM	6.23 $\pm$ 0.45	1.45 $\pm$ 0.48	0.43 $\pm$ 0.00*	4.85 $\pm$ 0.64**	3.92 $\pm$ 2.37**	3.85 $\pm$ 0.80**
VAMS	6.92 $\pm$ 1.02	3.05 $\pm$ 0.93	0.88 $\pm$ 0.18	4.70 $\pm$ 1.51	1.88 $\pm$ 0.31	2.71 $\pm$ 0.97

¹ Values are means $\pm$ SD.
The values represent the results of gene expression in 10 samples in each group (two embryo hearts per litter, five maternal rats in each group) for three replicates.
* p <0.01, ** p <0.05 compared with VAN group.

Fig. 1. Protein expression levels of RAR and RXR genes in E13.5 embryo heart tissues from five groups by Western blot analysis. Each group has 10 samples. The bars represent the mean ratio of RARs or RXRs to GAPDH for three replicate. Values are shown as means $\pm$ SE. * p <0.01, compared with VAN group.

Correlation analysis between maternal VA level and GATA-4 expression in embryo hearts. We made an analysis between maternal VA level and GATA-4 gene mRNA expression in E13.5 and E18.5 embryo hearts by using bivariate correlation statistics.

Ultrastructure and immunohistochemistry at E18.5. For transmission electron microscopy, the left ventricle tissues of E18.5 (about 1 mm³) were fixed in 4%

glutaraldehyde and were washed with PBS, fixed with osmic acid and washed again, and then embedded in VCD4206. Tissue sections were cut with an ultramicrotome in 40 nm, and then stained with uranyl acetate and lead citrate and examined and photographed using a transmission electron microscope (CM120 BioTWIN CRYO, Philip, Holland). The electron microscopy pictures were evaluated by two histologists with at least 10 y of experience.

The myocardium of the left ventricle at E18.5 was fixed in 10% buffered neutral formalin overnight at 4°C followed by paraffin embedding. Deparaffinized sections of tissue were dehydrated, and antigen retrieval was improved by microwave cooking for 20 min in 10 mmol/L citric acid, pH 6. Three grams per liter of H₂O₂ was used to inhibit the endogenous peroxidase reaction. The sections were then subjected to immunohistochemistry, using polyclonal mouse anti-rat GATA-4 antibodies (dilution 1 : 150; sc-25310; Santa Cruz Biotechnology Inc., Santa Cruz, CA) according to the manufacturer's instructions. All the slides were analyzed under a light microscope and reassessed by a pathologist. Sections were digitally photographed at 20× and 40× magnification.

Fig. 2. mRNA expression levels of GATA-4 gene at E13.5 and E18.5 embryo hearts. Each bar represents the average value of GATA-4 2^{-ΔΔCT} in 10 samples at different stages. Values are shown as means ± SE. **p* < 0.01, compared with VAN group.

A

B

Fig. 3. The relationship between the maternal vitamin A levels and GATA-4 expression in the offspring at E13.5 and E18.5. A: The concentrations of maternal VA were closely related to GATA-4 mRNA expression at E13.5 and E18.5 before conception ($R^2=0.8691$, $p=0.028$ at E13.5). B: No relationship was found between the maternal VA level and GATA-4 mRNA expression in offspring hearts after termination of pregnancy ($R^2=0.8282$, $p=0.024$ at E18.5).

A

B

Fig. 4. A: Transmission electron microscopy micrographs of embryo hearts at E18.5 in 5 groups ($\times 17,500$). In the VAD group, the muscle fibers exhibited a disoriented arrangement and lost normal structure. The arrows show that mitochondria were abnormal with matrix reduction, broken cristae and vacuoles. In the VAM group, the arrows indicate muscle broken fiber and mitochondrial cristae reduction. Arrows in the VADS figure refer to the irregular muscle fibers, while the muscle fibers exhibit a relative normality in the VAMS group. B: GATA-4 expression in left ventricle tissues of E18.5 in 5 groups. Immunohistochemistry exhibited brown staining in the cell nucleus. The sections in the bigger square were digitally photographed at $20\times$ magnification. The sections in the smaller square were at $40\times$ magnification. C: Each group contains 10 embryonic heart tissues. Count GATA-4 positive staining in left ventricle tissues of E18.5 in 5 groups within $1\times 1\text{ cm}^2$. Values are shown as means $\pm$ SE. * $p < 0.01$, ** $p < 0.05$ compared with VAN group.

Statistical analysis. The SPSS17.0 software package was used for statistical analysis. Data were presented as mean $\pm$ SD. One-way ANOVA was conducted on statistical comparisons of measurement data, while the χ^2 test and Fisher's exact probability test were used for numeration data. For real-time PCR data, the ΔCt was normalized to the control values by calculating $2^{-\Delta\Delta\text{Ct}}$. One-way ANOVA was conducted on the $2^{-\Delta\Delta\text{Ct}}$ values. Correlation analysis was used for bivariate correlation statistics. $p < 0.05$ or $p < 0.01$ was considered statistically significant.

RESULTS

Vitamin A concentration of maternal rats

From the fifth and sixth week of depletion of the VA diet, the maternal rats on the VAD diet showed all kinds of the VAD symptoms, such as hairloss, wizened hair, abnormal secretion in the eyes, and irritation in response to external stimuli, while the maternal rats fed the VAM diet exhibited frequent movements and xerosis cutis, without classical VAD symptoms. No maternal rats died during the 10 wk. Twenty-two out of 32 (68.8%) VAD group rats conceived, while all of the rats in the VAN group and VAM group conceived. Before copulation (70th day), the serum VA concentration of the VAD group and VAM group was lower than that of the VAN group ($p < 0.01$). After taking out the embryos (on E13.5 and E18.5), the serum VA concentrations of the VAD group, VAM group, and VADS group were still less than that of the VAN group while the VAMS group resumed normal levels. The VA concentration of maternal rats is shown in Table 3.

RAR and RXR gene expression in E13.5 embryo hearts

Here we detected both RAR and RXR expression at mRNA and their protein levels in E13.5 embryo hearts (Table 4 and Fig. 1). The data showed that the

VAD diet significantly decreased the expression levels of RAR α and RAR γ mRNA and protein, while it markedly increased the expression levels of RXR α and RXR β mRNA and protein in E13.5 embryo hearts. In the VAM group, the mRNA and protein levels of RAR γ were suppressed compared with the VAN group. However, all of the RXRs in the VAM group exhibited up-regulation both at mRNA and protein levels. In the VADS group, the suppression of all the RARs was improved and all RXR expression levels were reduced compared to the VAD group. The expression of RAR β in the VADS group was lower than that of the VAN group, while RXR α and RXR β levels were rather higher compared to the VAN group. The expression levels of RARs and RXRs in the VAMS group were similar to those of the VAN group through VA supplementation in the VAMS group.

Expression levels of GATA-4 gene in E13.5 and E18.5 embryo hearts

Real-time PCR was used to quantitate GATA-4 gene expression at E13.5 and E18.5, and the mRNA relative expression level which was quantified by the $2^{-\Delta\Delta\text{Ct}}$ method is shown in Fig. 2. The mRNA expression in the embryo hearts of the VAD group was the lowest among the 5 groups. Meanwhile, the embryo hearts from the VADS, VAM, and VAMS groups also exhibited moderately decreased GATA-4 gene expression compared to the VAN group. The expressions of the GATA-4 gene on E13.5 and E18.5 were improved in the two VA supplementary groups.

Relationship between maternal VA level and GATA-4 mRNA expression

The data showed that the concentrations of maternal VA were closely related to GATA-4 mRNA expression at E13.5 and E18.5 before conception ($R^2 = 0.8691$, $p = 0.028$ at E13.5. $R^2 = 0.8282$, $p = 0.024$ at E18.5). However, no relationship between the maternal VA level

and GATA-4 mRNA expression in offspring hearts was found after termination of pregnancy (Fig. 3).

Ultrastructure and immunohistochemistry at E18.5 embryo hearts

In the VAN group, cardiac muscle fiber showed a regular arrangement: Z-bands were equal and clear, and mitochondria were distributed beneath the sarcolemma or in the field of myofibrils. However, the muscle fibers exhibited a disoriented arrangement in the VAD group, and lost normal structure. Mitochondria were abnormal with matrix reduction, broken cristae and vacuoles. We also found muscle broken fibers and mitochondrial cristae reduction in the VAM group. The muscle fibers showed an irregularity in the VADS group and a relative normality in the VAMS group. We observed GATA-4 gene immunohistochemistry in a $1 \times 1 \text{ cm}^2$ sample. Immunohistochemistry performed on left ventricle tissues of E18.5 exhibited brown staining in the cell nucleus. We found by macrography that the expression of GATA-4 was less in the VAD group. Counting by positive staining, we found that the expression of GATA-4 in the VAD group was significantly less than that of the VAN group ($p < 0.01$). The expression of the GATA-4 gene in the two supplementation groups was improved compared with the VAD and VAM group, while the expression of GATA-4 in the VADS and VAM groups was also decreased compared to the VAN group ($p < 0.05$) (Fig. 4).

DISCUSSION

In our study, we showed that the embryo hearts from vitamin A insufficient groups exhibited ultrastructural changes and significantly lower expression of GATA-4, RAR α and γ , and higher expression of RXR α and β . GATA-4 gene expression in the offspring's ventricular cardiomyocyte was relevant to their maternal serum VA levels, while the results of the vitamin A supplemented groups were improved.

Aberrant heart development in embryos from maternal deficiency of VA during pregnancy was observed in the 1930s–1950s (24–26). The action of retinoids, which are mediated by two distantly related families of nuclear retinoid receptors, RARs and RXRs, has been implicated in controlling cell proliferation, differentiation and apoptosis (11, 19, 27, 28), including involvement in cardiac development and function (11, 21, 29). These studies show that RAR α is almost ubiquitously expressed in embryos at all stages, while the expressions of RXR α and RXR β are highest at E11.5–12.5 (30, 31). RA differentially up-regulates the gene expression of RAR α 2 and γ 2 in E11.5 embryos and adult rats (32). However, RAR β , RAR γ , and RXR γ exhibited more restricted expression patterns. GATA-4 was firstly identified as one of the key regulators of cardiac development (33, 34). In previous studies, VAD quail embryos showed obviously decreased levels of GATA-4 expression coincident with aberrant cardiovascular development, especially failure in the inflow tract and posterior heart tube development (19, 35).

In the present study, we found that the expressions

of RAR α and RAR γ were lower and the levels of RXR α and RXR β were much higher in the embryo hearts of the VAD group, and the embryo hearts from the VAM group exhibited less RAR γ expression with up-regulation of three RXR genes at E13.5. Overexpression of RXR α results in induction of cellular differentiation and control of aberrant cell growth (36). Here we speculated that such aberrant expression of RARs and RXRs at the early stage of heart development may result in abnormal appearances such as a disoriented arrangement of cardiac muscle fibers, unclear Z-bands, broken cardiac muscle fibers, and decreased expression of GATA-4 in left ventricle tissues for the VAD and VAM groups at E18.5. Noteworthy is the expression of RXR α in the VAD group at E13.5, which was much higher than that of the VAN group. Many other studies showed that the mutation of RXR α can lead to cardiac malformation and embryonic lethality (21, 37, 38). We recognized that a significantly high level of RXR α and RXR β expression in the VAD group was a compensation mechanism for a low level of RAR α expression because RXRs most probably served as heterodimeric partners for RARs (11, 39), and RARs may serve as “buffered receptors” to trap retinoic acid by changing their levels (32).

Another interesting finding in our study was the relationship between GATA-4 gene expression in the offspring's ventricular cardiomyocyte and their maternal serum VA levels. The data showed a positive relationship between the VA concentration of maternal rats before conception and GATA-4 mRNA expression in the offspring hearts at E13.5 and E18.5. In our study, the count of GATA-4 positive staining in left ventricle tissues at E18.5 significantly decreased in vitamin A insufficient groups. The myocardium is manifest as a critically thinned ventricular wall in several retinoid receptor knockouts (21, 37), and this phenotype is identical to a subset of the effects of embryonic VAD (37). The expression of GATA-4 is also obviously decreased in the heart-forming regions of a VAD embryo (40). Numerous observations strongly suggest the participation of retinoids via RARs and RXRs in the transcriptional regulation of the GATA genes during cardiogenesis. The supplementation of VA to the VAD diet restored GATA-4 expression in the VADS and VAMS groups, and even could rescue the vitamin A-deficient phenotype. The expression levels of GATA-4 in the VAM and VAMS groups were moderately increased between E13.5 and E18.5, although the expression level of GATA-4 in the VAD group and VADS group did not undergo any temporal change during this period.

VAD still remains a significant public health problem at the global level, especially in developing countries, hitting hardest young children and pregnant women. Maternal VAD, which lacks adequate attention, puts the rapidly developing fetus at a high risk of subclinical deficiency, resulting in resorptions, abortions, and congenital anomalies (41, 42). In our study, the expression of GATA-4 increased and the ultrastructure improved in the embryo hearts which were from the VAMS group after administration of enough VA (3.3 mg VA/kg diet).

Meanwhile, the expressions of RARs and RXRs in the VAMS group at E13.5 were similar to those of the VAN group. Both RXR α and RXR β expression in the VADS group exhibited a slight increase compared to that of VAN group, while they were significantly higher in the VAD group compared with the VAN group. The supplement of VA at the early stage of embryo development, which is the critical VA requirement period of heart formation, led to improved expressions of RARs and RXRs.

In summary, in this study, we show a down-regulated effect of RARs, and an up-regulation effect of RXR and GATA-4 gene coexpression with VAD. Maternal insufficiency of VA during pregnancy will down-regulate the expression of the RARs and up-regulate the expression of RXRs in the offspring hearts, while suppressing the expression of GATA-4.

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REFERENCES

- 1) World Health Organization. Nutrition. Micronutrient deficiencies. Vitamin A deficiency. [Online]. Available: <http://www.who.int/nutrition/topics/vad/en/> [accessed June 27, 2012]
- 2) Blomhoff R. 1994. Overview of vitamin A metabolism and function. In: Vitamin A in Health and Disease (Blomhoff R, Marcel Dekker, eds), p 1–35. Raven Press, New York.
- 3) Zile MH. 1998. Vitamin A and embryonic development: an overview. *J Nutr* **128**: 455s–458s.
- 4) Ross SA, McCaffery PJ, Dräger UC, De Luca LM. 2000. Retinoids in embryonal development. *Physiol Rev* **80**: 1021–1054.
- 5) Mark M, Ghyselinck NB, Chambon P. 2009. Function of retinoic acid receptors during embryonic development. *Nucl Recept Signal* **7**: e002.
- 6) Merki E, Zamora M, Raya A, Kawakami Y, Wang J, Zhang X, Burch J, Kubalak SW, Kaliman P, Belmonte JC, Chien KR, Ruiz-Lozano P. 2005. Epicardial retinoid X receptor α is required for myocardial growth and coronary artery formation. *Proc Natl Acad Sci USA* **102**: 18455–18460.
- 7) Giguère V. 1994. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. *Endocr Rev* **15**: 61–79.
- 8) Halilabac A, Ribes V, Ghyselinck NB, Zile MH, Dollé P, Studer M. 2007. Retinoids control anterior and dorsal properties in the developing forebrain. *Dev Biol* **303**: 362–375.
- 9) Ruberte E, Dollé P, Krust A, Zelent A, Morriss-Kay G, Chambon P. 1990. Specific spatial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. *Development* **108**: 213–222.
- 10) Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong AE, Kakizuka A, Evans RM. 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev* **6**: 329–344.
- 11) Kastner P, Messaddeq N, Mark M, Wendling O, Grondona JM, Ward S, Ghyselinck N, Chambon P. 1997. Vitamin A deficiency and mutation of RXR α , RXR β and RAR α lead to early differentiation of embryonic ventricular cardiomyocytes. *Development* **124**: 4749–4758.
- 12) Heikinheimo M, Scandrett JM, Wilson DB. 1994. Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev Biol* **164**: 361–373.
- 13) Arceci RJ, King AA, Simon MC, Orkin SH, Wilson DB. 1993. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol* **13**: 2235–2246.
- 14) Laverriere AC, MacNeill C, Mueller C, Poelmann RE, Burch JB, Evans T. 1994. GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J Biol Chem* **269**: 23177–23184.
- 15) Nemer G, Nemer M. 2003. Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. *Dev Biol* **254**: 131–148.
- 16) Molkentin JD, Lin Q, Duncan SA, Olson EN. 1997. Requirement of the transcription factor GATA-4 for heart tube formation and ventral morphogenesis. *Genes Dev* **11**: 1061–1072.
- 17) Grépin C, Robitaille L, Antakly T, Nemer M. 1995. Inhibition of transcription factor GATA-4 expression blocks in vitro cardiac muscle differentiation. *Mol Cell Biol* **15**: 4095–4102.
- 18) Grépin C, Nemer G, Nemer M. 1997. Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* **124**: 2387–2395.
- 19) Zile MH. 2004. Vitamin A requirement for early cardiovascular morphogenesis specification in the vertebrate embryo: insights from the avian embryo. *Exp Biol Med* **229**: 598–606.
- 20) Kostetskii I, Jiang Y, Kostetskii E, Yuan S, Evans S, Zile MH. 1999. Retinoid signaling required for normal heart development regulates GATA-4 in a pathway distinct from cardiomyocyte differentiation. *Dev Biol* **206**: 206–218.
- 21) Clark KL, Yutzev KE, Benson DW. 2005. Transcription factors and congenital heart defects. *Annu Rev Physiol* **68**: 97–121.
- 22) Romeih M, Cui J, Michaille JJ, Jiang W, Zile MH. 2003. Function of RAR γ and RAR α 2 at the initiation of retinoid signaling is essential for avian embryo survival and for distinct events in cardiac morphogenesis. *Dev Dyn* **228**: 697–708.
- 23) Reeves, PG, Nielsen FH, Fahey JGC. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**: 1939–1951.
- 24) Hale F. 1937. Relation of maternal vitamin A deficiency to microphthalmia in pigs. *J Med Texas State* **33**: 228–232.
- 25) Wilson JG, Warkany J. 1949. Aortic arch and cardiac anomalies in the offspring of vitamin A deficient rat. *Am J Anat* **85**: 113–155.
- 26) Wilson JG, Roth CB, Warkany J. 1953. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. *Am J Anat* **92**: 189–217.
- 27) Ghatpande S, Ghatpande A, Zile M, Evans T. 2000.

- Anterior endoderm is sufficient to rescue foregut apoptosis and heart tube morphogenesis in an embryo lacking retinoic acid. *Dev Biol* **219**: 59–70.
- 28) Collop AH, Broomfield JA, Chandraratna RA, Yong Z, Deimling SJ, Kolker SJ, Weeks DL, Drysdale TA. 2006. Retinoic acid signaling is essential for formation of the heart tube in *Xenopus*. *Dev Biol* **129**: 96–109.
 - 29) Hoover LL, Burton EG, Brooks BA, Kubalak SW. 2008. The expanding role for retinoid signaling in heart development. *The Scientific World Journal* **25**: 194–211.
 - 30) Dolle P, Fraulob V, Kastner P, Chambon P. 1994. Development expression of murine retinoid X receptor (RXR) genes. *Mech Dev* **45**: 91–104.
 - 31) Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM. 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoid acid. *Genes Dev* **6**: 329–344.
 - 32) Takeyama K, Kojiam R, Ohashi R, Sato T, Mano H, Masushige S, Kato S. 1996. Retinoic acid differentially up-regulates the gene expression of retinoic acid receptor α and γ isoforms in embryo and adult rats. *Biochem Biophys Res Commun* **222**: 395–400.
 - 33) Kelley C, Blumberg H, Zon LI, Evans T. 1993. GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* **118**: 817–827.
 - 34) Arcenci RJ, King AA, Simon MC, Orkin SH, Wilson DB. 1993. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol* **13**: 2235–2246.
 - 35) Kostetskii I, Yuan SY, Kostetskaia E, Linask KK, Blanchet S, Seleiro E, Michaille JJ, Brickell P, Zile M. 1998. Initial retinoid requirement for early avian development coincides with retinoid receptor coexpression in the pre-cardiac fields and induction of normal cardiovascular development. *Dev Dyn* **213**: 188–198.
 - 36) Tanaka T, Suh KS, Lo AM, De Luca LM. 2007. P21WAF1/CIP1 is a common transcriptional target of retinoid receptors: pleiotropic regulatory mechanism through retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimer and RXR/RXR homodimer. *J Biol Chem* **282**: 29987–29997.
 - 37) Sucov HM, Dyson E, Gumeringer CL, Price J, Chien KR, Evans RM. 1994. RXR α mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev* **8**: 1007–1018.
 - 38) Gruber PJ, Kubalak SW, Pexieder T, Sucov HM, Evans RM, Chien KR. 1996. RXR α deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J Clin Invest* **98**: 1332–1343.
 - 39) Maden M. 2001. Vitamin A and the developing embryo. *Postgrad Med J* **77**: 489–491.
 - 40) Grépin C, Dagnino L, Robitaille L, Haberstroh L, Antakly T, Nemer M. 1994. A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol Cell Biol* **14**: 3115–3129.
 - 41) Miller M, Humphrey J, Johnson E, Marinda E, Brookmeyer R, Katz J. 2002. Why do children become vitamin A deficient? *J Nutr* **132** (9 Suppl): 2867S–2880S.
 - 42) Underwood BA. 1994. Maternal vitamin A status and its importance in infancy and early childhood. *Am J Clin Nutr* **59** (Suppl): 517S–524S.