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Original Article

Methanal results in heart malformations by dysfunction of the β_2-spectrin-based pathway

Zhirong Jiang, Yu Tian, Xiaofan Wang, Qifeng Lv, Xiaoxia Song

Cardiac Ultrasound, Qingdao University Hospital, Qingdao 266000, China

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Abstract: Methanal (formaldehyde gas) has been regarded as potential health risk resulting from widespread environmental exposure. Evidence has shown that methanal can lead to heart malformations by hindering heart development. However, the potential mechanism of methanal-induced heart malformations has not yet been understood. In this study, we explored the possible mechanism of heart malformations induced by methanal in pregnant rats. Here, we report that β_2-spectrin and TBX5 protein levels are significantly altered in human cardiovascular malformations. We show that methanol induced heart dysmorphogenesis during pregnancy in rats. Expression levels of β_2-spectrin were decreased in the ventricle and atria tissues in pregnant rats treated with methanal compared to the control group. Methanal exposure produced a significant depletion of glutathione (GSH) in both the embryo and VYS. Inhibition of β_2-spectrin synthesis decreased GSH synthesis and exacerbated methanal embryotoxicity. Mechanistically, we show that methanol decreased TBX5 and induced calpain-dependent loss of β_2-spectrin downstream effector proteins, including ankyrin-B in the heart. In conclusion, these findings illustrate a role for methanal in heart malformations through regulation of the β_2-spectrin-based pathway.

Keywords: Methanal, heart malformations, β_2-spectrin, TBX5

Introduction

Methanal (CH_2O) is colorless gas with a particularly pungent smell, which has been regarded as a noxious gas [1]. A previous study evaluated the harmful effects of exposure to formaldehyde and results of the survey indicated that experimental animals exposed to formaldehyde vapor (1.2 mg/m^3) reached the threshold limit value [2]. The report indicated that the greatest gain would thus be obtained by respecting the current occupational exposure limit which is currently set at 0.75 ppm. This level can be considered as safe for virtually all workers [3]. Additionally, the effects of methanal on model development, experimental evaluation, and the impact of environmental factors has been investigated and provide a powerful tool to evaluate the performance of the chamber testing systems [4]. Currently, methanal is an economically important chemical but it is classified as a human carcinogen that causes nasopharyngeal cancer, and probably leukemia [5].

In recent years, the effects of methanol on fetal development have generated great concern for developmental biology [6]. Interestingly, methanal caused increased malformation, while embryos treated with HCOONa/BSO did not have any developmental deformities [7]. A previous study has implicated the harmful effects of methanal on generation of embryotoxic metabolites, dysmorphogenesis, alterations of normal growth parameters, and embryonic lethality [8]. However, the effects of methanal on heart malformations have not yet been well elaborated.

β_2-Spectrin is one of the critical integral membrane proteins and the cytoskeletal domain proteins are expressed in excitable and nonexcitable cells [9]. A study has showed that β_2-spectrin defects associated with protein partner ankyrin-B were identified in congenital forms of human arrhythmia [10]. Importantly, Lim et al. indicated that loss of β_2-Spectrin prevents cardiomyocyte differentiation and heart development and data demonstrated that

Table 1. Primers for RT-qPCR

Gene Name	Sequence	
	Reverse	Forward
β_2-spectrin	5'-CCCTGAATGGTTTTACTCCACTGC-3'	5'-GGCCAGACTCTGTTATAGCTTGG-3'
TBX5	5'-GAGACAGCTTTTATCGCTGTG-3'	5'-CATCGCTGCCCCGGAATCCCT-3'
GSH	5'-TTGTAAACATCAGGGGCAAA-3'	5'-ATGGGCCAAGATCTTTCTGTAA-3'
Dystrophin	5'-GAGTCGCCTCTATGGAAAAGCA-3'	5'-GGTCAGATAAGTACTTGGCACGTAA-3'
F-actin	5'-GCTAAGAAGGCGATCA-3'	5'-AGAATGAGGACTGGGTG-3'
α-SMA	5'-TGTGCTGGACTCTGGAGATG-3'	5'-GATCACCTGCCCATCAGG-3'
β-actin	5'-CAAGAGATGGCCACGGCTGCT-3'	5'-TCCTTCTGCATCCTGTCGGCA-3'

β_2-Spectrin is a potential underlying cause of congenital heart defects [11]. However, the relationship between methanal and β_2-Spectrin is not well understood.

TBX5 is a key regulator of heart development, and a study has demonstrated integral roles for TBX5 throughout cardiac development and in understanding human cardiac morphology and function [12]. Ghosh et al. have identified functional interaction between a T-box protein and a MADS box factor, which may be crucial in cardiomyocyte differentiation [13]. Notably, differential regulation of TBX5 protein expression and sub-cellular localization during heart development contributed to better understanding of the molecular basis of hand/heart birth defects [14]. However, the associations between methanal and Tbx5 protein expression have not been investigated.

Therefore, in this study we investigated the possible signaling pathway involved in methanal-induced heart malformations. Using experimental animals, we found that methanal could induce heart malformations resembling human cardiovascular malformations, through alterations in β_2-spectrin and TBX5 protein levels. Findings in the current study illustrate that methanal can induce heart malformations by regulation of the β_2-spectrin-based pathway.

Materials and methods

Ethical statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Qingdao University Hospital. The protocol was approved by Chinese Association for Laboratory Animal Operation. All surgery and euthanasia were performed under sodium pentobarbital anesthesia (40 mg/kg).

Animal study

Pregnant female Sprague-Dawley rats (n = 6 in per group, gestational ages: E6) were purchased from Shanghai Slack experimental animals Co., LTD (Slack, Shanghai, China). Rats were settled in the environment of methanal (CH_2O, 5.8 mg/kg) or air for a total of 10 days. Experimental rats were sacrificed with 100 mg/kg i.p. sodium pentobarbital (Invitrogen, CA, USA) and the embryos obtained at E16 gestational ages described previously [15]. The embryos were then prepared for observation of heart malformations.

Quantitative real time PCR (qRT-PCR) analysis

Total RNA was extracted from ventricle and atria tissues by using RNAeasy Mini Kit (QIAGEN, Gaithersburg, MD). Expressions levels of β_2-spectrin, TBX5, dystrophin, F-actin, α-SMA, ankyrin-B were measured by qRT-PCR with β-actin as an endogenous control [16] (Invitrogen, CA, USA). All the forward and reverse primers were synthesized by Invitrogen (**Table 1**). The PCR thermocycler conditions were as follows: 95°C for 5 min, then 35 cycles of 95°C for 20 sec, 56.2°C for 20 sec and 72°C for 20 sec, and a final extension at 72°C for 5 min. Relative mRNA expression changes were calculated by $2^{-\Delta\Delta Ct}$ [17]. The results are expressed as the n-fold way compared to control.

Western blotting

Cells from the ventricle and atria tissues were homogenized in lysate buffer containing pr-

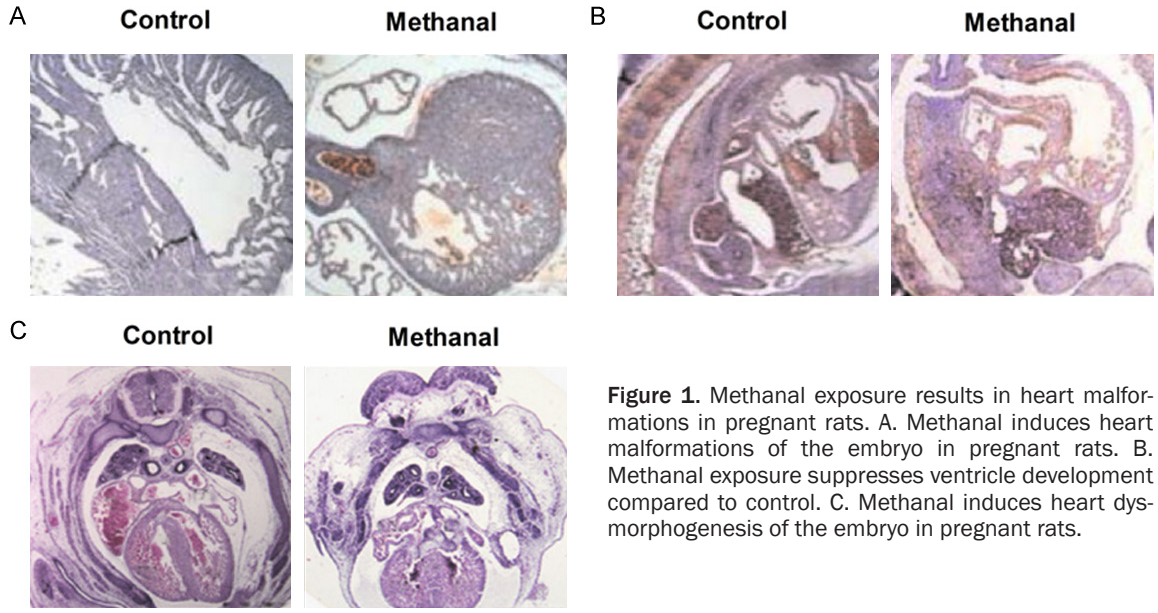

Figure 1. Methanal exposure results in heart malformations in pregnant rats. A. Methanal induces heart malformations of the embryo in pregnant rats. B. Methanal exposure suppresses ventricle development compared to control. C. Methanal induces heart dysmorphogenesis of the embryo in pregnant rats.

otease inhibitor and centrifuged at 8000× g at 4°C for 10 min. SDS assays were performed as described previously [18]. Subsequently, rabbit anti-rat primary antibodies: β_2-spectrin (1:1000, ab692, Abcam), TBX5 (1:1000, ab32503, Abcam), and β-actin (1:1000, ab5694, Abcam) were added to membranes after blocking in 5% skim milk for 1 h at 37°C. Membranes were then incubated with HRP-conjugated goat anti-rabbit IgG mAb (PV-6001, ZSGB-BIO, Beijing, China) secondary antibodies for 24 h at 4°C. A Ventana Benchmark automated staining system was used for analyzing protein expression (Olympus BX-51, Olympus; Tokyo, Japan).

Knockdown of β_2-spectrin

Myocardial cells were isolated from embryos as previously reported [19] and cultured in cultured in MEM (Sigma-Aldrich) medium (Gibco, CA, USA) supplemented with 10% fetal calf serum. The siRNA targeting for the β_2-spectrin gene sequence was designed and synthesized by Invitrogen (Invitrogen, CA, USA). The siRNA oligonucleotides were the following sequences: β_2-spectrin, 5'-ACCATCTGGCAGGAGCTGA-3', with the sequence 5'-ACGTAGATCCTTCAGCACC-3' was designed as negative control. Both siRNA-β_2-spectrin or siRNA-vector were transfected into myocardial cells using lipofectamine 2000 (Sigma-Aldrich) according to manufacturers' instructions [20].

Transmission electron microscopy

The embryos from experimental rats (n = 4 in each group) were used for transmission electron microscopy analyses as described previously [21]. Briefly, embryos were fixed in 5% paraformaldehyde and 4% glutaraldehyde in phosphate buffer at 4°C for 12 h. The embryonic heart was then cut into small 4 μm-thick tissue samples. The tissue sections were prepared from the ventricle and atria from two rats groups. The tissues were then post-fixed in 1% OsO_4 in 0.1 M phosphate buffer at 37°C for 1 h, dehydrated in a graded series of ethanol. Tissues were subsequently embedded in Araldite and double stained with lead citrate and uranyl acetate. The tissues were viewed under a Philips CM120 electron microscope.

Histology

Embryonic tissues were stained with hematoxylin and eosin for 2 h at 37°C. Tissues were viewed with a light microscope (Olympus BX-51, Olympus, Japan).

Statistical analysis

Data are presented as mean ± SD of triplicate. All data were analyzed by SPSS 13.0 software (SPSS, Chicago, IL, USA). Comparison between groups was assessed by Student's *t* test or one-way analysis of variance (ANOVA).

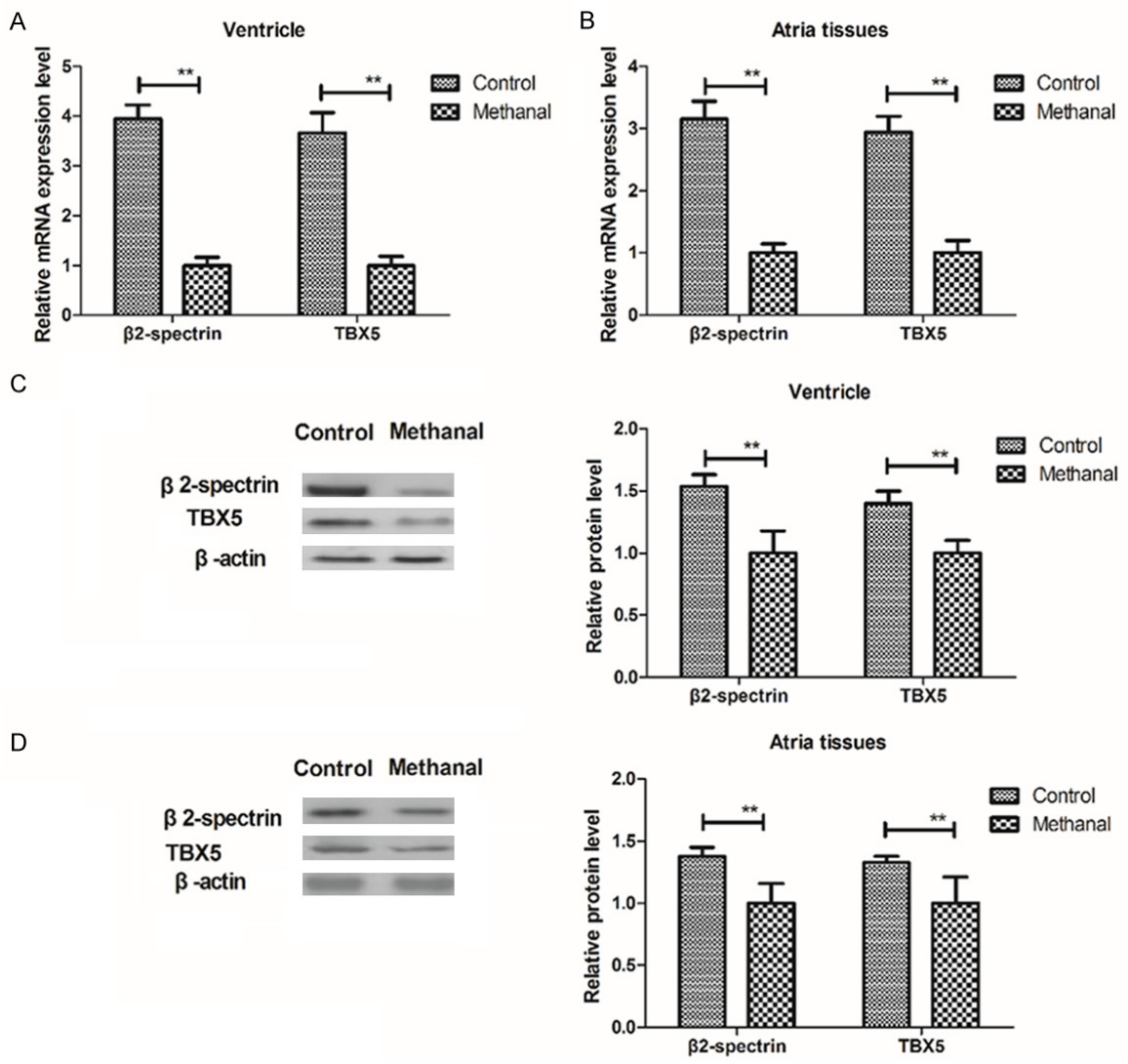

Figure 2. Methanal regulates β_2-spectrin and TBX5 expression in ventricle and atria tissues in the embryo of pregnant rats. (A, B) Methanal decreases gene expression levels of β_2-spectrin (A) and TBX5 (B) in ventricle and atria tissues in the embryo. (C, D) Methanal decreases protein expression levels of β_2-spectrin (C) and TBX5 (D) in ventricle and atria tissues in the embryo.

A *P*-value of <0.05 and <0.01 was considered to indicate a statistically significant result.

Results

Methanal leads to embryo heart malformations in pregnant rats

We first analyzed the effects of methanal on embryo heart development in pregnant rats. As shown in **Figure 1A**, methanal could induce embryo heart malformations. Histological analyses demonstrated that methanal suppressed ventricle development compared to control (**Figure 1B**). We showed that methanol induced heart dysmorphogenesis (**Figure 1C**). These results suggest that methanol exposure can result in heart malformations in pregnant rats.

Methanal regulated β_2-spectrin and TBX5 expression in ventricle and atria tissues in the embryos of pregnant rats

Reports have indicated that β_2-spectrin and TBX5 play essential roles heart malformations [22, 23]. We report that gene expression levels of β_2-spectrin and TBX5 were decreased in

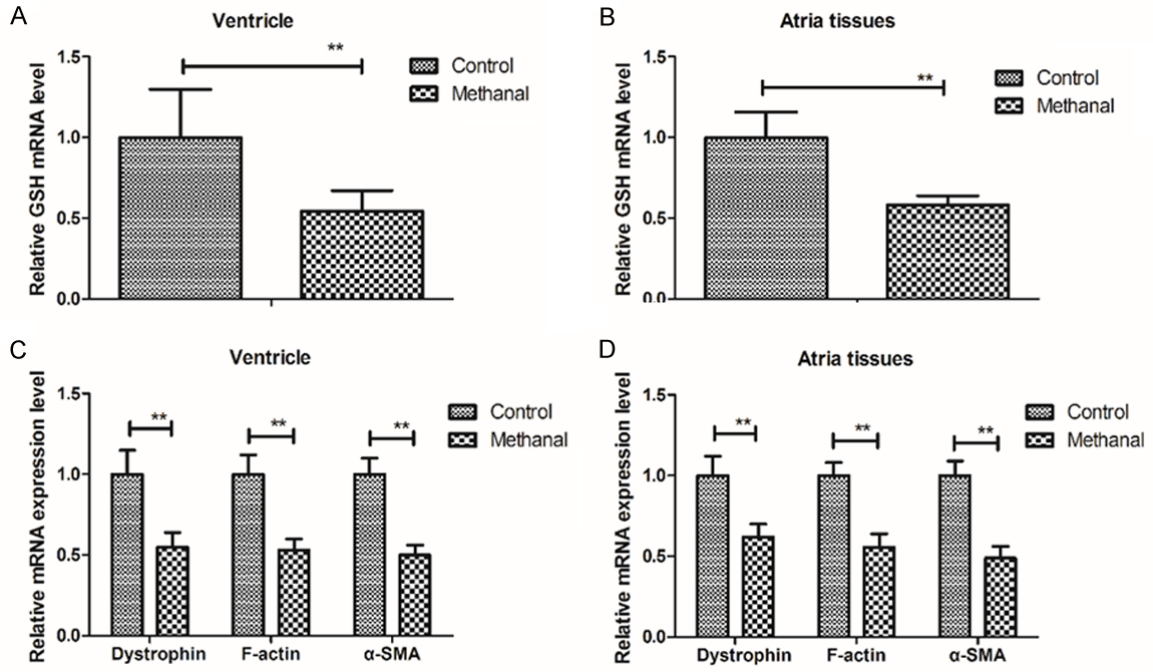

Figure 3. Methanal exposure produces a significant depletion of GSH and cytoskeletal networks in ventricle and atria tissues in the embryo of pregnant rats. (A, B) Methanal exposure decreases GSH gene expression in ventricle (A) and atria tissues (B) in the embryo of pregnant rats. (C, D) Methanal exposure downregulates gene expression of dystrophin, F-actin, and α-SMA in ventricle (C) and atria tissues (D) in the embryo of pregnant rats.

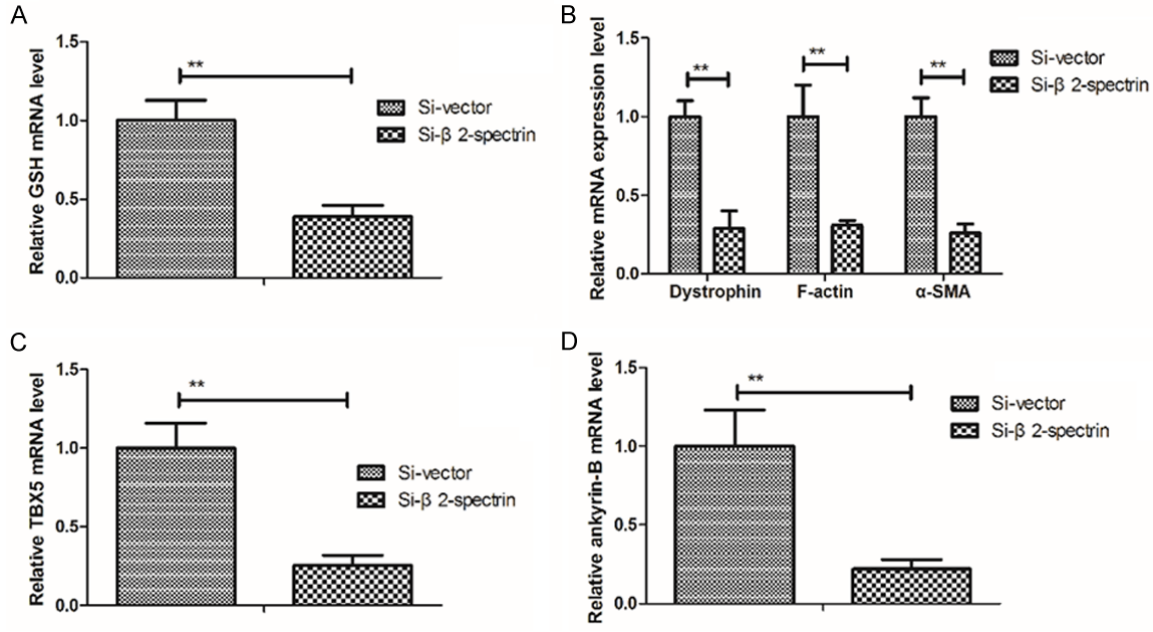

Figure 4. Methanal induces heart malformations by dysfunction of the β_2-spectrin-based pathway. A. Inhibition of β_2-spectrin synthesis decreases GSH synthesis. B. Inhibition of β_2-spectrin synthesis decreases the cytoskeletal networks of dystrophin, F-actin, and α-SMA in myocardial cells. C. β_2-spectrin knockdown decreases TBX5 in myocardial cells. D. β_2-spectrin knockdown decreases calpain-dependent loss of β_2-spectrin downstream effector ankyrin-B in myocardial cells.

the ventricle and atria tissues in newborn rats (**Figure 2A**, **2B**). Western blot demonstrated that protein expression levels of β_2-spectrin and TBX5 were decreased in ventricle and at-

ria tissues in the embryo (**Figure 2C**, **2D**). These results suggest that methanal downregulated β_2-spectrin and TBX5 expression.

Methanal exposure produced a significant depletion of GSH and cytoskeletal networks in ventricle and atria tissues from embryos of pregnancy rats

Previous study has indicated that cytoskeletal networks play a crucial role in the heart development. Therefore, we analyzed the GSH and cytoskeletal networks of dystrophin, F-actin, and α-SMA in ventricle and atria tissues. As shown in **Figure 3A**, **3B**, methanal exposure decreased GSH gene expression in ventricle and atria tissues. We demonstrated that methanal down-regulated gene expression of dystrophin, F-actin, and α-SMA in ventricle and atria tissues (**Figure 3C**, **3D**). Taken together, these results indicate that methanal exposure produced a significant depletion of GSH and cytoskeletal networks in ventricle and atria tissues in embryos of pregnant rats.

Methanal induced heart malformations by dysfunction of the β_2-spectrin-based pathway

Finally, we investigated the possible mechanism of heart malformations mediated by methanal. We found that inhibiti-on of β_2-spectrin synthesis decreased GSH synthesis and cytoskeletal networks of dystrophin, F-actin, and α-SMA in myocardial cells (**Figure 4A**, **4B**). Mechanistically, we identified that β_2-spectrin knockdown decreased TBX5 and calpain-dependent loss of β_2-spectrin downstream effector ankyrin-B in myocardial cells (**Figure 4C**, **4D**). Collectively, these results indicate that methanal exposure induces heart malformations by dysfunction of the β_2-spectrin-based pathway.

Discussion

Evidence supports a link between methanal exposure and increased risk of congenital heart malformations [24]. In addition, data demonstrate that loss of β_2-spectrin can prevent cardiomyocyte differentiation and impair heart development [11]. Furthermore, developmental biology studies have explained the physical interaction between TBX5 and early heart development and indicate that TBX5 is a key regulator of heart development [25]. In this study, we are the first to investigate the potential mechanism of methanal-induced heart malformations. Here, we report that methanal can induce heart malformations by dysfunction of the β_2-spectrin-based pathway.

In a preliminary study, methanal exposure decreased antioxidant enzyme activities and lipid peroxidation products in heart tissue of subacute and subchronic formaldehyde exposed rats [26]. We demonstrated that methanal exposure decreased GSH gene expression in ventricle and atria tissues, which may inhibit the metabolism of myocardial cells and further lead to heart malformations [27]. Notably, findings in the current study indicate that methanal exposure down-regulated gene expression of dystrophin, F-actin, and alpha-SMA in ventricle and atria tissues, which disturbed the development of cardiomyocytes.

TBX5 is reported to be associated with infarcted hearts by reprograming fibroblasts directly into functional cardiomyocytes [28]. Results in this work demonstrate that TBX5 is down-regulated by methanal exposure in ventricle and atria tissues compared to the control group. Although another study suggested that TBX5 could regulate the differentiation of cardiomyocyte by controlling STAT3, no report investigated the relationship between methanal and TBX5 expression [29]. We report that methanal induces heart malformations through the β_2-spectrin-based signal pathway.

Recently, research has identified β_2-spectrin as an important protein required for the biogenesis and maintenance of the cardiomyocyte submembrane cytoskeleton [11]. In this study, we demonstrated that methanal exposure decreased β_2-spectrin expression in ventricle and atria tissues in pregnant rats. Results have indicated that inhibition of β_2-spectrin synthesis decreased GSH synthesis and TBX5 expression in the ventricle and atria tissues in pregnant rats. We also found that β_2-spectrin knockdown decreased Ca^{2+} and its downstream effector ankyrin-B in ventricle and atria tissues and was calpain-dependent in β_2-spectrin knockdown rats. These findings suggest that the β_2-spectrin-meidated pathway may be a potential molecular pathway induced by methanal exposure.

In conclusion, this study explored the possible mechanism of heart teratogenicity induced by methanal exposure in animals. The findings

indicate that methanal exposure increases the risk of heart malformations, through modulation of the β_2-spectrin-mediated pathway in myocardial cells. However, further study should be performed to identify additional target of methanal exposure leading to heart malformation.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhirong Jiang, Cardiac Ultrasound, Qingdao University Hospital, Qingdao, 59 HaiEr Road, LaoShan District, Qingdao 266000, China. Tel: +86-53276587735; E-mail: jiangzhirongprof@outlook.com

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