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Inhibition of histone acetylation by curcumin reduces alcohol-induced expression of heart development-related transcription factors in cardiac progenitor cells

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ABSTRACT

Alcohol exposure during pregnancy may cause congenital heart disease (CHD). In our previous studies, we found that alcohol selectively increased acetylation of histone H3 at lysine 9 (H3K9) and enhanced the expression of heart development-related genes in cardiac progenitor cells. The objective of this study is to investigate the protective effects of histone acetyltransferases (HATs) inhibitor, curcumin, on histone hyperacetylation and the over-expression of heart development genes induced by alcohol. Western blot analysis was employed to detect the acetylation levels of histone H3K9 and real-time PCR was applied to measure the expressions of heart development-related transcription factors, GATA4, Mef2c and Tbx5 (GMT). Our results showed that alcohol increased the acetylation of H3K9 by 2.76-fold ($P < 0.05$) and significantly enhanced the expression of GATA4 and Mef2c ($P < 0.05$). When cells were treated with alcohol plus 25 μM curcumin, the hyperacetylation of H3K9 and over-expression of GATA4 and Mef2c by alcohol was reversed. These data indicate that curcumin can correct the over-expression of cardiac genes by reversing the alcohol induced hyperacetylation of histone H3 at lysine 9 in cardiac progenitor cells, suggesting that curcumin is protective against alcohol-induced cardiac gene over-expression that may result in heart malformations.

1. Introduction

Birth defect, caused by interaction between genetic and lifestyle factors, has become a leading cause of domestic perinatal death. The clinical observation and retrospective studies showed that alcohol drinking during pre-pregnancy and pregnancy caused a birth delay and induced several teratogenic effects on fetal development, leading to the so called Fetal Alcohol Syndrome which is characterized by hypoevolutism, craniofacial abnormal morphology, central nervous system malformation and congenital heart disease (CHD) [1,2]. It is well known that CHD is one of the most common congenital malformations in children [3]. However, the mechanisms underlying the interactions between genetic and nutritional effectors in CHD are hard to reveal.

With the development of epigenetic research, scientists try to find out how exogenous factors transmit genetic information through the expression of selected genes in the body. Histone acetylation plays an important role in regulating gene expression and chromatin structure during the development of the heart [4]. Cardiogenesis is a precise process regulated by sequential gene expression. At a specific time point during development, the sequential activation and expression of development-related genes is critical for the formation of a normal heart [5]. Histone acetylation plays an important role in the process of heart development by acting as a switch for gene expression regulation [6,7]. Histone acetyltransferases (HATs) and histone deacetylase (HDACs) are a pair of antagonistic enzymes that can adjust the electrostatic attraction and stereospecific blockade between DNA and histone to either compact or loose the chromatin, and then either reduce or increase gene transcription [8]. Our preliminary study found that imbalance of histone acetylation affected the expression of cardiac development-related genes, and prevented the differentiation of mesenchymal stem cells into myocardial cells [9,10]. We also discovered that alcohol caused the hyperacetylation of histone H3K9, resulting in an over-expression of heart development-related genes in cardiac progenitor cells [11]. In addition, we demonstrated that curcumin, a HATs enzyme inhibitor, could inhibit histone acetylation [12]. Consequently, we hypothesize that curcumin can correct the over-expression of the heart development-related transcription factors by reversing histone hyperacetylation induced by alcohol in the heart.

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To test the hypothesis, we determined histone acetylation levels and measured the expression levels of the heart development-related transcription factors, GMT (GATA4, Mef2 and Tbx5), in cultured cardiac progenitor cells with or without the treatment of alcohol and curcumin. The results of our study indicate that curcumin can correct the transcription factor expression levels by reversing the histone hyperacetylation induced by alcohol in cardiac progenitor cells.

2. Materials and methods

2.1. Culture and treatment of cardiac progenitor cells

The cardiac progenitor cells were donated from Molecular Oncology Laboratory at the University of Chicago Medical Center [13]. After recovery from nitrogen, the cardiac progenitor cells were cultured with Dulbecco's Modified Eagle Medium (DMEM)/High Glucose (Thermo, Waltham, MA USA) containing 10% Fetal Bovine Serum (FBS)(Thermo, Waltham, MA, USA) and 1% penicillin (Thermo, Waltham, MA, USA). When the cells reached 90% confluence, they were subcultured for three passages, and the fourth generation of cardiac progenitor cells was taken for our studies. Our preliminary study found that 200 mM alcohol was an effective concentration resulting in hyperacetylation of cardiac progenitor cells [11]. Our preliminary data of the cytotoxicity tests showed that 25 μM curcumin was the highest concentration that the cardiac progenitor cells could tolerate. The experiments were designed and the cells were divided into three groups: alcohol treated group with 200 mM alcohol; curcumin treated groups with 5, 15, 25 μM curcumin dissolved in the 200 mM alcohol; control group of the cardiac progenitor cells without any treatment. The culture flasks were closed with a hermetic seal to avoid the evaporation of alcohol. After 24 h of the treatment, the cells were prepared for subsequent experiments.

2.2. MTT assay

Cell viability and mitochondrial activity were measured as reported previously [11] using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in spectrophotometric assays (KeyGEN, Nanjing, China).

2.3. Western blotting

After 24-h treatment of drugs, nucleoproteins were extracted by using extract kit (KeyGEN, Nanjing, China), protein concentration was detected using a bicinchonininc acid (BCA) protein assay kit (Pierce, Rockford, Illinois, USA). Nucleoproteins with the sample volume of 50 mg were run on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. After 1.5-h blocking with 5% nonfat dried milk, the membrane was incubated with primary antibody of H3K9 (ab61231, (Abcam, Cambridge, UK) at the dilution of 1:500, another membrane was incubated with antibody for β-actin 1:200 (BOSTER, Wuhan, China), which was used as an internal control. After overnight incubation at 4 °C, the membranes were washed and incubated with horseradish-conjugated secondary antibody (both anti-mouse and anti-rabbit) (ZhongShan-Golden Bridge, China) at the dilution of 1:5000 for 1 h at room temperature. The target proteins were detected using chemiluminescence analyzer (KeyGEN, Nanjing, China). The density of the bands was analyzed using Quantity One Version 4.4 software (Bio-Rad, Richmond, California USA).

2.4. Quantitative real-time PCR analysis

Total RNAs were extracted using RNA extract kit (Biotake, Beijing, China) and reverse-transcribed (RT) to cDNA by using RT kit (Takara, Otsu Shiga, Japan). The cDNA was detected using quantitative RT-PCR assay with SYBR Green RealMasterMix kit (Tiangen, Beijing, China) to determine the transcriptional expression of heart development-related genes including GATA4, Mef2c, Tbx5. β-Actin was used as the endogenous "house-keeping" gene to normalize the RNA sample levels. The specific primers were designed as follows:

GATA4: 5′-CCCTCCCGCACGATTTCT-3′ (forward) 5′-AGAGGCCCA ACTCGCTCAA-3′ (reverse);

Mef2c: 5′-GCGCAGGGAATGGATACGG-3' (forward) 5′-TGCCAGG TGGGATAAGACG-3' (reverse);

Tbx5: 5′-CCAAAGACAGGTCTTGCGATTCG-3′ (forward) 5′-TTCTC CTCCCTGCCTTGGTGAT-3′ (reverse).

The results were analyzed as $2^{-\Delta\Delta Ct} \pm$ standard deviation (SD) in which $\Delta\Delta Ct = \Delta Ct_{\text{experimental group}} - \Delta Ct_{\text{control group}}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$.

2.5. Statistical analysis

All data were expressed as mean ± SD, and statistically analyzed by one way ANOVA. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effect of curcumin on expression levels of heart development-related transcription factors in cardiac progenitor cells treated by alcohol

Using real-time PCR assays, we first detected the expression levels (mRNA) of the heart development-related transcription factors, GATA4, Mef2c, and Tbx5 in cardiac progenitor cells treated by alcohol, curcumin or both. 200 mM alcohol significantly enhanced the expression level of GATA4 (2.69 ± 0.89 folds, alcohol vs. controls, $P < 0.05$) and Mef2c (6.35 ± 0.99, alcohol vs. controls, $P < 0.01$). However, there was no significant increase of the expression level of Tbx5 in alcohol-treated group (1.60 ± 0.63, alcohol vs. controls, $P > 0.05$). The alcohol-induced increases of the expression levels of GATA4 and Mef2c were reversed by 25 μM curcumin to levels similar to controls (alcohol/curcumin vs. controls, $P > 0.05$) (Fig. 1).

3.2. Effect of curcumin on histone H3 acetylation at lysine 9 in cardiac progenitor cells treated by alcohol

We then analyzed the effect of alcohol and curcumin on H3K9 in cardiac progenitor cells using Western blotting assays. 200 mM alcohol increased 2.76-fold acetylation of H3 at lysine 9 significantly (2.76-folds, alcohol vs. controls, $P < 0.01$) in cardiac progenitor cells. The alcohol-induced hyperacetylation in these cells was corrected by curcumin when the cells were treated with both alcohol and curcumin. Fig. 2 shows a dose-dependent inhibitory effect of curcumin on alcohol-induced H3K9 acetylation in cardiac progenitor cells (Fig. 2). Five micromolar curcumin was not enough to completely reverse the hyperacetylation induced by alcohol (2.76-fold, alcohol alone vs. controls and 2.22 folds, alcohol/5 μM curcumin vs. controls, $P > 0.05$). But curcumin could significantly correct the alcohol-induced H3 acetylation at lysine 9 in cardiac progenitor cells when its concentrations reached 15 or 25 μM (Fig. 2).

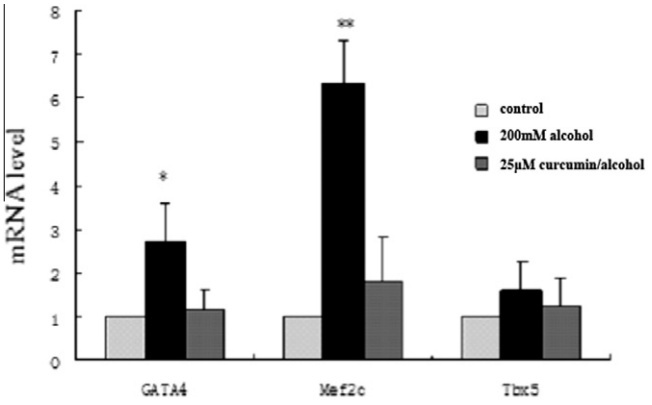

Fig. 1. Effect of curcumin on mRNA expression of heart development-related transcription factors in cardiac progenitor cells treated with alcohol. Cardiac progenitor cells were treated with 200 mM alcohol and 25 μM curcumin for 24 h. The control was treated with vehicle solution. The results are expressed as mean ± SD from at least three separate experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ as compared to the controls.

3.3. Cytotoxic effect of curcumin in cardiac progenitor cells

Our previous study showed that application of 200 mM alcohol to the same type of cells did not produce significant toxic effects in the cells [11]. In the present study, we further measured cytotoxicity and cell death using a MTT-based spectrophotometric assay in tested cells treated with either alcohol, curcumin or both. The cell viability was slightly reduced by 200 mM alcohol. However, when various concentrations of curcumin (5, 15, and 25 μM) were added to the cells in addition to the 200 mM alcohol, no additional toxic effects of curcumin was evident in these cells as no significant difference in cell viability was observed between the cells treated with alcohol alone and the cells treated with alcohol plus various concentrations of curcumin ($P > 0.05$) (Fig. 3).

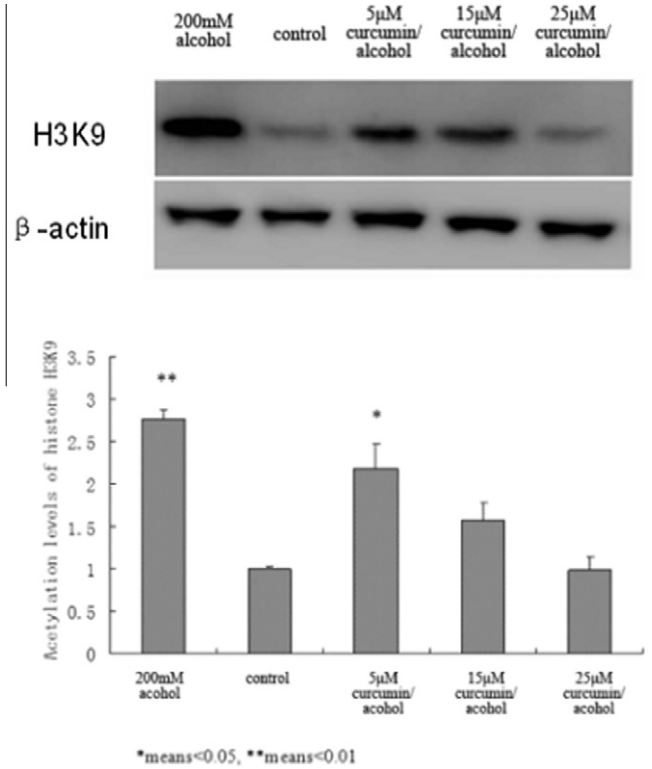

Fig. 2. Effect of alcohol and curcumin on histone H3 acetylation at lysine 9. Cardiac progenitor cells were treated with 200 mM alcohol and curcumin at 5, 15, or 25 μM for 24 h. The control was treated with the vehicle solution without drugs. Values calculated as the ratio between acetylated H3 lysine 9 and β-actin are presented as mean ± SD, $n = 3$ experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ as compared with control.

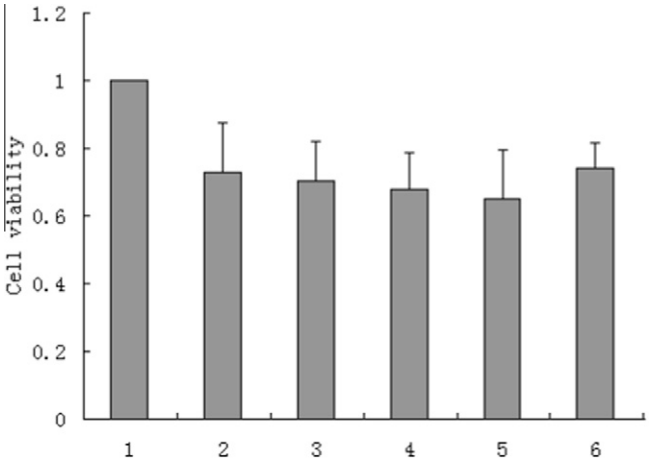

Fig. 3. Effect of different concentrations of curcumin and alcohol on mitochondrial activity of cardiac progenitor cells. Cardiac progenitor cells were treated with 200 mM alcohol and curcumin at 5, 15, or 25 μM. After the treatment, mitochondrial activities were determined using the MTT assay (see Section 2). Data represent mean ± SD from three separate experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ as compared to the controls. 1. Control cells without treatment of drugs; 2. Cells treated with 200 mM alcohol; 3. Cells treated with 5 μM curcumin plus 200 mM alcohol; 4. Cells treated with 15 μM curcumin plus 200 mM alcohol; 5. Cells treated with 25 μM curcumin plus 200 mM alcohol; 6. Cells treated with 25 μM curcumin alone.

4. Discussion

It is well known that CHD is caused by the interaction of genetic and environmental factors including lifestyle. It is becoming clearer that chromatin plays a fundamental role in the control of gene transcription in multicellular organisms. Epigenetic modifications are responsible for chromatin dependent regulatory mechanism and are essential for the regulation of gene expression during embryonic development. Epigenetics involve changes in gene and protein expression levels, not due to DNA sequence, but to genomic imprinting, DNA methylation, histone modification, non-coding RNA, etc. [14,15]. As an important way of epigenetic modifications, histone acetylation regulates the state of chromatin to decide inhibition or activation of gene expression [16]. In the course of embryonic development, it is necessary for specific genes to be expressed accurately, at a proper sequence, time and space. The inappropriate expression or repression of these genes can change trajectories in the developmental process and result in developmental malformation [17].

Embryonic development is a huge and precise project, which needs the expression of all the genes involved to strictly follow a strict order of time and space and involve the regulation of many enzymes. Some adverse environmental factors may disturb this process. Such disturbance may break the balance of gene expression, and lead to abnormal morphology. As the most important transcription factors during the early stage of heart development, GATA4, Mef2c, Tbx5, play critical roles in cardiac cell development by interacting with each other, or by regulating the genes downstream from them in the form of complex [18,19]. Qualitative or quantitative abnormalities in the expression of these transcription factors will lead to cardiac malformations. In this study, the expression levels of several heart development-related transcription factors such as GATA4, Mef2c, but not Tbx5, were found to be significantly enhanced by alcohol in cardiac progenitor cells.

However, the alcohol-induced increases of the expression of GATA4 and Mef2 were corrected by curcumin in these cells when treated with both alcohol and curcumin. These data indicate that curcumin is protective against alcohol-induced over-expression of GATA4 and Mef2.

Our previous study and other reports showed that alcohol could increase the acetylation level of H3 at lysine 9 in different cells and tissues [11,20,21]. Since histone hyperacetylation is related to gene over-expression, we further determined the histone acetylation levels in our study. We found, indeed, that alcohol could enhance significantly the acetylation of H3 at lysine 9 in cardiac progenitor cells. Interestingly, alcohol-induced histone hyperacetylation in cardiac progenitor cells was completely reversed by curcumin, suggesting that curcumin can reduce histone acetylation by inhibiting P300/HDAC1, enzymes that cause histone acetylation. It is known that the hyperacetylation can loosen chromatin structure, and activate gene transcription. In particular, the acetylation of H3 at lysine 9 is considered as a sign of gene transcription activation [22]. Based on our results, we make an assumption that alcohol enhances the expression of heart development-related transcription factors by increasing the acetylation level of H3 at lysine 9 in cardiac progenitor cells. This change can be reversed by curcumin through the inhibition of P300/HDAC1 that results in a decreased histone acetylation and decreased gene expression in the cells.

Recently, studies show that curcumin can decrease the hyperacetylation of histone and reverse the cardiac hypertrophy of animal model *in vivo* [23]. In our study, the data also suggest that curcumin can reduce the hyperacetylation of H3 at lysine 9 by inhibiting HATs enzyme, resulting in a decreased expression of cardiac transcription factors. However, we don't know whether the observed effect of curcumin from *in vivo* and *in vitro* systems comes from a similar mechanism. It is well known that the transcription factors, GATA4, Mef2 and Tbx5 (GMT), play critical roles in cardiac cell growth and heart development [18]. However, in our study, we only observed the change in expression levels of GATA4 and Mef2, not Tbx5, in cardiac progenitor cells treated by alcohol or curcumin. It is not clear for us for the moment why Tbx5 expression is not influenced by histone acetylation change caused by alcohol or curcumin. More studies are needed in the future to determine the relationship between alcohol-induced histone hyperacetylation and the transcriptional expression of other cardiac genes.

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