ORIGINAL RESEARCH

The Effect of Methamphetamine on Ventricular Myocytes of Neonatal Rats

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ABSTRACT

Objective • The paper studied the effect of Methamphetamine (MDMA) on myocardial gap junction protein to further reveal the molecular toxicological mechanism of Methamphetamine directly inducing cardiotoxicity,does it affect the expression, distribution, and phosphorylation status of connexin 43 in myocardial cells, inducing cardiac dysfunction.

Methods • Select 1-day SPF grade neonatal rats, obtain purified ventricular myocytes, and undergo acute exposure for 1 hour to establish an in vitro model of MDMA acute exposure.An in vitro MDMA acute exposure model was constructed to observe the effects of acute exposure to different concentrations of MDMA on the expression and transcription level of CX43 protein in ventricular myocytes; the effect on the content and distribution of mouse ventricular myocyte Connexin 43; the influence on the phosphorylation status of Ser368 at the phosphorylation site of protein 43; and the effects on the intracellular calcium oscillation mode and the intracellular calcium ion concentration.

Results • The content of total Connexin 43 in cardiomyocytes of the acute MDMA exposure group decreased significantly. Immunofluorescence detection of MDMA found that the content of Connexin 43 in the gap junction decreased with abnormal distribution. MDMA can lead to changes in the phosphorylation status of Connexin 43, which is manifested as an increase in the proportion of non-phosphorylation status, and can also increase the phosphorylation level of site Ser368. MDMA can change the calcium oscillation mode and rise of calcium ion concentration in cardiomyocytes.

Conclusion • MDMA can destroy the calcium homeostasis in cardiomyocytes and affect the expression, distribution, and phosphorylation state of cardiomyocyte Connexin 43. This is also one of the potential mechanisms concerning the direct myocardial toxicity of MDMA.

Practical Application • MDMA is a kind of amphetamine derivative, a class of synthetic non-catecholamine sympathomimetic drugs. It can selectively act on the central nervous system above the brainstem to stimulate the neurosynaptic precursors to release serotonin (5-HT) and the monoamine neurotransmitters dopamine (DA) and norepinephrine (NE). After taking it, people may experience elevated mood, euphoria, sexual impulse, and involuntary head shaking caused by sympathetic nerve excitement. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

MDMA has been proven to cause damage to multiple organs of the human body, including neurotoxicity, cardiovascular toxicity, and liver toxicity. It acts on synaptic endings, stimulating the release of monoamine neurotransmitters while blocking the reuptake of neurotransmitters, inhibiting the activity of monoamine oxidase, leading to a significant increase in dopamine and norepinephrine content in synaptic gaps, generating excitatory and hallucinogenic effects, causing high fever, and changes in speech and behavior. Acute MDMA poisoning can cause arrhythmia, hypertension, high fever, and liver damage symptoms. Chronic poisoning usually manifests as seizures, mood disorders, and cognitive impairment.[1-3]

Cardiac hypertrophy, dilated cardiomyopathy and hypertrophic cardiomyopathy caused by long-term use of MDMA,[4] as well as the sudden death of arrhythmia induced by acute MDMA poisoning have also been continuously reported.[5,6] Although MDMA can cause a variety of

cardiovascular diseases and acute cardiac dysfunction has been confirmed by extensive experimental results and clinical data, the direct toxic effect of MDMA on cardiomyocytes, especially the mechanism of inducing arrhythmia, has not yet been explored. Our research attempts to explore the direct toxic effects of MDMA on myocardial cells and investigate the potential mechanisms that lead to arrhythmia.

METHODS

Establishment of primary culture of neonatal rat ventricular myocytes and MDMA in vitro exposure model

SPF grade 0 day or 1 day newborn rats were obtained. Heart extraction and preparation of ventricular muscle cell tissue block were performed. Ventricular muscle tissue stepwise digestion, cardiomyocyte purification, counting, and inoculation were conducted. On the 6th day of cell culture, the ventricular myocytes were cultured and incubated with serum-free DMEM medium for 12 hours. A micropipette was used to draw the MDMA-concentrated stock solution (100 mmol/L), which was added to the culture wells in proportion to make 3 different concentrations of MDMA-exposed groups of 10, 50, and 250 μM/L, respectively. The intervention time is 1 hour, and the Control group was added with serum-free DMEM medium for 1-hour incubation, 10 holes per group.

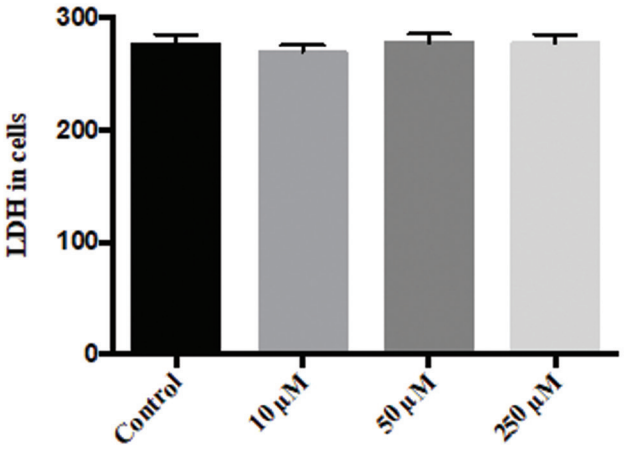
Detection of cell activity after MDMA exposure.

LDH (LDH-Cytotoxicity Colorimetric Assay Kit [BioVision, K311-400, USA]) lactate dehydrogenase detection method was applied to detect cell viability after MDMA infection.

Western blot protein immunoblotting method for detecting various proteins

cells were collected from all rats, and washed with PBS for twice. Each flask was added with 400 μl lysate, then 40 μl PMSF at 10 mmol / L density. The flasks were agitated gently, and put on ice for 10 min to lyse the cells uniformly. The cells were repeatedly drawn with a sterile syringe. The lysate was added to an EP tube, which was then ice-bathed for 30 min, and centrifuged at 12000 g for 15 min. The supernatant was moved to a new EP tube. Protein density was measured with a microplate reader and BCA assay. Each tube was added with 20 μl 6 × Buffer for every 100 μl, boiled for 5 min, mixed, and then stored under -80°C.The above samples were obtained to do electrophoresis in 12% SDS-PAGE. The isolated protein bands were transferred to a PVDF membrane using the Wet method and closed under room temperature for 1 h. The membrane was added with the primary antibody, Total Cx43 (Item No: IBSBIOA-1, Sigma, USA), Cx43 Ser368 (Item No: PA130037, Sigma, USA). The density of all the antibodies was 1:1000. The membrane was incubated overnight under 4°C, and washed with PBST for thrice. Following this, the membrane was added with the secondary antibody (Item No: 562-1, Shanghai Zemai Biotech Co., Ltd) at the density of 1:1000, incubated for 1 h, and washed with PBST for three times. Color developing and fixing was completed using a chemiluminescence assay.

Figure 1. The effect of MDMA on cell viability



Immunofluorescence quantitative analysis to detect the content and distribution status of connexin 43.

The inverted laser confocal scanning microscope was used to observe the content and distribution of Connexin 43 between the gap junctions of ventricular myocytes in the different MDMA concentration-exposed groups and the Control group. The quantitative analysis of Connexin 43 was performed using the area percentage method (SI= Detecting the area of connexin 43 in the field of vision/ Detecting the area of muscle cells in the center of the visual field).

Detection of intracellular Ca^{2+} in ventricular myocytes.

The ventricular myocytes cultured to day 6 were taken out of the incubator and added with serum-free medium for 16 hours pretreatment. The myocytes were added with 250 μM MDMA-containing serum-free medium, incubated for 30 minutes, and then added with 1000 μM Fluo-4 fluorescent load solution. The myocytes were placed in incubator for 30 minutes. A laser confocal scanning microscope was used to detect the changes of calcium ions in cardiomyocytes in real-time.

Statistical analysis

All data were expressed as mean ± standard deviation. Multi-sample comparisons of 3 or more groups were conducted using a one-way analysis of variance, and the least significant difference test was used for multiple comparisons pairwise. If $P < .05$, it is considered a statistically significant difference.

RESULT

MDMA does not affect LDH cell activity

The results showed that, compared with the Control group, the exposure to different concentrations of MDMA for 1 hour had no significant effect on the viability of primary cultured neonatal rat ventricular myocytes, as shown in Figure 1.

MDMA significantly affects the phosphorylation state of cardiomyocyte Connexin 43

MDMA of 250 μM significantly affects the phosphorylation state of cardiomyocyte Connexin 43, causing non-phosphorylated

Connexin 43, that is, an increase in the proportion of non-phosphorylation, which is significantly different from the Control group. However, under the intervention of 10 μM and 50 μM MDMA, the ratio of non-phosphorylated Connexin 43 was not statistically different from that of the Control group, and phosphorylated Ser368 was significantly increased compared to the Control group, as shown in Figure 2.

The effect of MDMA on the content of Connexin 43 at the gap junction of cardiomyocytes.

In the Control group, the positive markers of Connexin 43 were arranged in a dotted line, distributed clearly and neatly between adjacent cardiomyocytes. Some Connexins were found in the cytoplasm of the cardiomyocytes around the nucleus. In the acute MDMA exposure group, including three concentration groups of 10, 50, and 250 μM, it can be seen that Connexin 43 is scattered in the myocardial cells in dots, the arrangement is disordered, and some Connexin 43 between adjacent cells disappears. In the high-concentration MDMA acutely exposed group, it is found that the positive fluorescent label is significantly reduced, as shown in Figure 3.

Changes in Ca^{2+} concentration in ventricular myocytes.

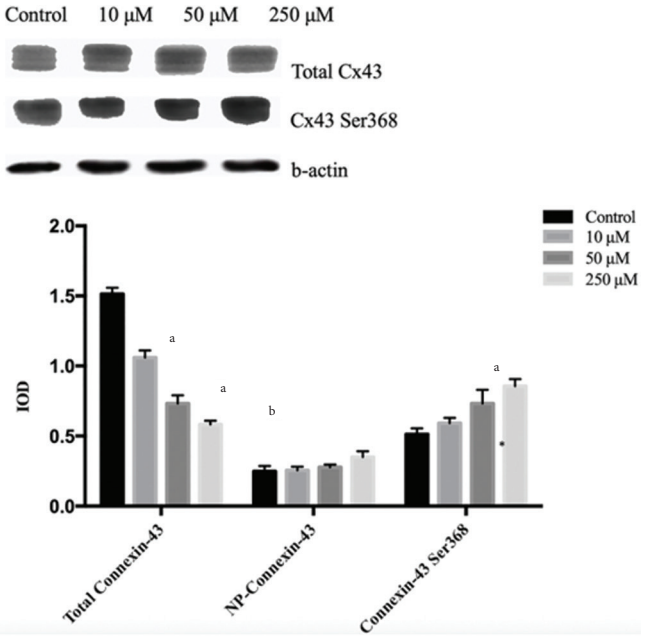
The pictures were taken when the Ca2+ concentration in the cardiomyocytes is at the peak and trough. It is worth noting that, regardless of the experimental group or the Control group, the changes in the Ca^{2+} fluorescence brightness under the microscope are all synergistic, meaning that the changes in the intracellular Ca^{2+} concentration under all the fields of view are consistent, as shown in Figure 4.

DISCUSSION

MDMA is a kind of amphetamine derivative, a class of synthetic non-catecholamine sympathomimetic drugs. It can selectively act on the central nervous system above the brainstem to stimulate the neurosynaptic precursors to release serotonin (5-HT) and the monoamine neurotransmitters dopamine (DA) and norepinephrine (NE). After taking it, people may experience elevated mood, euphoria, sexual impulse, and involuntary head shaking caused by sympathetic nerve excitement.

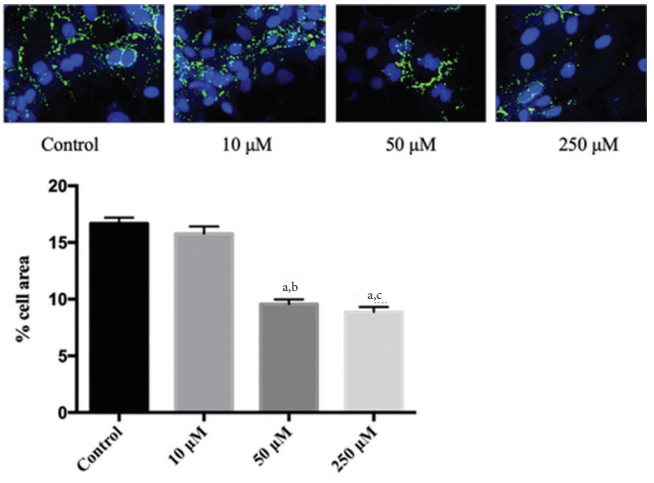
Previous research on cardiovascular toxicity of MDMA found that the main mechanisms for MDMA to induce cardiovascular dysfunction or disease include indirect and direct pathways. The indirect pathway refers to the fact that MDMA acts on the nerve center in the body to cause the increase of monoamine neurotransmitters in the nerve terminals, and the sympathetic nerve excitation indirectly leads to abnormal cardiovascular function, which is called the MDMA catecholamine-dependent pathway.[7,8] However, the direct pathway is still unclear. Some studies argue that MDMA and its oxidative metabolites can destroy the antioxidant defense system of cells and generate a large number of reactive oxygen free radicals,[9] thus leading to cellular oxidative stress, lipid oxidation, and abnormal intracellular calcium concentration.

Figure 2. The effect of MDMA on the content of total Connexin 43 in cardiomyocytes.



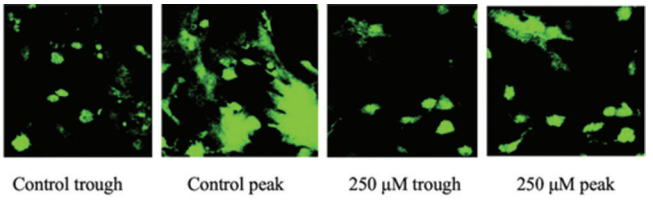
[a]$P < .05$ vs Control
[b]$P < .01$ vs Control

Figure 3. The effect of MDMA on the content of Connexin 43 at the gap junction of cardiomyocytes.



[a]$P < .01$ vs Control
[b]$P < .05$ vs 10 μM
[c]$P < .01$ vs 10 μM

Figure 4. The effects of MDMA on the change of Ca^{2+} concentration.



The heart's normal beating requires that the ECG signal spreads between the cardiomyocytes in an orderly manner until it propagates to the entire heart, forming synchronized contractions and expansions. Among them, the cardiomyocyte gap junction plays an important role in maintaining the normal contraction rhythm of the myocardium and ensuring the rapid and consistent propagation of ECG signals between cells.[10] A complete gap junction is a channel composed of two opposing structures on the cell membrane between two adjacent cells, which play a role in cell-to-cell communication, allowing small molecular weight substances and ions to be rapidly transferred between adjacent cells.[11]

The hemichannel gap junction is formed by six Connexins (Cx). A total of 4 major Connexins were found on mammalian cardiomyocytes: Cx37, Cx40, Cx43, and Cx45. Among them, Connexin 43 (Cx43) is the most important and abundant Connexin in the ventricular muscle of adult mammals.[12] In our experiments, high concentrations of MDMA caused a significant decrease in the expression of total Connexin 43 in cardiomyocytes. It is speculated that the following two reasons caused the different results. Firstly, the experimental subject was different, and we adopted different primary cells of ventricular myocytes, maybe with different mechanisms at the gene level. The second is that NF-κB plays an important role in the mechanism of immunity, inflammation and tumor formation. Experiments have confirmed that MDMA can activate NF-κB to increase the transcription of the Cox-2 gene. Cox-2 is a protease that codes for inducing a myocardial inflammatory response, which can cause an increase of myocardial inflammatory cytokines. This mechanism inhibits the expression of Connexin 43 in myocardial cells,[13] Please refer to the relevant figures and the observed phosphorylation changes.

Ca^{2+} is an important second messenger in cells and is extremely important in signal transduction pathways. The change of intracellular Ca^{2+} oscillation frequency has been confirmed by a large number of studies, which has a very important regulatory effect on certain Ca^{2+} oscillation frequency-dependent intracellular signal transduction reflections. It is argued that the activity of these Ca^{2+} sensitive proteins is affected by the intracellular " Ca^{2+} spark" frequency.[14] In cells, the activity of many enzymes depends on the activation of the Ca^{2+} pathway. PKC is a Ca^{2+}-dependent protein kinase,[15] which is very sensitive to changes in the intracellular Ca^{2+} oscillation pattern. The increase of intracellular Ca^{2+} concentration and the change of Ca^{2+} oscillation mode can promote the transport of PKC from the cytoplasm to the cell membrane and assist the activation of PKC.[16] In our experiments, it is found that the concentration of Ca^{2+} in the ventricular myocytes of neonatal rats in the primary culture in vitro increased one hour after acute MDMA exposure.

The preliminary research results of this topic can serve as a valuable reference in the field of MDMA myocardial toxicity mechanism research, promote the improvement of relevant theories and mechanisms, and provide a foundation for related research in forensic and clinical medicine.

CONCLUSION

To sum up, MDMA can destroy the calcium homeostasis in cardiomyocytes and affect the expression, distribution, and phosphorylation state of cardiomyocyte Connexin 43. This is also one of the potential mechanisms of the direct myocardial toxicity of MDMA. Is there a change in the activity of calcium channel proteins related to the mechanism of MDMA induced disruption of intracellular calcium homeostasis in myocardial cells; Does MDMA still have a regulatory effect on the phosphorylation status of other specific phosphorylation sites of connexin 43. The above-unexplored issues require further research to clarify. I hope that the preliminary research results of this topic can play a role in the field of MDMA myocardial toxicity mechanism research, promote the improvement of relevant theories and mechanisms, and provide a foundation for related research in forensic and clinical medicine. This fills the gap in the research on the molecular mechanism of MDMA myocardial toxicity in forensic toxicology and proposes new research ideas.

ETHICAL COMPLIANCE

Research experiments conducted in this article with animals or humans were approved by the Ethical Committee and responsible authorities of our research organization(s) following all guidelines, regulations, legal, and ethical standards as required for humans or animals.

CONFLICTS OF INTEREST:

There are no conflicts to declare or mention it if there is any to declare.

AUTHOR CONTRIBUTIONS

Jiesong Yan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing Review and Editing. Yanhu Shi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing Review and Editing.

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