Reduced Glycolysis Contributed to Inhibition of Testis Spermatogenesis in Rats After Chronic Methamphetamine Exposure

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Background: Previous reports suggested that methamphetamine (METH) exposure could lead to inhibition of rat testis spermatogenesis. Glycolysis and glucose metabolism as well as oxidative stress have been implicated in testis spermatogenesis. Here we explored the underlying mechanism of local metabolism and glycolysis of testis after METH exposure.

Material/Methods: METH was intraperitoneally injected into rats with different doses and duration of METH exposure to establish short-term and chronic exposure models. The serum 8-hydroxy-2-deoxyguanosine (8-OHdG) level of rats was detected by enzyme-linked immunosorbent assay. Untargeted gas chromatography-mass spectrometry analysis was applied to identify differential metabolites and metabolic signature. The mRNA expression of hypoxia inducible factor 1α (HIF1α), glucose transporter 1 (GLUT1), hexokinase 1 (HK1) and lactate dehydrogenase C (LDHC) in rat testes were detected by polymerase chain reaction. Further, we determined the 4 proteins with western blotting and immunohistochemistry.

Results: Decreased testes index and sperm counts were showed in the chronic METH group. The metabolome revealed that the main differential metabolites impacted were associated with glycolysis and glucose metabolism. The mRNA and protein expression of GLUT1, HK1, and LDHC were reduced in the chronic METH group but elevated in the short-term METH group, whereas HIF1α was upregulated in the short-term METH group but remained at baseline in the chronic METH group.

Conclusions: Overall, glucose metabolism was regulated by HIF1α after short-term METH exposure. Reduced glycolysis in the testis led to impaired spermatogenesis after chronic METH exposure.

MeSH Keywords: Enzymes • Glucose Metabolism Disorders • Glycolysis • Methamphetamine • Spermatogenesis • Testis

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Background

Addictive psychostimulant drugs, including methamphetamine (METH), can be an important cause of male factor infertility [1]. Additionally, as previously reported in studies mainly focused on apoptosis or hormone alteration, METH exposure can lead to the inhibition of rat testis spermatogenesis [2,3]. Disorders of hypothalamic-pituitary-adrenal and hypothalamic-pituitary-testicular axes were associated with the adverse effect of testis spermatogenesis [4,5]. However, we consider that the alteration of endocrine axes indirectly affected testis spermatogenesis after METH exposure. In fact, no reports have explored the local metabolism of testis. Glycolysis can provide lactate or ATP production for acrosome reaction or sperm motility in the process of sperm maturation [6]. Thus, we hypothesized that the inhibition of testis spermatogenesis after METH exposure might be related to changes in local glucose metabolism, which is the main energy source of organs and tissues.

After METH exposure, oxidative stress induced abnormal sperm morphology and low sperm concentration by increasing apoptosis in the testis [3], and hypoxia inducible factor 1α (HIF1α) is an important regulatory molecule associated with oxidative stress [7]. A previous study suggested that the activation of HIF1α could mediate metabolic reprogramming and induce glycolysis in tissues [8]. Thus, this might be explained as HIF1α regulating local glycolysis of testis to inhibit spermatogenesis after METH exposure. As the metabolome is a sensitive measure of local metabolism and a dynamic indicator of genetic changes [9], we detected differential metabolites by an untargeted metabolomics approach and further determined the expression of glycolysis-related proteins.

Although previous studies shown that 14 or 15 days and maximum dose 6 mg/kg (4 times per day) of METH exposure could inhibit spermatogenesis [2,3], longer duration and larger dose per time of METH exposure for spermatogenesis and these underlying mechanisms were still unclear. But on the other hand, spontaneous ejaculation has been reported to occur after METH exposure [10], which might be associated with alteration of endocrine hormones related to testis spermatogenesis. Therefore, we need to elucidate whether the inhibition of testis spermatogenesis emerges after early METH administration. Furthermore, further investigation is required to determine the impacts on testis spermatogenesis of METH exposure at different doses and duration. Our study of METH exposure in rats were divided 2 stages, including short term (15-days) METH exposure and chronic (28-days) METH exposure, to investigate the effects on testis spermatogenesis, which could provide a novel prospective for understanding the adverse effects of METH abuse.

Material and Methods

Animals and METH exposure model establishment

Eight-week-old male (220 to 250 g weight) Sprague-Dawley rats, 40 rats in total, were obtained from the Hunan Slack Jingda Experimental Animal Co., Ltd. and bred in Animal Experiment Center of Kunming Institute of Zoology. All rats were housed with an environment with controlled temperature (24°C) and humidity (55%) and a 12-hour light/dark cycle. Animals were randomly divided into 4 groups: 1) short-term METH exposure group (15-days of METH exposure, called “short-term METH group” or “ST METH group” in brief); 2) short-term control group (15-days control group, called “ST Control group” in brief); 3) chronic METH exposure group (C METH group); 4) chronic control group (C Control group).

Purified powder of METH, which was soluble in physiological saline, was obtained from Yunnan Anti-Drug Committee. The METH solution, whose concentration was 1 mg/mL, was intraperitoneally injected into the cavity of rats in the METH exposure groups. Equal volume of saline was injected in the same way in the corresponding control groups. The short-term METH and chronic METH groups consisted of different doses and duration of exposure listed in Table 1, which based on previous reports [11,12]. In the short-term exposure group, the treatment doses were with 5 mg/kg at 6-hour intervals again on the first day, but treated as 2 mg/kg at 6-hour intervals again on the second day, and increased 2 mg/kg/per time gradually to 10 mg/kg/per time for 2 times per day, and the duration of exposure was 15 days. In the C METH group, the treatment doses were with 10 mg/kg only 1 time on the first day and treated with 2 mg/kg per day on the second day and increased 2 mg/kg gradually to 10 mg/kg, then the duration of exposure was 28 days. This study was conducted in accordance with the Helsinki declaration and the guidelines for Care and Use of Laboratory Animals promulgated by the National Institutes of Health.

Determination for serum 8-hydroxy-2 deoxyguanosine (8-OHdG) levels in rats

We quantified the level of 8-hydroxy-2 deoxyguanosine (8-OHdG) by competitive enzyme-linked immunosorbent assay (ELISA) with rat ELISA kits (ab201734, Abcam, Cambridge, UK). After establishing the METH exposure model, orbital sinus blood was sampled with mild suction from rats under isoflurane anaesthesia and 5 mL of blood was collected from each rat. The microtiter plates were coated with capture 8-OHdG diluted in coating buffer (1: 250) at 4°C overnight. The absorbance at 450 nm was measured in each well by a plate reader. The antibody concentrations were calculated according to standard curve obtained from the OD values and all samples were assayed in triplicate.
Table 1. The doses and duration of exposure in the ST METH and the C METH group, and the volume of saline treated in corresponding control groups.

| Short-term groups | Day 1 | Day 2–3 | Day 4–6 | Day 7–9 | Day 10–12 | Day 13–15 |
|-------------------|-------|---------|---------|---------|-----------|-----------|
| ST METH group (10:00) (METH, mg/kg) | 5 | 2 | 4 | 6 | 8 | 10 |
| ST METH group (16:00) (METH, mg/kg) | 5 | 2 | 4 | 6 | 8 | 10 |
| ST Control group (10:00) (saline, mL) | 1.3 | 0.5 | 1.1 | 1.7 | 2.3 | 3.0 |
| ST Control group (16:00) (saline, mL) | 1.3 | 0.5 | 1.1 | 1.7 | 2.3 | 3.0 |

| Chronic groups | Day 1 | Day 2–3 | Day 4–6 | Day 7 | Day 8–13 | Day 14 | Day 15–20 | Day 21 | Day 22–28 |
|----------------|-------|---------|---------|------|----------|--------|-----------|--------|-----------|
| C METH group (10:00) (METH, mg/kg) | 10 | 2 | 4 | Free | 6 | Free | 8 | Free | 10 |
| C Control group (10:00) (saline, mL) | 2.5 | 0.5 | 1.1 | Free | 1.7 | Free | 2.4 | Free | 3.1 |

Weighing, sperm counts, and preparation of tissue samples

Before blood sampling, the body weight of rats was measured and after being sacrificed with injecting air into blood vessel, the testes of rats were weighted. The testis index was calculated as: Ti=testes weight/body weight. Then, the left epididymis of each rat was placed in 0.5 mL phosphate-buffered saline (PBS) and homogenized for 30 seconds. Each sample was diluted in 10 μL aliquots, and sperm counts were determined by a hemocytometer [13].

Sodium pentobarbital (3%, 45 mg/kg) was used to euthanize the rats after blood sampling. Parts of left testicular tissues were analyzed by untargeted gas chromatography-mass spectrometry (GC-MS), and the remaining tissues were used in real-time polymerase chain reaction (PCR) and western blot. Right testicular tissues were prepared for immunohistochemistry.

Untargeted GC-MS analysis of acute METH exposure and control groups

Untargeted GC-MS analysis was employed to examine the comprehensive metabolic signatures and differential metabolites in the testes of rats in the short-term METH group and the short-term control group. The extractions from testis samples were derivatized and performed on an Agilent 7890A gas chromatography-mass spectrometry (GC-MS), and the remaining tissues were used in real-time polymerase chain reaction (PCR) and western blot. Right testicular tissues were prepared for immunohistochemistry.

The structural identification of differential metabolites was performed by AMDIS software. Deconvolute mass spectra from raw GC-MS data were analyzed and the purified mass spectra were automatically matched with an in-house standard library that included RT, mass spectra, KEGG Metabolome Database and Agilent Fiehn GC/MS Metabolomics RTL Library [14]. According to differential metabolites, the following proteins were selected for further verification: HIF1α, glucose transporter 1 (GLUT1), hexokinase 1 (HK1), and lactate dehydrogenase C (LDHC).

Quantitative real-time PCR and western blotting

Specific primers were designed to amplify the target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control to normalize gene expression (Table 2). Total RNA was isolated from samples using a RNeasy Kit with TRIzol (Invitrogen), and reverse-transcribed into cDNA using a reverse transcription kit with TRizol (Invitrogen), and reverse-transcribed into cDNA using a reverse transcription kit with M-MLV polymerase (Promega). Then, the target mRNA was amplified, and the samples were conducted in triplicate.

The proteins of testis samples were transferred to polyvinylidene fluoride membranes, blocked with blocking buffer, and then incubated with the primary antibodies (HIF1α, 1: 500, Abcam; GLUT1, 1: 800, Abcam; HK1, 1: 1000, Proteintech; LDHC, 1: 500, Proteintech) overnight at 4°C. Afterwards, the membranes were incubated with the secondary antibody goat anti-rabbit IgG (sc-2004, 1: 1000, Santa Cruz Biotechnologies). The membranes were reprobed with β-actin (1: 2000; Abcam, Cambridge, UK) antibodies and each blot was then exposed to the Pierce Fast Western Blot Kit ECL Substrate (Cat No. 35055,
Results

Reduction in the TI and sperm counts of the C METH group

We found that the body weights of rats in the short-term METH group and C METH group were lower than those of rats in control groups (Figure 1A). The TI and sperm counts were not significantly different in the short-term METH group and short-term control group (Figure 1C, 1D), but these measurements were reduced in the C METH group compared with the C Control group (Figure 1D).

Increased serum 8-OHdG levels in METH exposure groups

The serum 8-OHdG level was significantly higher in the short-term METH group than in the short-term control group ($P = 0.024$) and was significantly higher in the C METH group than in the C Control group (Figure 1E).

Metabolism profiling of the short-term METH and short-term control groups

The model quality was described by the R2X (model cumulative interpretation rate) of PCA-X score plot, which indicated that the current model was suitable for characterizing differences between the 2 groups (R2X > 0.5). The 2 groups were located on the left and right sides of the score map of PLS-DA statistical analysis, which highlighted significant metabolic differences (R2Y and Q2 values were closer to 1). The OPLS-DA score plot indicates that the samples from the 2 groups can be effectively distinguished. The permutation test indicated that the current model was reliable (Figure 2A).

### Immunohistochemistry

Fixed tissue sections were deparaffinized in xylene, rehydrated with graded concentrations of alcohol, incubated with 3% $H_2O_2$, and then blocked with 3% bovine serum albumin (BSA). Subsequently, the HIF1α (1: 100), GLUT1 (1: 100), HK1 (1: 100), and LDHC (1: 100) antibodies were separately added to the sections and incubated at 4°C overnight. After exposure to biotinylated goat anti-rabbit IgG (1: 200) (Boster, Wuhan, China), the sections were incubated in the streptavidin-biotin complex (SABC; Boster, Wuhan, China). Staining was terminated by distilled water in case of visible brown staining and nuclear counterstaining was conducted with hematoxylin. Finally, the sections were visualized with an imaging system (DM1000, Leica, Germany) and analyzed with Image-Pro Plus 6.0 software.

### Statistical analysis

All the data are expressed as the mean ± standard deviation (SD) and analyzed using SPSS 22.0 (IBM, Armonk, NY, USA). Statistical charts were generated by GraphPad Prism version 5.0. One-way analysis of variance (ANOVA) was performed to compare multiple groups of quantitative data. For all analyses, the significance level $\alpha$ was set to 0.05 (2-tail), and $P<0.05$ (*) or $P<0.01$ (**) or $P<0.001$ (*** ) was required to claim statistical significance.

Table 2. Characteristics of the designed primers and the cycling for gene amplification with q-PCR.

| Gene   | Primer sequence (5'-3') | Product size (bp) | Annealing temperature (°C) |
|--------|------------------------|-------------------|----------------------------|
| HIF1α  | F: ACCCTAACTAGCGAGGAAGA | 176               | 58.76                      |
|        | R: GCACCAAGCAGCGATAGGT |                   | 60.89                      |
| GLUT1  | F: CTGGCATCAAAGCGCTTCCTC | 193              | 59.62                      |
|        | R: AACACCCGACAGCAGTCAA |                   | 59.39                      |
| HK1    | F: TAAACGGAGCGGGATGGAA | 188              | 60.18                      |
|        | R: ACGTGCGTGGCTCGATAGC |                   | 60.32                      |
| LDHC   | F: CTTGACCTTCATGATCTCTC | 184              | 59.96                      |
|        | R: TCTGAGTCCACAGGTAT  |                   | 59.90                      |
| GAPDH  | F: ACATCAAGAAGTGTTGAGGA | 111              | 60.16                      |
|        | R: TGGAAGATGGAGTGGTGT |                   | 60.16                      |

HIF1α – hypoxia-inducible factor 1α; GLUT1 – glucose transporter 1; HK1 – hexokinase 1; LDHC – lactate dehydrogenase C; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; F – forward primer; R – reverse primer.
We screened and characterized 46 different substances. To show the correlation between the different metabolite concentrations, we conducted Pearson correlation analysis and heat map analysis on the R platform to obtain a correlation matrix of differential metabolites (Figure 2B, 2C). According to the correlation matrix diagram and the heat map analysis, glucose metabolism and glycolysis were mainly significant differences between the two groups. We summarized some of the highly significant differential metabolites or metabolic pathways, including glucose-6-phosphate (G-6-P), the galactose metabolic pathway, the fructose and mannose metabolic pathways and the glycolytic pathway (Figure 3).

Expression of the 4 genes and proteins in testis

HIF1α is closely related to oxidative stress [15] and regulates the glycolysis involved in testicular spermatogenesis [16]. GLUT1 is a transmembrane protein involved in the regulation of cellular glucose metabolism in the testis, which is also regulated by HIF1α [17]. Therefore, we evaluated the mRNA and protein expression of HK1, LDHC, HIF1α, and GLUT1. As indicated in Figure 4, the mRNA and protein expression levels of HK1, LDHC, HIF1α, and GLUT1 in rat testes were increased in the short-term METH group compared with the short-term control group. However, the expression levels of HK1, LDHC, and GLUT1 were decreased in the C METH group compared with the C Control group. The transcript levels and protein expression of HIF1α were not significantly different between the C METH group and the C Control group, and they were upregulated in the short-term METH group but were maintained at the baseline level in the chronic METH group.

Immunohistochemistry of 4 selected proteins

HIF1α protein was increased in the short-term METH group compared with the short-term control, C Control and C METH groups, which displayed no marked differences in staining intensity (Figure 5). GLUT1 was increased in the short-term METH group compared with the short-term control group, but significantly reduced in the C METH group compared with the C Control group (Figure 4). Concerning HK1 and LDHC, both were mainly located in the sperm flagellum and spermatogonia layer (Figure 5). Our results illustrated that HK1 and LDHC staining was increased in the short-term METH group compared with the short-term control group but decreased in the C METH group compared with the C Control group (Figure 5).

Discussion

In our work, we confirmed that chronic METH exposure could affect spermatogenesis by reducing glycolysis in rat testes. Our results also indicated that the glucose metabolite G-6-P increased after short term METH exposure but decreased in response to chronic METH exposure. Moreover, the TI, sperm counts, and testicular cell apoptosis were not significantly different between the short-term METH and short-term control groups but were decreased in the C METH group compared...
with the C Control group. This phenomenon might be explained by alterations in glucose metabolism and energy exhaustion in testes, which contributed to impediment of spermatogenesis after METH exposure.

Oxidative stress, an important biochemical mechanism, can mediate the physiological function of the central nervous system and peripheral tissues after METH exposure [18]. Our results mean that oxidative stress increases with raising doses and prolonged time after METH exposure. Although the use of METH by humans was reported promote feelings of energy, euphoria, impulsivity, and spontaneous locomotor activity [19], and depression and fatigue can occur after repetitive actions as a result of excessive energy consumption [20]. HIF1α can

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**Image Description:**

The image contains a figure with two panels A and B. Panel A shows three graphs titled PCA-X, PLS-DA, and OPLS-DA, each with a scatter plot. The graphs compare data points labeled as C or M, possibly indicating control or METH-exposed groups. Panel B is a heatmap with various metabolites represented in red and green colors, indicating their respective levels or changes. The metabolites listed include Glucose-6-phosphate, Mannose, Xanthine, Ornithine, Tyrosine, Serine, Dulcitol, Methionine, Phenylalanine, Glutamine, Valine, Glutamate, Spermine, Spermidine, Glutathione, Hypoxanthine, N-acetylgalactosamine, Xylitol, Myristic acid, Pantothenic acid, Glycolic acid, Myo-inositol, DHAP, Putrescine, AMP, Glycerol-2-phosphate, Thymine, Guanosine, and Inosine. The heatmap uses a color scale from -1.00 to 1.00, indicating the magnitude of change in metabolite levels.
Figure 2. Metabolism profiling of the short-term METH and short-term control groups. (A) Evaluation of metabolic characteristics in rat testes between the short-term control group and the short-term METH group (n=10): PCA-X scores plot (R²X=0.58 >0.5); PLS-DA scores plot (R²X=0.511, R²Y=0.977, Q²=0.813); OPLS-DA scores plot (R²X=0.609, R²Y=0.999, Q²=0.855); permutation test. (B) The correlation matrix of differential metabolites between the short-term control group and the short-term METH group. Each row and column represent a differential metabolite. The correlation coefficient metric is shown on the right side of the figure. The size and color depth of the circles are related to the correlation between the different metabolites. (C) Color map representation of 46 metabolites found between the 2 groups (class 1: C group; class 2: M group). Each row represents a differential metabolite, and each column represents a sample number. The tree structure on the left represents the clustering of similarity for different metabolites between the 2 groups. C group – the short-term control group; M group – the short-term METH group; t[1] – the first principal component; t[2] – the second principal component; METH – methamphetamine.
Reduced glycolysis inhibits spermatogenesis after chronic METH exposure

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Reduced glycolysis inhibits spermatogenesis after chronic METH exposure. Significant differential metabolites detected between the two groups were labeled in green and chose to explore. Arrow represented the reaction direction.

Figure 3. Metabolic pathways of differential metabolites and enzymes related to glycolysis in rat testes. Significant differential metabolites detected between the two groups were labeled in green and chose to explore. Arrow represented the reaction direction.

Enhanced glycolytic activity in rat testes after short-term METH administration seemed to induce testicular spermatogenesis. Conversely, consecutive intraperitoneal injection of METH for 14 days led to the adverse effects on testes structure and spermatogenesis indices in rat testes tissues [25], which might be associated with continuous treatment and moderate or high doses of drug. However, previous studies had reported that acute METH exposure (5, 10, and 15 mg/kg) did not affect the motility and morphology of sperms [26], and until 35 days, METH exposure could cause significant changes in sperm morphology, sperm chromatin abnormalities, DNA integrity impairment [27]. Similar to these situations, the sperm motility and counts were not significantly increased after short-term METH exposure in the light of our results. In this regard, we speculated that body and testes weight declined due to the effect of METH exposure and enhanced glycolytic activity, which were in response to the regulation of HIF1α with increased oxidative stress. Even if oxidative stress was continuously increased, chronic METH exposure caused the downregulation of glucose metabolism and GLUT1, and the glycolytic activity in rat testes was weakened and the inhibition of testicular spermatogenesis was indicated by the reduction in the TI and sperm counts. To some extent, the exhaustion of energy and glucose metabolism might account for the aforementioned results. A recent study also showed that after METH exposure, the compensatory upregulation of gamma-aminobutyric acid (GABA), GABA-A receptors and GABA transporter 1 (GAT1) in rats testes tissues appeared [28], which might represent a homeostatic response to the adverse effects of METH exposure by inhibiting local mechanism and reducing energy consumption. Moreover, enzymes related to glycolysis, including HK1 and LDHC, might be broken down because of the reduction of GLUT1 and G-6-P according to the theoretical basis of enzyme catalysis [29]. Therefore, the alteration of testicular...
Figure 4. Relative mRNA and protein expression levels of HIF1α, GLUT1, HK1, and LDHC in each group (n=6). (A) The relative mRNA expression levels (fold) of HIF1α, GLUT1, HK1, and LDHC. (B) The protein expression levels of HIF1α, GLUT1, HK1, and LDHC; ns – not significant; * P<0.05, ** P<0.01, *** P<0.001. HIF1α – hypoxia-inducible factor 1α; GLUT1 – glucose transporter 1; HK1 – hexokinase 1; LDHC – lactate dehydrogenase C.
spermatogenesis affected by METH might be a process that was mediated by energy consumption in testes, from excessive energy consumption at short term stage to low energy supply at chronic stage.

The limitations of this study are the followings: firstly, untargeted GC-MS analysis was not performed in the chronic METH exposure group and the chronic control group. We only used metabolomics analysis to discover main different metabolites and metabolic pathways for the further investigation.
In addition, the 4 selected proteins related to glycolysis were not evaluated at different times of the 2 stages so as to lack of exploring for dynamic changes in glycolytic activity of the testes. Furthermore, the low level of HIF1α transcription in the C METH group was not explained, and the cause of this observation might be related to weakened hormonal stimuli [30]. Finally, our results might not be directly applied to human population because of numerous metabolic factors and inconsistent purity of drugs. Future research might address these limitations by thorough detection and investigation with a longitudinal design.

However, our experiment revealed that glucose metabolism and glycolysis in rat testes changed after METH exposure. We provided some valuable evidence on inhibition of testis spermatogenesis after chronic METH exposure but no significant alteration after short-term METH exposure. Importantly, our results indicated underlying mechanism differs from previous research perspectives and help to lay a foundation for further study.

Conclusions

Overall, our study confirmed that glucose metabolism varied with oxidative stress, was regulated by HIF1α after short-term METH exposure, and reduced glycolysis in the testis might lead to impaired spermatogenesis after chronic METH exposure. Understanding the mechanism of glucose metabolism disorder and glycolysis unbalance will help to obtain deep recognition on effect from METH abuse.

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Conflict of interest

None.

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