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Background : *Methamphetamine (METH) is a highly addictive psychostimulant drug which can distribute and toxic to multiple organs including liver. There have been reported that methamphetamine increase glycogen storage, mitochondrial aggregation and microvascular lipid, on the other hand, decrease total protein and glutathione peroxidase in the liver.*

Objectives : *This study aimed to investigate morphological changes as well as the changes of protein and DNA contents in the liver cell (hepatocyte) in the liver of methamphetamine-induced male rat.*

Methods : *Male Sprague-Dawley rats induced addiction by receiving methamphetamine was studied for morphological changes in the liver cells by Hematoxylin and Eosin staining. Study of protein contents was performed by Bromophenol blue staining, while the study of DNA contents performed by Feulgen staining. ImageJ software was applied for morphological study, and protein and DNA intensities were measured and calculated in comparison with control.*

Results : *Qualitative study demonstrated abnormal morphologies in hepatocyte including nuclear enlargement, nuclear shrunken and nuclear fragmentation in methamphetamine-induced rats. In quantitative study, the percentage of the number of abnormal hepatocytes was significantly increased in methamphetamine-induced rats. In addition, the relative optical density (ROD) of protein and DNA contents were significantly decreased in methamphetamine-induced rats when compare with control.*

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Conclusion : *This study demonstrated structural and functional changes in hepatocytes of METH-induced rats. These could also reflect abnormal liver function in human with METH.*

Keywords : *Methamphetamine, liver, hematoxylin & eosin, bromophenol blue stain, Feulgen stain.*

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เหตุผลของการทำวิจัย : *เมทแอมเฟตามีนเป็นสารเสพติดประเภทกระตุ้นระบบประสาท แล้วก่อให้เกิดความเป็นพิษจากการแพร่กระจายไปยังหลายอวัยวะภายในร่างกายรวมถึงตับด้วย ซึ่งมีหลายงานวิจัยได้รายงานว่า เมทแอมเฟตามีนส่งผลให้เกิดการเพิ่มการสะสมของไกลโคเจน การเกาะกลุ่มกันของไมโตคอนเดรีย และการสะสมของถุงไขมันขนาดเล็กภายในเซลล์ตับ ในทางตรงกันข้ามส่งผลลดปริมาณของโปรตีน และเอนไซม์ glutathione peroxidase ในตับ*

วัตถุประสงค์ : *เพื่อศึกษาการเปลี่ยนแปลงทางโครงสร้าง ตลอดจนปริมาณของโปรตีนและดีเอ็นเอในเซลล์ตับ ในหนูเพศผู้ที่ถูกเหนี่ยวนำด้วยเมทแอมเฟตามีน*

วิธีการทำวิจัย : *หนูเพศผู้สายพันธุ์ Sprague-Dawley ถูกเหนี่ยวนำให้เกิดการติดสารเสพติดจากการได้รับเมทแอมเฟตามีน นำมาศึกษาการเปลี่ยนแปลงทางโครงสร้างของเซลล์ตับ โดยการย้อม hematoxylin and eosin staining ศึกษาปริมาณของโปรตีนโดยการย้อม bromophenol blue staining ขณะที่การศึกษาปริมาณของดีเอ็นเอย้อมด้วย Feulgen staining และใช้โปรแกรม ImageJ ในการวิเคราะห์ลักษณะทางโครงสร้าง การวัดค่าความหนาแน่นของโปรตีน และดีเอ็นเอ โดยทำการเปรียบเทียบกับกลุ่มควบคุม*

ผลการศึกษา : *จากการศึกษาเชิงคุณภาพแสดงให้เห็นลักษณะของเซลล์ตับที่ผิดปกติประกอบด้วย นิวเคลียสมีขนาดที่ใหญ่ นิวเคลียสหดหรือเหี่ยว และนิวเคลียสเกิดการแตกหักในหนูที่ได้รับเมทแอมเฟตามีน จากการศึกษาเชิงปริมาณพบว่าเปอร์เซ็นต์ของเซลล์ตับที่ผิดปกติเพิ่มขึ้นแตกต่างอย่างมีนัยสำคัญทางสถิติในหนูที่ได้รับเมทแอมเฟตามีน นอกจากนี้ยังพบว่าค่าความหนาแน่นสัมพัทธ์ (ROD) ของโปรตีน และดีเอ็นเอลดลงในหนูที่ได้รับเมทแอมเฟตามีนเมื่อเปรียบเทียบกับหนูกลุ่มควบคุม*

สรุป : *การศึกษาในครั้งนี้แสดงให้เห็นถึงการเปลี่ยนแปลงทางโครงสร้าง และหน้าที่ของเซลล์ตับของหนูที่ได้รับเมทแอมเฟตามีน ซึ่งสามารถสะท้อนการทำงานของตับที่ผิดปกติได้ในคนได้รับเมทแอมเฟตามีน*

คำสำคัญ : *เมทแอมเฟตามีน, ตับ, Hematoxylin & eosin, Bromophenol blue stain, Feulgen stain.*

Methamphetamine (METH) is highly addictive and toxic drug of abuse. It has been reported approximately 33 million people being abused worldwide. [1] The toxic effects of METH on the brain are well recognized including feelings of alertness, wakefulness, energy, well-being, and suppression of appetite. [2] However, methamphetamine can also affect the visceral organs such as the heart by causing arrhythmias and cardiomyopathy; in the lung, hypertension and shortness of breath; in the kidney, renal failure; and, especially in the liver, acute hepatic failure. Accordingly, it is important to examine the effects of methamphetamine to the liver. Therefore, we designed this experiment to investigate morphology changes in the hepatocytes as well as determine protein and DNA contents inside the cells.

Methods

Animal treatment

Male Sprague-Dawley rats weighing 280 - 350 g were obtained from National Laboratory Animal Center of Mahidol University, Thailand. They were acclimatized to the laboratory conditions for 7 days in Center for Animal Research, Naresuan University, Thailand. The animals were maintained under conditions of controlled temperature ($22^{\circ} \pm 1^{\circ}C$) and 12-hour light and dark cycle; they were given access to food and water. After 7 days of acclimatization, all animal was accustomed to treatment before treatment for 2 days, with 0.9% saline 2.0 mg/kg (i.p. 3 times / day). All protocols have been approved by Naresuan Animal Ethics Committee (NUAE01E).

The rats were divided into two groups. Control group (n = 4), animals were injected with 0.9% saline 2.0 ml/kg (i.p. 3 times /day) for 15 days, while animals in METH addiction group (n = 4) were injected with d-Methamphetamine HCl (in saline), the initial dose was 0.1 mg / kg and increased of 0.1 mg / kg / times (i.p. 3 times /day) for 13 days. Day 14 was injected with d-Methamphetamine HCl 4.0 mg/kg (i.p. 3 times /day) and was injected binge dose of d-Methamphetamine HCl 6.0 mg/kg (i.p. 4 times /day) in day 15. [3,-5]

Specimen preparations

At the end of the experiment, animals were killed by cervical dislocation under CO_2 anesthesia and their livers were removed. Under light microscope, the livers were fixed in 10% neutral formalin, then dehydrated in gradual series of ethanol, embedded in paraffin wax and sectioned at 5 μm thickness.

Morphological study

Slides of liver tissue were stained by hematoxylin and eosin technique for histological examination and characterization of abnormal cells (quantitative analysis). Two sections from each sample were deparaffinized with xylene and rehydrated with series of alcohol concentrations such as 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol and distilled water, respectively. After that, the sections were stained in hematoxylin for 10 minutes, washed in running tap water, and dipped with 1% Lithium carbonate for 10 dips. Then, the section were washed in running tap water and 95 % ethanol for 1 minutes, stained with Eosin for 20 seconds and washed in running tap water. Finally, the sections were dehydrated with series of alcohol concentrations such as 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol and cleared with xylene respectively. All sections were mounted with mounting media.

Each slide was examined under a light microscope at 20X magnification. The total number of hepatocytes were analyzed by imageJ software. [7] The criteria for hepatocytes classification are as follows;normal hepatocytes have a round shape of nucleus and abnormal hepatocytes have an Irregular shape of nucleus such as nuclear enlargement, nuclear shrunken and nuclear fragmentation. The quantitative data were presented as the percentages of normal and abnormal hepatocytes.

Protein and DNA intensity study

The studies of protein contents were conducted by bromophenol blue staining technique. Two sections for each sample were deparaffinized with xylene and rehydrated in decreasing concentration of alcohol and distilled water. Then, the sections were stained in Bromophenol blue 7 minutes and washed in distilled water, followed by dehydration in increasing concentration of alcohol, clearing in xylene and mounting.

The studies of DNA contents were performed by using Feulgen staining techniques. After deparaffinization and rehydration, all sections were kept warming at 60°C in 1 N hydrochloric acid for 1 hour for a hydrolysis process, followed by distilled water for 3 minutes. Then, the sections were immersed in Schiff's reagent for 1 hour, dipped with bisulfite solution for 3 dips, washed in distilled water and counterstained with light green 3 minutes, followed by dehydration in increasing concentration of alcohol, clearing in xylene and mounting.

The protein and DNA contents were examined under a light microscope at 20X magnification. Each section was taken ten pictures for analysis. The protein and DNA intensities were analyzed by imageJ software. [7] The results of protein and DNA contents were presented by Relative optical density (ROD); from ROD = log (255 / intensity mean).

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was determined through *t*-test to compare between the groups and the threshold for statistical significance was set at $P < 0.05$. All statistical analyses were performed using SPSS statistical software (IBM SPSS Statistics for window, version 17.0).

Results

Effect of methamphetamine on morphology changes of the rat liver

In the qualitative study, the results showed abnormal morphology in hepatocytes of METH-induced rats including nuclear enlargement, nuclear shrunken and nuclear fragmentation (Figure 1B & D) when compared with normal form hepatocytes in the control (Figure 1A &C).

Effect of methamphetamine on hepatocytes in rat liver

In the quantitative study, the results showed an increase in the percentage of abnormal morphology of hepatocytes in METH-induced rats compared with control group (Figure 2, $P < 0.05$).

Effect of methamphetamine on protein contents in hepatocytes.

Qualitatively, the protein distribution in hepatocytes was analyzed by bromophenol blue method. Numerous dark blue stained proteins were seen in cytoplasm of hepatocytes in both control (Figure 3A) and METH-induced groups (Figure 3B). Quantitatively, the protein intensity was found a significant decrease in METH-induced group when compare with control group (Figure 5).

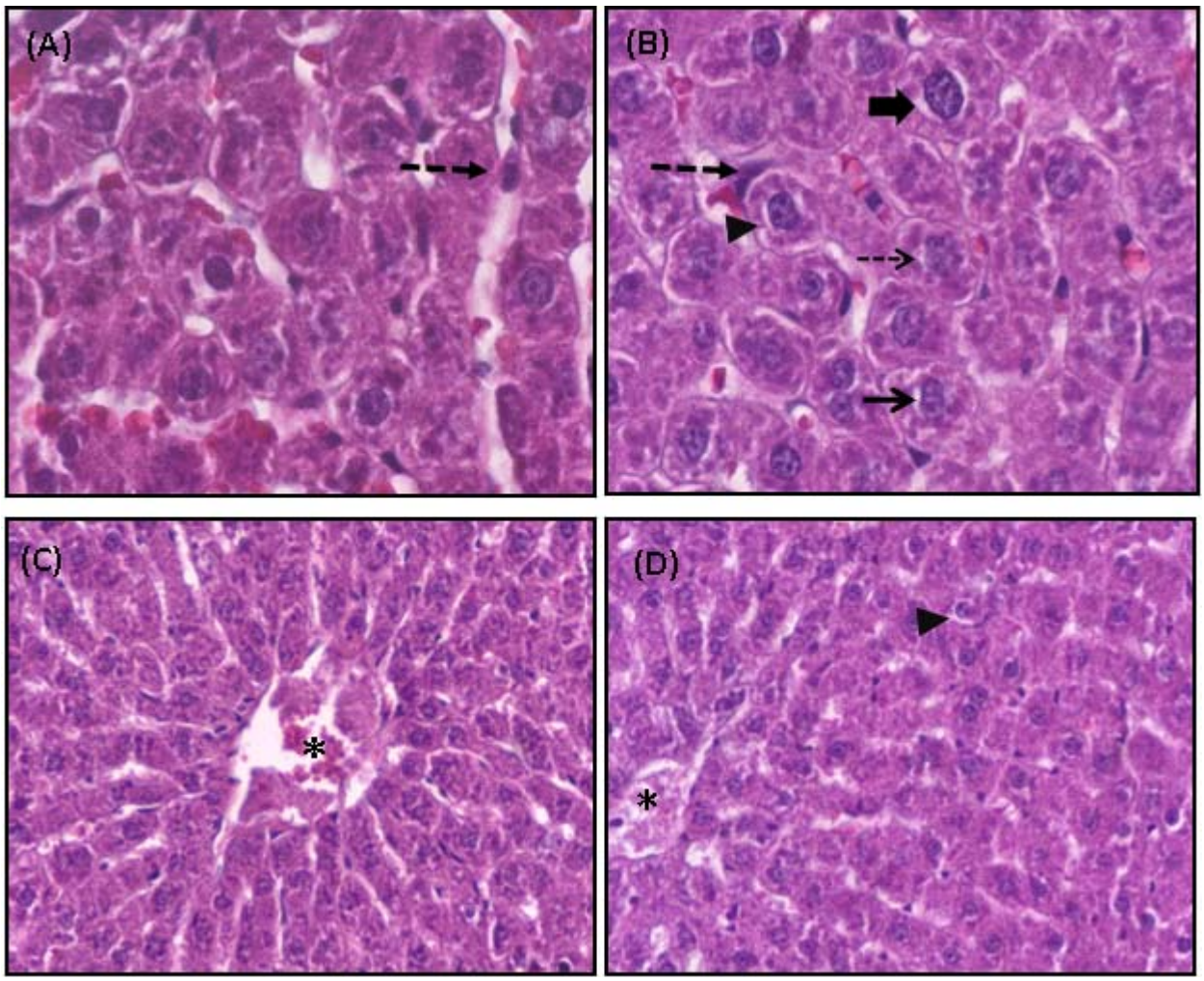

Figure 1. Hematoxylin-eosin (H&E) staining for revealing morphological changes of liver cells in rat treats for 15 days; (A) control group in (40x), (B) METH-induced group (40x), (C) Control group in (20x) and (D) METH-induced group (20x). (▲ : vacuoles, ⬆ : Kupffer cell, ⬆ : nuclear enlargement, ⬆ ; nuclear shrunken, ⬆ : nuclear fragmentation, and *: central vein).

Effect of methamphetamine on DNA contents in hepatocyte.

DNA contents in hepatocytes were analyzed by Feulgen staining method demonstrating by pink stained nuclei in hepatocytes of control and METH-induced groups (Figure 4A&B). Quantitatively, DNA intensity was found a significant decrease in METH-induced group when compare with control group (Figure 5).

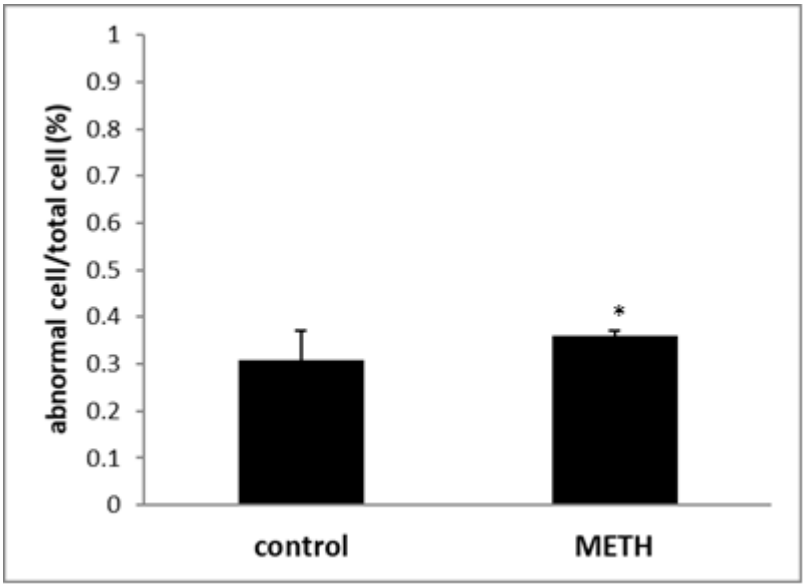

Figure 2. Percentage of abnormal hepatocytes in control and METH-induced groups (n = 4). * $P < 0.05$ versus control group.

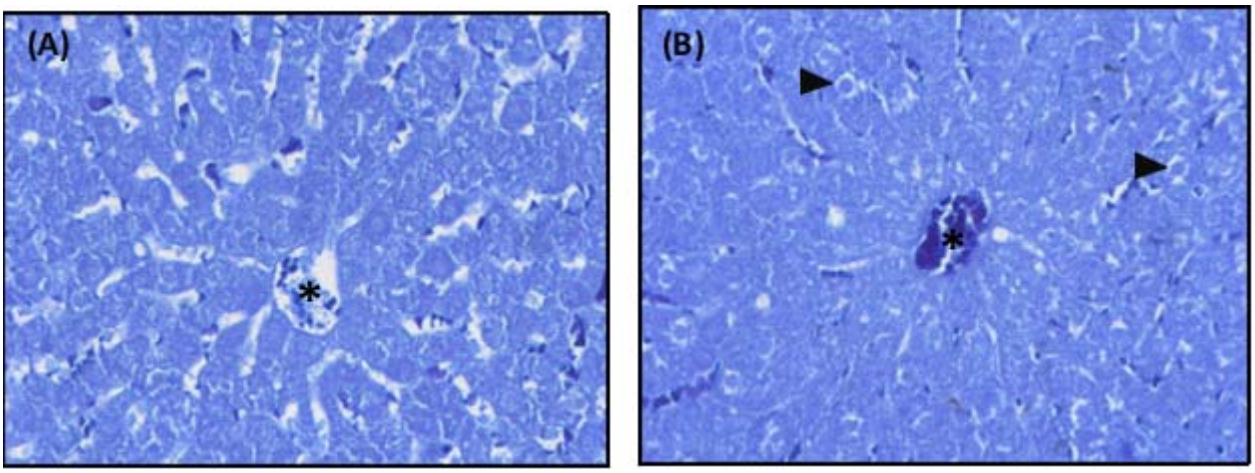

Figure 3. Morphological changes of protein distribution in 15 days of rat liver were analyzed by bromophenol blue staining technique; (A) control group in (20x), (B) METH-induced group (20x). (▲: vacuoles, *: central vein).

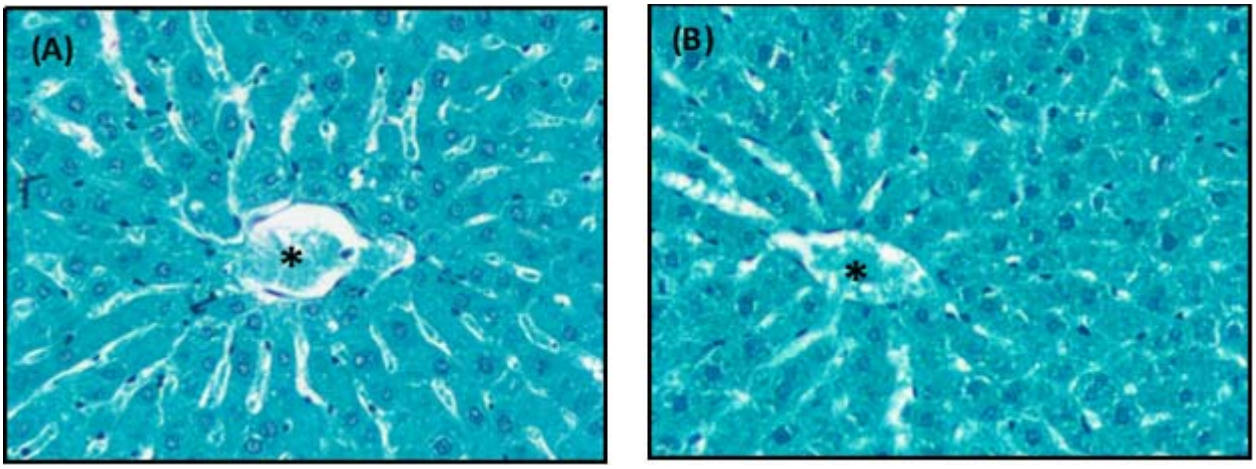

Figure 4. Morphological changes of DNA contents ; (A) control group in (20x), (B) METH-induced group (20x). (*: central vein).

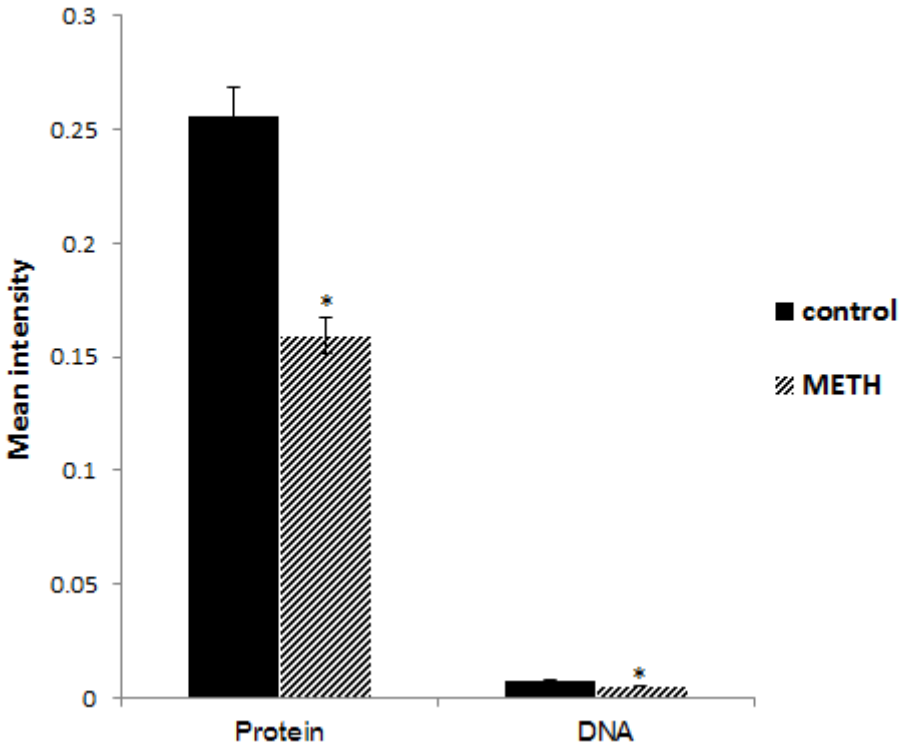

Figure 5. Protein and DNA intensity in control and METH-induced groups. Data were presented as mean ± SEM. * $P < 0.05$ versus control group.

Discussion

In the present study, we demonstrated structural changes of hepatocytes in METH-induced rats included nuclear enlargement, nuclear shrunken and nuclear fragmentation. The results are consistent with the study of Suphakong K, *et al* [8] who reported abnormalities of liver tissue and cells after exposure to an abused drug, Dextromethorphan (DEX).

Moreover, a significant decrease in the number of hepatocytes with normal morphology may reflect the effect of METH in development of hepatocytes. It has been suggested that addictive substances such as DEX or METH might induce an increase of ROS production leading to inhibition of cell regeneration.[8, 9]

The present study also demonstrated that protein and DNA contents in hepatocytes were decreased in METH-induced rats. These may reflect the effects of METH on the changes of cellular contents and their functions. It has been suggested that an increase of free radicals from various causes can induce protein cross-linking or protein fragment leading to protein production deficits.[10, 11] Moreover, an increase of ROS production inside the cell can induce DNA fragmentation leading to an inhibition of cell proliferation.[9]

The results of this study provide evidence to support abnormalities in morphology as well as DNA and proteins contents of liver cells in METH addiction. However, mechanisms of these abnormalities need to be studied further.

Conclusion

In summary, the present study showed that METH can induce morphological abnormalities of hepatocytes as well as decreases in protein and DNA contents inside the cell. Therefore, the results from the present study could convincingly reflect the adverse effects of METH in other organ systems rather than to the brain and should direct our attention in awareness of using this drug.

Acknowledgement

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