

## EXPERIMENTAL STUDY

# Nod-like receptor protein 3 and nod-like receptor protein 1 inflammasome activation in the hippocampal region of postmortem methamphetamine chronic user

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**ABSTRACT**

**OBJECTIVE AND BACKGROUND:** Methamphetamine (Meth) is one of the most important central nervous system (CNS) stimulant abuse drugs that cause long-term or permanent damage to different regions of the brain, particularly hippocampus, by neuronal apoptosis and inflammation. In this study, we evaluated Nod-like Receptor Protein 3 (NLRP3) and Nod-like Receptor Protein 1 (NLRP1) Inflammasome Activation in the Hippocampal Region of postmortem Meth Chronic User.

**METHODS:** Molecular and histological analyses were conducted on the brain of 14 non-addicted and 11 Meth users separately. The expression level of NLRP1, NLRP3 was measured using western blotting and immunohistochemistry (IHC) techniques. Histopathological assessment was performed with stereological Cell Counting of hippocampal cells stained with hematoxylin and eosin (H&E). Moreover, Tunel staining was carried out in order to detect any kind of DNA damage.

**RESULTS:** Based on our findings using western blotting and immunohistochemistry assay, overexpression of NLRP1 and NLRP3 proteins in the hippocampal region of Meth addicts was observed. The stereological analysis in the hippocampus of the human brain revealed increased neurodegeneration. Furthermore, the increased rate of apoptosis and cell death were significant and confirmed by Tunel assay in the hippocampus of Meth groups.

**CONCLUSION:** Chronic Meth abuse could result in increases of NLRP1 and NLRP3 and induction of inflammation and apoptosis in the hippocampus in Meth groups (Tab. 1, Fig. 9, Ref. 40). Text in PDF [www.elis.sk](http://www.elis.sk).

**KEY WORDS:** methamphetamine, CA1, NLRP1, NLRP3.

**Introduction**

The abuse of Methamphetamine (Meth) has become widespread. Actually and it is considered as a growing problem. Meth is an extremely very addictive stimulant of the central nervous system (CNS). Its abuse is associated with many adverse side effects from which particularly, the dopaminergic and serotonergic systems

of the brain are more prominent could be disrupted (1). Meth is a new recreational drug that causes severe damage to different parts of the brain (2). Meth is also known as sympathomimetic drug that can cause several physical and psychological side effects such as uncontrollable repetitive movements, sweating, pupil dilation and also severe behavioral reactions (3). According to the US Food and Drug Administration, about 38 million people reported having an addiction to Meth and related derivatives (4). Meth is the most commonly abused substance after cannabis. Chronic Meth abuse leads to neurodegeneration of cortex, hippocampus and midbrain areas which are related by memory deficits (5). Severe symptoms of Meth abuse appear to be due to changes in the hippocampus (6). Not only Meth inhibits neurogenesis in the ventral spatial processing region, but it is also apparent that it could prevent cell death in the behavior-regulatory region of the hippocampus (7). Microglia are the primary CNS immune cells. They are derived from the myeloid lineage and they are involved in the pathogenesis of many neurological disorders and in neurologic pathologies as well (8). Meth use can abuse could result in neurotoxicity which leads leading to microglial activation. This can which could be either be neuroprotective or pro-neurotoxic in the brain. Based on imaging studies, microglial activation is seen in areas of dopaminergic and serotonergic innervation in the brain of patients in Meth users

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**Acknowledgements:** We are grateful for the funding provided by Skull Base Research Center, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences and Clinical Research Development Center of Loghman Hakim hospital, Tehran, Iran.

addicts (9). Activation of Nod-like Receptor Protein 3 (NLRP3) inflammasome can cause neuroinflammation mediated by microglia- and degeneration of dopaminergic neuron(10). Meth is known to reason microglial stimulation and activation of NLRP3 which is a cytosolic protein complex and secretion of interleukin-1 $\beta$  (IL-1 $\beta$ ) in an NLRP3 inflammasome-dependent way can induce secretion of several proinflammatory cytokines that are mediators of CNS inflammation during chronic stress and they play a key role in the induction of inflammation and the immune response development (11). Not only microbial infection but also endogenous signals of danger can cause the production of the NLRP3 inflammasome consisting of cytosolic sensor NLRP3, bridge protein apoptosis-associated speck-like protein containing a CARD (ASC), and cysteine protease caspase-1(12-15). Meth-induced NLRP3 inflammasome signaling pathway and its contribution to microglial activation is still unclear. This pathway is implicated in the neuroinflammatory process and in mediating neuroinflammation in the CNS based on recent studies. The NLRPs have many subfamilies and they are associated with the development of several neurodegenerative disorders such as multiple sclerosis (MS), Alzheimer’s disease (AD) and Parkinson’s disease (PD). Many studies have been conducted in order to explain the role of NLRs in their pathogenesis. NLRP3 is a primary mediator of Meth-induced inflammasome activation and in this review we mainly focus on its role in microglia (14). Strong evidence indicates that NLRP3 inflammasome participate actively in chronic sterile CNS inflammation and cause harmful

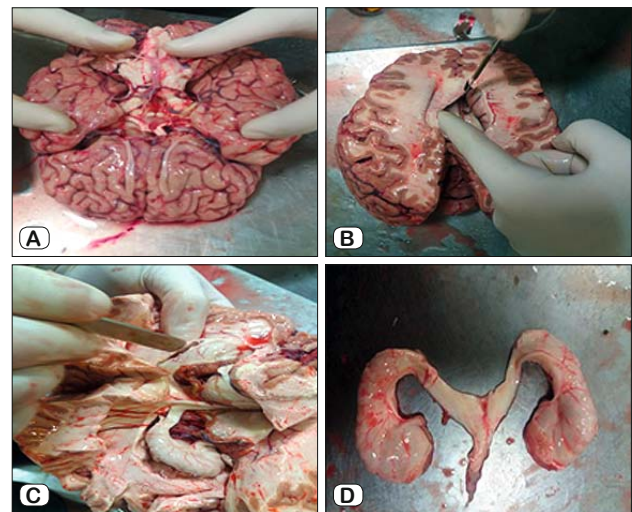


Fig. 1 shows the brain dissection and isolation of hippocampus in control and Meth groups.

effects (16). The critical character of NLRP3 in promoting neuronal injury was extra demonstrated in traumatic-induced injury (17). Damage-associated molecular patterns (DAMPs) may be released in neurodegenerative disorders and acute neuronal injury, and are able to act as endogenous danger signals and initiate a cas-

Tab. 1. shows the profile of the participants in the study. These individuals are divided into two groups.

The ultimate cause of death	Healthy dental pattern	Suicide attempt history	Duration of the consumption of meth (years)	Smoking history (years)	BMI	Age (years)	Sampling time after death (h)	Sample number
MI	29	–	–	3	26.4	41	1	N1
Accident	30	–	–	–	21.9	26	4	N2
MI	27	–	–	2	26.3	31	2	N3
MI	28	1	–	7	30.7	35	1	N4
Accident	29	–	–	–	19.8	28	1	N5
Accident	29	–	–	6	19.7	33	2	N6
Accident	32	–	–	–	20.8	31	13	N7
Accident	28	–	–	–	23.4	27	11	N8
MI	27	–	–	2	24.6	32	12	N9
Accident	30	–	–	1	21.3	23	10	N10
Accident	29	–	–	3	23.7	34	13	N11
Meth overdose	26	1	3	10	17.9	36	12	M12
Respiratory insufficiency after Meth	13	3	4	16	17.3	39	11	M13
Cardiac arrest after taking Meth	18	–	5	23	18.4	45	16	M14
Cardiac arrest after taking Meth	23	1	7	11	17.6	42	13	M15
Cardiac arrest after taking Meth	26	–	2	8	17.2	27	17	M16
Meth overdose	19	1	3.5	12	18.7	43	16	M17
Meth overdose	16	3	4	12	16.4	39	16	M18
Cardiac arrest after taking Meth	24	–	3	7	18.3	29	15	M19
Advanced respiratory infection following long-term Meth consumption	22	1	3	9	17.7	37	16	M20
Meth overdose	21	–	3	4	18.9	43	9	M21
Drowning following high Meth intake	27	–	1.5	3	19.4	22	14	M22
Brain hemorrhage following excessive Meth consumption	25	–	3.5	11	17.8	40	13	M23
Falling after taking Meth	14	1	4	15	17.1	46	9	M24
Meth Overdose	22	–	3	11	17.8	34	14	M25

N: control group, M: Meth group

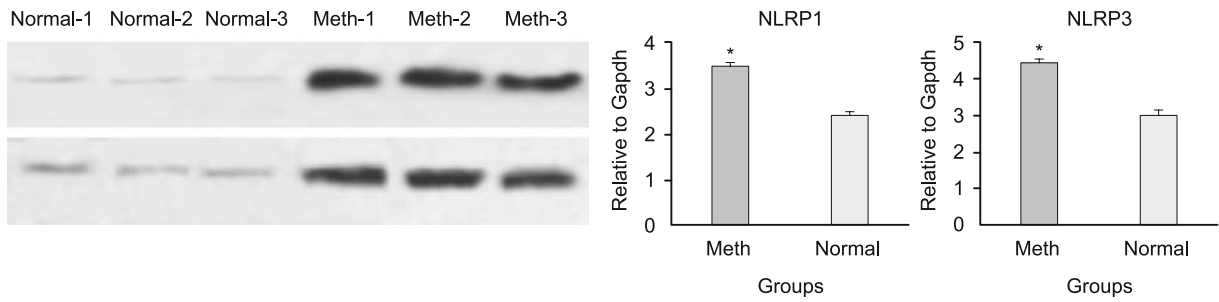


Fig. 2. Western blot analysis of protein expression level of NLRP3 (upper row) and NLRP1 (lower row) in the hippocampal CA1 region in Meth and control group. NLRP3 and NLRP1 expressions were increased in the Meth group Compared to the control group ( $p < 0.05$ ).

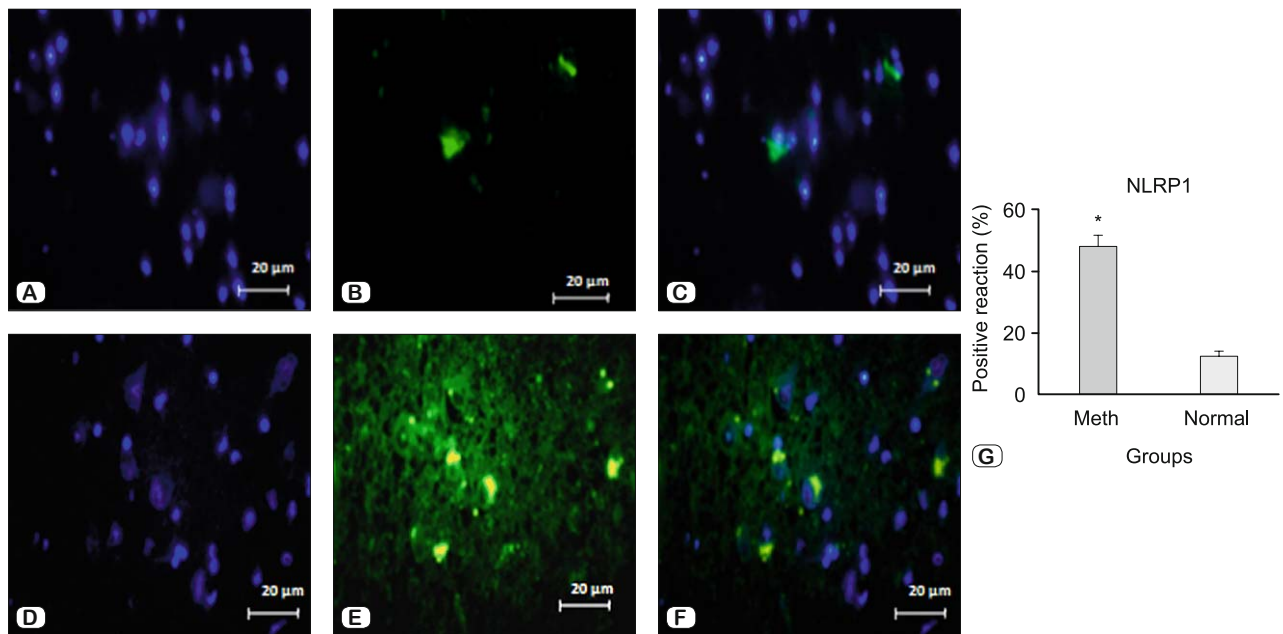


Fig. 3. Immunofluorescence staining in the hippocampal CA1 regions of Control (upper row) and Meth (lower row) group. NLRP1 protein levels increased in the CA1 region of hippocampus of Meth group compared to control group ( $p < 0.05$ ). A&D: nuclei stained by DAPI (blue). B&E: primary antibody to NLRP1 (green). C&F: Merge.

cade of danger-associated intracellular signaling pathways subsequently, which activate and promote the NLRP3 inflammasome that induces the noninfectious inflammatory signaling in response to neuronal danger signals (18). Generally, the NLRP3 inflammasome signaling pathway is a specific inflammatory pathway implicated in the initiation and perpetuation of neurotoxicity. Thus, blockade of NLRP3 inflammasome activation might be a promising therapeutic strategy (19). Based on the before mentioned information, the purpose of this study was to explore whether lifetime Meth abuse can induce NLRP1 and NLRP3 upregulation and neuronal apoptosis in the CA1 region of a postmortem hippocampus.

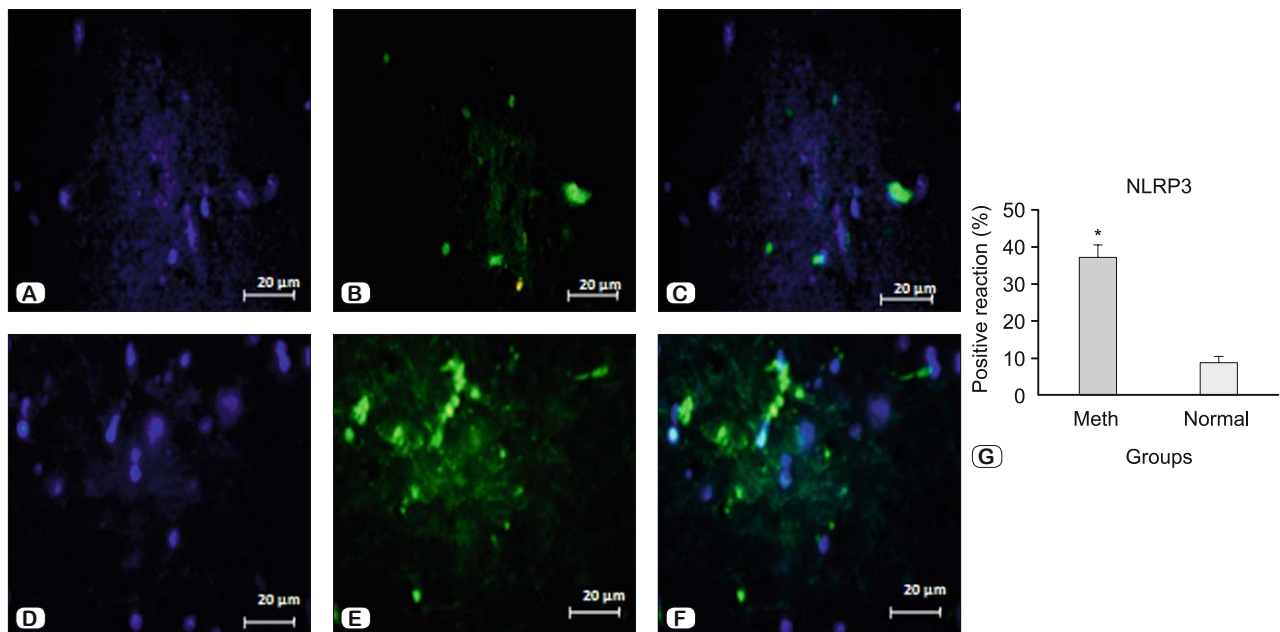
### Material and methods

Postmortem human brains were collected from Iranian Legal Medicine Organization. Our ethical consideration in the preservation and sampling of the human brain was based on the Dec-

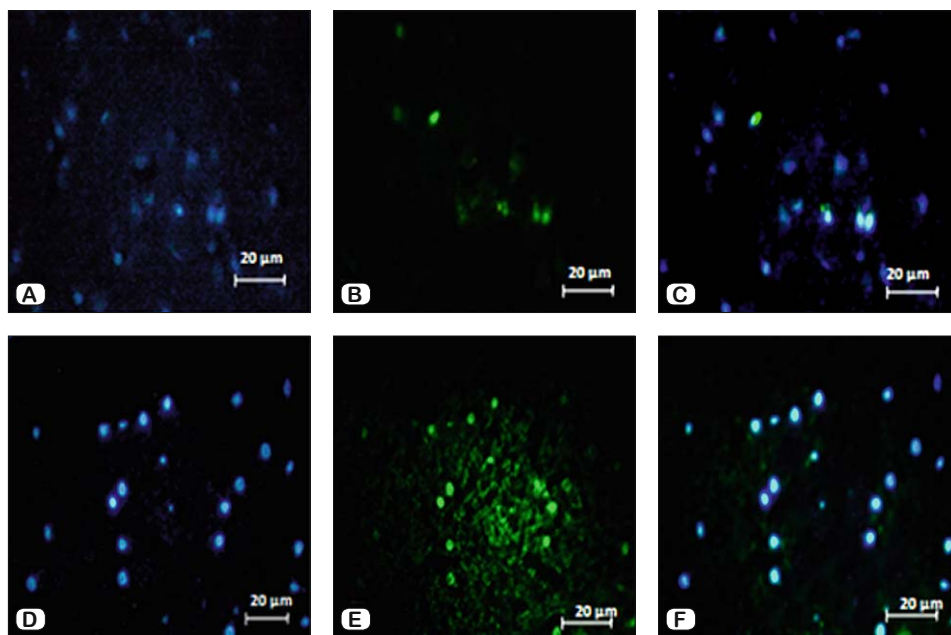
laration of Helsinki. All procedures were accepted by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1396.542). The relatives of the deceased persons provided oral consent for the brain donation for purpose of research. The analysis was performed on 14 chronic male Meth users (aged  $39 \pm 1.9$  years) with a drug overdose death. The control group consisted of 11 male adults without any exposure to Meth (aged  $38 \pm 2$  years). In order to prevent post mortem delay, all Control and Meth groups were analyzed within a minimum period of time after death. None of the Meth users had a history of mental illness or neurodegenerative disorders. In addition, none of them was HIV-positive. Methamphetamine drug testing kits were used to detect Meth in the urine samples of both groups.

### Sampling and tissue preparation

After obtaining informed consent from the family of the deceased, freshly isolated brain tissues of 14 documented Meth



**Fig. 4.** Immunofluorescence staining in the hippocampal CA1 regions of Control (upper row) and Meth (lower row) group. NLRP3 protein levels increased in the CA1 hippocampus in Meth group compared to control group ( $p < 0.05$ ). A&D: nuclei stained by DAPI (blue). B&E: primary antibody to NLRP3 (green). C&F: merge.



**Fig. 5.** Examination of apoptosis and DNA damage by TUNEL assay in Control (upper row) and Meth (lower row) group. Apoptosis increased in the CA1 region of hippocampus of Meth group compared to control. A& D: nuclei stained by DAPI (blue). B&E: apoptotic cells (green). C&F: Merge.

users and 11 non-addicted from the control group were transported to the lab in lactated Ringer’s solution. After removing blood clots and necrotic tissue, all samples were weighed. For Hematoxylin and Eosin (H&E), Nissl and Golgi staining, immunohistochemistry and tunel assay after dissociation of hippocampus from the brain, it was fixed in 10 % formalin for 1

week. Hippocampus was embedded into paraffin blocks and a microtome was used to cut the hippocampus in its longitudinal axis into 5 and 25 μm thick sections (Fig. 1).

*Western blotting*

Proteins expressions of NLRP1, NLRP3 were analyzed in the CA1 region of the hippocampus using Western blotting assay. First, tissues were washed by PBS two times and disrupted and homogenized with a sonicator, combined with buffer and transferred to micro-centrifuge tubes and the mixture was centrifuged for 15 minutes. Then it was applied to gel electrophoresis in Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) to sort the proteins. Afterwards, the separated protein bands were transferred

to polyvinylidene difluoride (PVDF) and finally they were examined by primary antibody including rabbit polyclonal anti-NLRP1 (1:200), and human polyclonal anti-NLRP3 (1:200) for detection. The secondary antibodies were conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology, USA). For confirming effective protein transfer during the test, the loading



control antibody GAPDH generation signal 21 was used.

#### Immunohistochemistry

The brains were Post-fixed (1 week) in 4 % paraformaldehyde and then transferred to 30 % sucrose (Sigma-Aldrich, Germany) solution and were allowed to equilibrate. Afterwards the hippocampuses were sectioned (8 µm in thickness) and stored at -20 °C in a cryoprotectant buffer containing 25 % ethylene, 25 % glycerine and 0.05 M phosphate buffer (all from Sigma-Aldrich, Germany). All slices were incubated with human monoclonal anti-NLRP1 and anti-NLRP3 (Abcam, USA) antibody and diluted to 1:100 overnight in the primary reaction. This process was followed by a similar washing using PBS, and one-hour incubation with goat anti-mouse FITC-conjugated secondary antibody (ab6785, Abcam, USA) at a 1:100 dilution in the second reaction. Finally, the tissue sections were washed with PBS, and the nuclei counterstained using DAPI.

#### Stereological study

##### The mean volume of neuron

The neurons volume was measured by using the nucleator method and calculated with the following equation:

$$V_N = 4\pi/3 \times L_n^3$$

In this formula  $L_n$  is two horizontal directions and was considered from the central point within the nucleolus to the cell or nucleus borders.

#### TUNEL assay

In situ Cell Death Detection kit was used to perform TUNEL assay (fluorescence, Roche, CH). After fixing the CA1 region of the hippocampus, it was embedded in paraffin, and mounted on glass slides. Then, the paraffin was removed using xylene and it was rehydrated in a piecemeal series of ethanol. After washing with water, TUNEL staining was performed and the TUNEL protocols were applied. The results of Meth and control groups were evaluated by Image J software 24.

#### Statistical analysis

Statistical analysis was conducted using IBM SPSS software, version 20.0. The significance level was analyzed using the analysis of variances (ANOVA). Also, for the other statistical comparisons of multiple means in the groups, one-way ANOVA and Tukey's post hoc test were used. Data in the histograms are presented as mean±SEM. p values <0.05 were considered statistically significant.

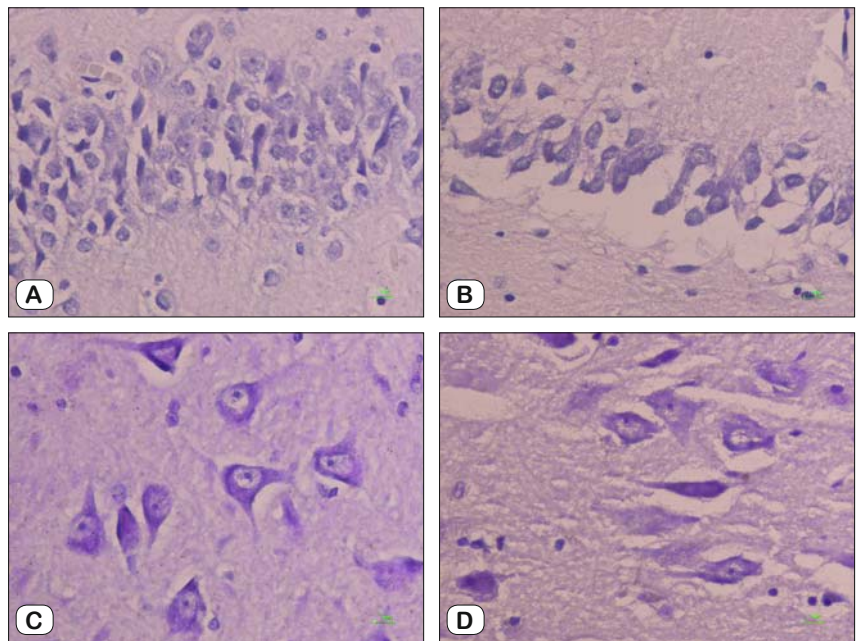


Fig. 6. Photomicrograph of the hippocampus stained with Nissl staining. The neurons in CA1 in Meth group are shrank and piknotic. (A and C) Control group; (B and D) Meth group (magnification: 40X).

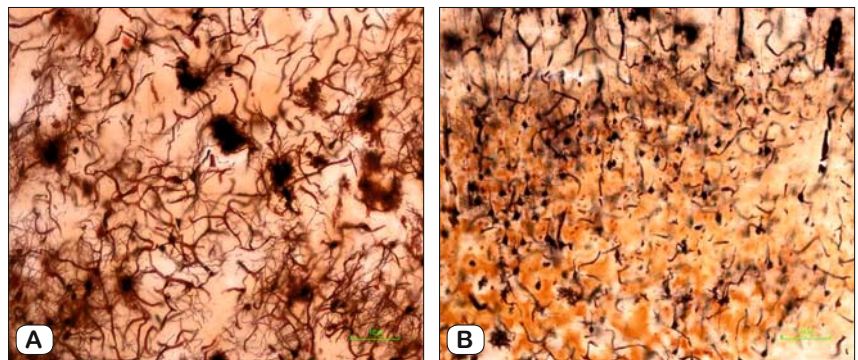


Fig. 7. Photomicrographs of the hippocampus stained with Golgi (magnification: 10X). (A) Control group and (B) Meth group; the total length of dendrites was also decreased in Meth groups in comparison with the control group.

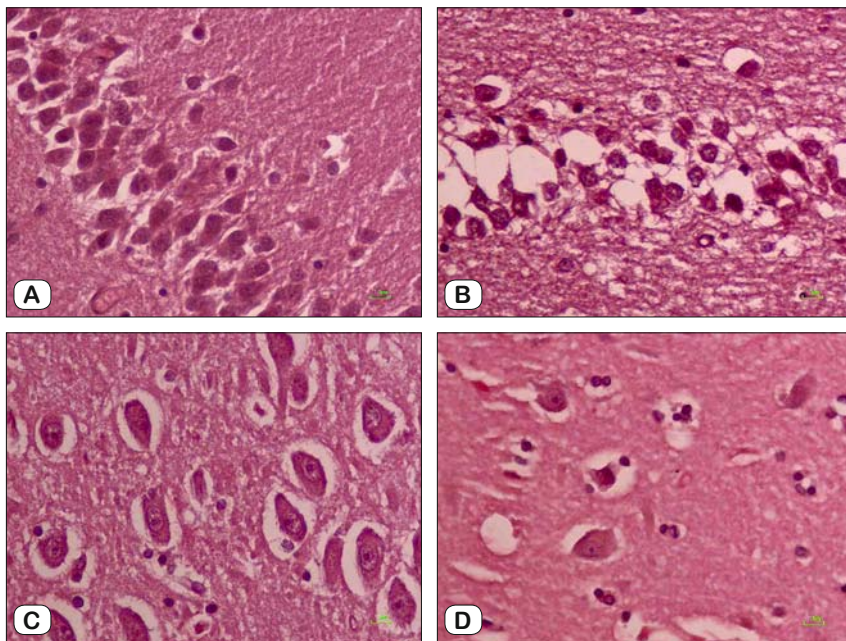
## Result

#### Western blot analysis

NLRP1 and NLRP3 protein expression was determined by Western blot assay. Figure 2 shows the expression of NLRP3 and NLRP1 in the CA1 region of the hippocampus in Control and Meth groups. Western blot analysis showed that NLRP1 and NLRP3 protein expression was significantly increased in the Meth group compared to the control group ( $p < 0.05$ ). These results confirmed that protein expression level of NLRP3 and NLRP1 increased 0.5-fold in the area CA1 of the hippocampus in the Meth group compared to control group.

#### Immunostaining analysis

The immunohistochemical analyses showed inflammation in the CA1. Level of anti-NLRP1 and anti-NLRP3 antibodies were



**Fig. 8. Photomicrograph of the hippocampus stained with H&E. The total number of neurons and glial cells in Meth groups decreased in comparison with the control groups. (A&C) Control groups and (B&D) Meth groups. (Magnification 40X).**

analyzed in the CA1 region of hippocampus in Meth and control groups in order to detect the presence of these markers (Figs 3 and 4). As shown in these figures, expression of these protein markers was elevated in the CA1 of the Meth group in comparison with the control group.

#### *Tunel assay*

After staining the nuclei with DAPI fluorescent dye, the apoptotic cells became Tunel positive and nuclei were merged with positive reaction cells (Fig. 5). Based on the results of Tunel assay (Fig. 5) the average of Tunel positive cells (count)/103 mm<sup>2</sup> was 50 % in the Meth and 15 % in the control group.

#### *Hematoxylin and Eosin (H&E), Nissl and Golgi staining analysis*

The result of H&E and Nissl staining showed that the densities of cells are significantly different based on staining results in the Meth and the control groups. The number of cells in control group was significantly higher than in the Meth group. The result of Golgi staining showed that the total length of dendrites was also decreased in Meth groups in comparison with the control groups (Figs 6, 7 and 8).

#### *The volume of neurons*

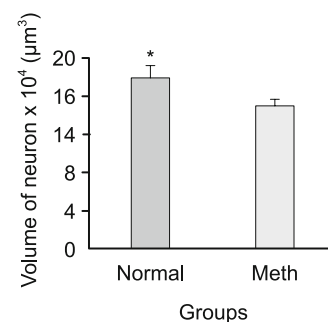
The result of H&E staining showed that the volume of the neuron was significantly reduced in the Meth groups in comparison with the control groups ( $p < 0.001$ ) (Fig. 9).

### **Discussion**

In this study, we have clarified the role of Meth in activating NLRP1, NLRP3 in the hippocampal region. Our results showed

that the expression of these markers in Meth group will in long term cause neurodegenerative changes in the hippocampal region. The activation of NLRP3 inflammasome is implicated in microglia-mediated neuroinflammation and dopaminergic neurodegeneration (22). Since in the rodent model exposed to Meth, activated NLRP3 inflammasome was observed, targeting or inhibiting the NLRP3 inflammasome could be a promising strategy for the treatment of drug abuse (23). Abusing Meth which is a strong CNS stimulant may induce neuroinflammation and microglial activation and leads to behavioral and cognitive changes. The precise mechanisms underlying Meth-mediated microglial activation continue to await clarification (24). In this study, after exposing cells to Meth an increase in the level of NLRP1, NLRP3 expression and activation of microglia were reported based on evidence demonstrating morphological changes in cells. Based on our immunostaining analyses, two possible mechanisms

for Meth-induced changes to the hippocampus have been proposed. Within the hippocampus, we found increases of NLRP1, NLRP3 expression in Meth group. By interruption of intracellular calcium signaling, Meth could change neural hemostasis leading to tissue damage. Growing evidence has suggested that activating NLRP3 inflammasome may induce neuroinflammation mediated by activated microglia in neurodegenerative disorders such as Parkinson's disease (25) and Alzheimer's disease (26). Surprisingly, there is an association between NLRP3 and depression-like behavior according to recent findings (27). After knowing that Meth could activate the NLRP3 inflammasome, the next step was clarifying the upstream mechanisms of regulating NLRP3 activation. Inflammasomes are cytosolic protein complexes that mediate the activation of caspase-1 and IL-1 $\beta$  maturation. The complex regulatory mechanisms remain unclarified. Potassium influx (28),



**Fig. 9. Volume of the neuron in control and Meth group. Result showed that the volume of the neuron was significantly decreased in the Meth groups in comparison with the control groups.**



endoplasmic reticulum stress (29, 30), reactive oxygen species (ROS) and nitric oxide (31, 32) have been considered contributors to activation of the NLRP3 inflammasome. Consistently, Meth increased ROS production in microglia based on our findings (33). Meth abuse can cause cognitive deficits by its neurotoxic effects that are able to induce long-lasting structural and functional changes to the brain (34). Most of the studies of Meth on the human brain come from applying magnetic resonance imaging (MRI) techniques in cross-sectional studies of adult Meth users. In the brain of adult Meth users, several structural abnormalities were evident in the frontal lobe, including decreased gray matter density or volumes (35), increased white matter volumes (36), and increased white matter hyperintensities (37). On the other hand, in the temporal lobe, smaller volumes are evident (38), including the hippocampus (39); but in the parietal cortex and striatal regions, larger volumes have been observed (40). Histologic and stereological results showed that the neurons quantity was decreased in the brain tissue, but glial cells increased. The neurons were shrunk and some of the cells suffered cell death and apoptosis. This study focuses on the effects of Meth in both white and the grey matter of the brain; In fact, Meth may have several effects on the brain using different signaling pathways. Thus, combined immunohistological studies on meth exposed mice are required to elucidate the molecular and cellular mechanisms underlying hippocampus changes. Our results recommend that investigation of histological changes and microglial NLRP3 activation in the hippocampus which that useful in human studies and could be targeted in the treatment of Meth abusers.

## Conclusion

Chronic Meth abuse could result in up regulation of NLRP1 and NLRP3 and induction of neuronal apoptosis in the CA1 of the hippocampus in postmortem human brain.

## Study limitations

Inadequate access to Meth user's corpses, lack of information on accurate amount of consumption, and late access to the corpses after death are among the limitation of the study.

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Received May 11, 2019.

Accepted June 17, 2019.