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Proteasome inhibition by MG-132 protects against deltamethrin-induced apoptosis in rat hippocampus

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ABSTRACT

Aims: Deltamethrin (DM), a type II synthetic pyrethroid insecticide, is widely used in agriculture and home pest control. The evaluation of their toxic effects is of major concern to public health. However, the molecular mechanism of DM-induced neurodegenerative disease is still far from clear. This study was designed to investigate the potential role of ubiquitin proteasome system (UPS) in DM-induced neurotoxicity where the proteasome inhibitor MG-132 could mitigate the neurotoxic effects.

Main methods: Male Sprague-Dawley rats were divided into two batches. The first batch of rats was administrated with a single dose of DM (12.5 mg/kg) by intraperitoneal injections (i.p.) and the animals were then euthanized at 5, 24, and 48 h post injection. The second batch was treated as follow: control group, DM (12.5 mg/kg) groups for 24 h, MG-132 (0.5 mg/kg, i.p.) 2 h plus DM 24 h group, and MG-132 alone group. Ubiquitinatied proteins, DNA damage and apoptosis were investigated.

Key findings: DM treatment induced the ubiquitinated proteins expression with the peaks at 5 h. Moreover, DM increased DNA damage, early apoptotic rate, the expression level of Cleaved Caspase-3, caspase-3 activity and decreased the expression level of Bcl-2 at DM 24 h group. Compared to DM 24 h group, MG-132 pretreatment significantly down-regulated ubiquitinated proteins, lowered the DNA damage and apoptosis by decreasing Caspase-3 and increasing Bcl-2 expression.

Significance: These results indicate that MG-132 effectively alleviates DM-induced DNA damage and apoptosis by inhibiting ubiquitinated proteins. UPS may play a role in DM-induced neurodegenerative disorders.

1. Introduction

Pyrethroid pesticides are widely used because of their high insect selectivity, and low persistence of residues [1]. Nonetheless, exposure to pesticides can cause neurotoxic effects to mammals [2]. Deltamethrin (DM), one of the type II pyrethroids, is reported to have toxic effects from both epidemiological and experimental studies [3,4]. In recent decade, the possibility has been raised that DM exposure is linked to neurodegenerative diseases. It was indicated that exposure to DM was associated with poorer neurocognitive ability in six-year old children [5]. Besides, DM is a potent inducer of Brain-derived neurotrophic factor (BDNF) expression in the hippocampus leading to neuronal hyperexcitation [6]. Furthermore, DM could cause spatial learning and memory deficits and neuronal loss in the hippocampus indicating that DM exposure might induce Alzheimer's disease (AD)-like pathology and cognitive abnormality in rats [7]. Previous studies in our lab have demonstrated that DM altered the expressions of P53, Bax, Bcl-2 and cytochrome C in rat hippocampus [8,9] suggesting that apoptosis may play a crucial role in neurotoxicity induced by DM. Yet the underling mechanisms are poorly understood.

The ubiquitin proteasome system (UPS) is a vital target for cell cycle regulation, and is the major extra-lysosomal pathway responsible for intracellular protein degradation in eukaryotes [10]. Alterations in the UPS may result in neurological disorders [11]. For instance, UPS activity diminishes in the cortex and hippocampus of the AD brain and also decreases with ageing [12], indicating that protein clearance mechanisms are directly linked to ageing and age-associated neurodegenerative diseases [13,14]. In addition, proteasome inhibition plays a role in mediating proteins observed in both AD and Parkinson's disease (PD) [15]. Based on these properties, UPS may play an important role in

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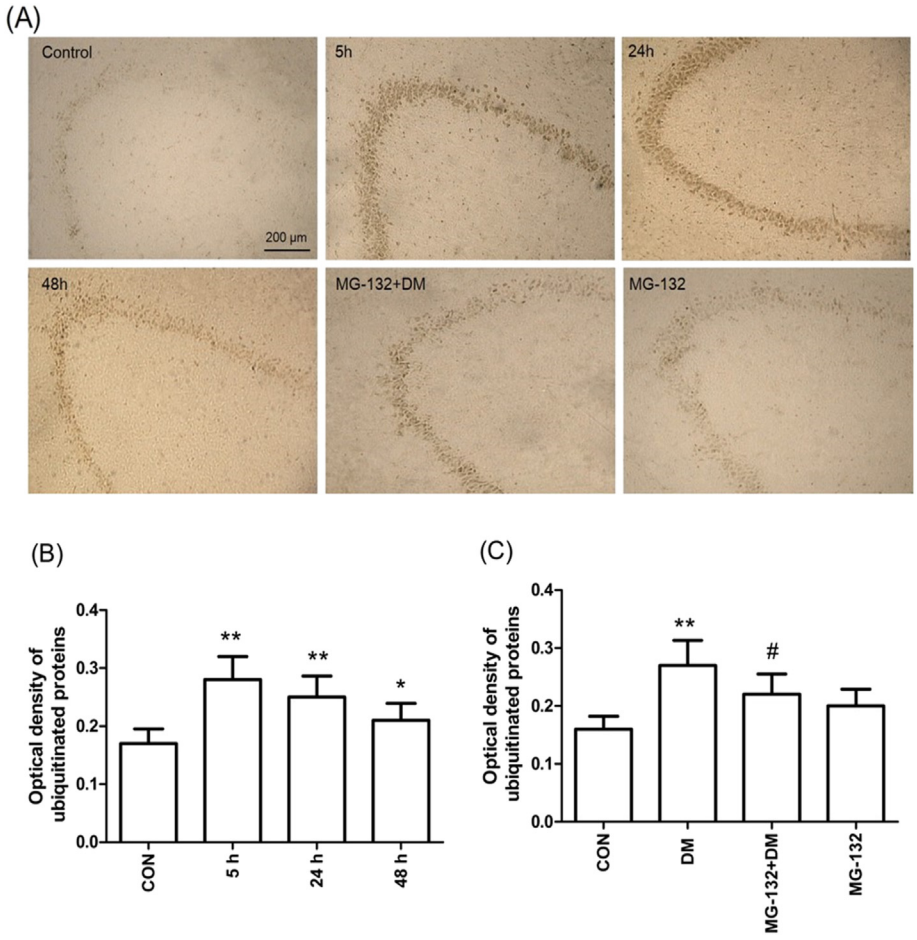

Fig. 1. Representative ubiquitinated proteins immunohistochemistry in rats hippocampus of different experimental groups. (A) Positive ubiquitinated proteins were stained in the CA3 region. Graph B indicated the increase number of ubiquitinated proteins induced by DM (12.5 mg/kg) in the whole region of hippocampus. Graph C displayed the impact of the proteasome inhibitor MG-132 on DM's inductive effect. When pretreated with MG-132 (0.5 mg/kg) for 2 h then administrated with DM, ubiquitinated proteins were decreased significantly. Scale bars: 200 μm. The values represent mean ± SD from 5 animals per group. Compared to the control group, $^{**}P < 0.01$, $^{*}\ P < 0.05$. Compared to DM 24 h group, $\#\ P < 0.05$.

DM-induced neurodegenerative disease. However, whether the UPS is involved in DM-induce toxicity and proteasome inhibition can alter its toxic effects remain to be elucidated. The present study is aimed to investigate effects of proteasome inhibitor MG-132 on the neurotoxicity induced by DM in a rat model.

2. Materials and methods

2.1. Materials

Deltamethrin (98.5% purity) was purchased from (Romainville Cedex, France). Corn oil was “Golden dragon fish” brand purchased from Carrefour supermarket (Wuhan, China). MG-132 (0.5 mg/kg, dissolved in DMSO) was purchased from Sigma (USA). Annexin-V-FITC/PI kits were purchased from Bender MedSystem, Austria. Primary antibodies (Bcl-2, Ubiquitin, GAPDH, Santa Cruz Biotechnology, Dollas, USA; Caspase-3, Cell Signal Technology, USA) respectively dissolved in the same diluted buffer (Beyotime Biotechnology, Shanghai, China). The peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dollas, USA). Polyvinylidene difluoride (0.22 μm, PVDF) membrane and enhanced chemiluminescent (ECL) were purchased from Merck group, Germany. All other chemicals were reagent grade of the highest laboratory purity available.

2.2. Animals and treatments

Eighty male Sprague-Dawley rats, of a specific pathogen-free grade (SPF), weighing 200–220 g were provided by the Animal Experimental Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, Hubei, China), and housed in the animal facility complied with the Guide for the Care and Use of Laboratory Animals published by Ministry of Health of People's Republic of China. After acclimated for one week, rats were randomly divided into two batches. Each batch had four groups with 10 rats/group. The first batch of rats received 12.5 mg/kg DM (dissolved in corn oil) by intraperitoneal injection (i.p.). Then animals were then euthanized 5 h, 24 h, or 48 h post the injection. The second batch of rats was administrated as follow: corn oil as control, DM (12.5 mg/kg, i.p.) for 24 h, MG-132 (0.5 mg/kg dissolved in DMSO, i.p.) 2 h prior to the 24 h-DM (12.5 mg/kg) treatment and MG-132 alone for 24 h. The dose of DM used in this study is 1/10th of LD_{50} based on our prior study [9] and did not cause any classic signs of toxicity, including tremor, salivation, and ataxia in rat. The dose of MG-132 was referred to the work of Wojcik et al. [16]. Rats were euthanized by decapitation. The whole brains of five animals from each group were frozen at −80 °C for immunohistochemical analysis later. The whole brains from the other five animals were dissected to harvest hippocampus from both hemispheres immediately. Hippocampus from one hemisphere was prepared into the unicellular suspension for flow cytometry analysis and comet assay. The other hemisphere's hippocampus was frozen at −80 °C for protein detection using Western blotting.

2.3. Immunohistochemical assay

The whole brain tissue samples were collected after decapitation and immediately frozen with liquid nitrogen. Prior to immunohistochemical assay, 15 μm frozen sections were prepared with a cryostat microtome (Leica Jung CM 1800, Leica, Germany) at −20 °C and fixed with acetone. The sections of hippocampus were incubated with polyclonal antibody against ubiquitin (Santa Cruz Biotechnology,

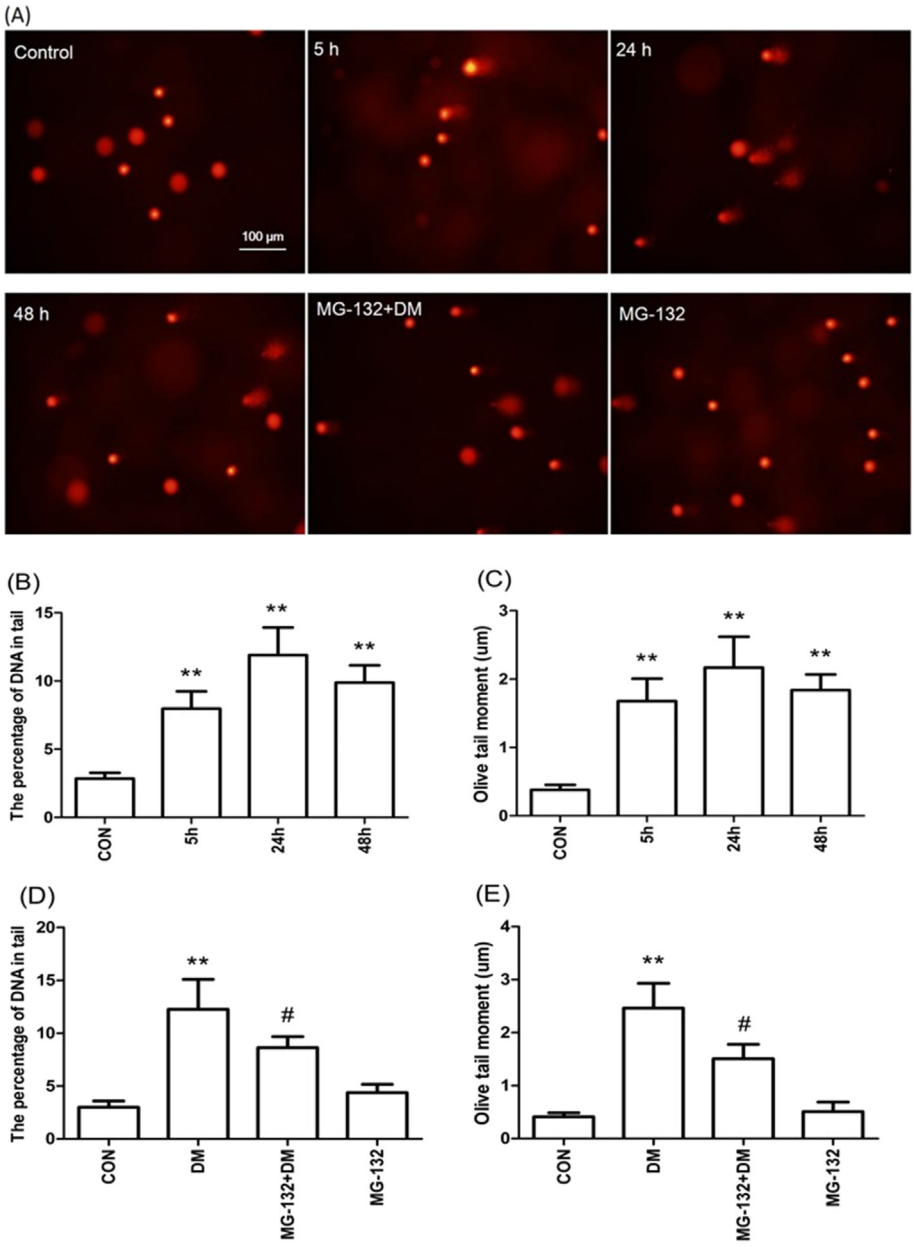

Fig. 2. DM treatment causes DNA damage in hippocampal cells by comet assay. The hippocampal cells were processed using single cell gel electrophoresis (comet) assay as described in Materials and methods. Fig. 2A shows that the number of comet cell (with comet tail) was increased significantly. (B, C) The percentage of DNA in tail was increased and OTM were longer following the DM treatment as compared to the control group ($P < 0.01$). (D, E) The pretreatment with MG-132 mitigated these damage effects induced by DM remarkably. MG-132 treated alone group didn't show any effect on DNA damage. The values represent mean ± SD from 5 animals per group. Compared to the control group, $^{**}P < 0.01$, Compared to DM 24 h group, # $P < 0.05$. Scale bars: 100 μm.

Dollas, USA, 1:400 in TBS) overnight at 4 °C, followed by secondary antibody at 37 °C for 1 h. The avidin-biotin-peroxidase complex immunohistochemical assay was carried out according to the protocol published by Wu's [8] (Santa Cruz Biotechnology, Dollas, USA). The ubiqutinated proteins were detected by DAB kit. Images were captured with a Leica Microsystems (TYPE DM LB 2, Germany). To measure the area contains ubiquitinated proteins positive cells in the hippocampus, we obtained five images each hippocampus region (CA1, CA2, CA3 and CA4) each animal. The quantification of the positive cells was measured automatically with the Microsystems at a total magnification of ×100 with CMIAS system.

2.4. The comet assay

Fully frosted microscope slides were dipped into 1% hot (50 °C) normal melting agarose (NMA) prepared in PBS. The slides were dried overnight at room temperature and stored at 4 °C for later use. Second layer were dipped into 0.5% low melting point agarose (LMA) and placed on coverslips. Twenty minutes later, the coverslips were removed, and the prepared cells (10,000) were mixed with 0.7% LMA and placed on the second layer. The slides were maintained at 4 °C for 15 min to solidify. Slides were then carefully immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) with 1% TritonX-100 and 10% DMSO added just before use, for at least 90 min at 4 °C. Following lysis, all slides were left in fresh electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13) for 20 min to denature the DNA. Subsequently, the DNA was electrophoresised for 25 min at 300 mA and 25 V. After electrophoresis, the slides were taken out and left in Tris buffer (0.4 mM, pH 7.5) to neutralize the excess alkali. To prevent additional DNA damage, all steps described above were conducted in dark and at 4 °C. After neutralization, the slides were stained with 50 μL of 5 μg/mL ethidium bromide solution and covered with coverslips and analyzed with fluorescence microscopy (Olympus, Japan) at a total magnification of ×200 with an excitation filter of 549 nm and a barrier filter of 590 nm. One hundred randomly chosen cells for comet assay were collected from two replicated slides per treatment. All the process described above was executed in the dark. The experiments were repeated three times independently. It has been shown that the

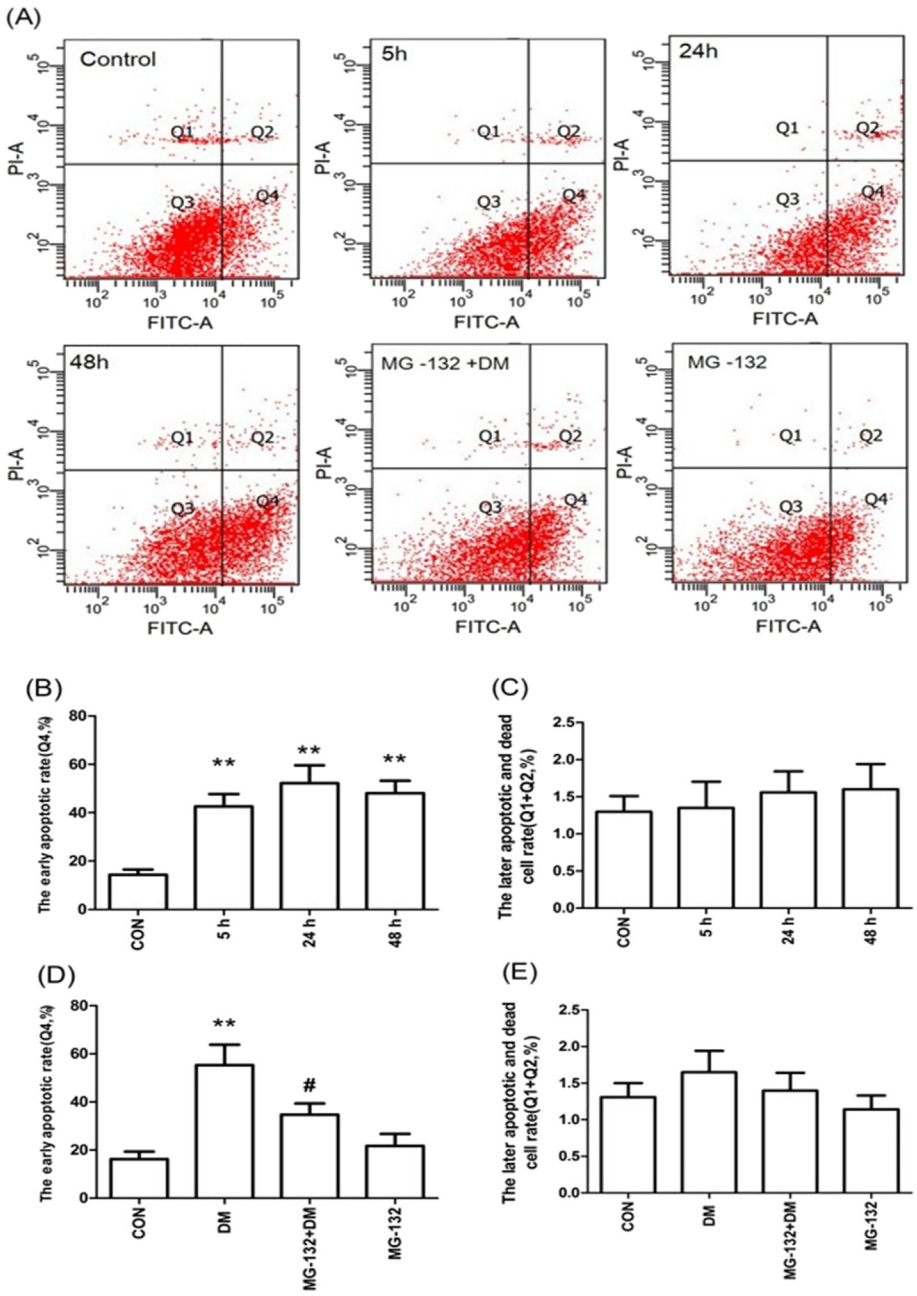

Fig. 3. Apoptotic change detected by Annexin V/PI assay. (A) Q1 = death cells; Q2 = the later apoptotic cells; Q3 = viable cells; Q4 = the early apoptotic cells. (B) The percentage of the early apoptotic rate (Q4) was induced by DM for 5 h, 24 h, 48 h respectively. (C) The percentage of later apoptotic and dead cells rate (Q1 + Q2) didn't change significantly. (D) The percentage of the early apoptotic rate (Q4) in MG-132 pretreated hippocampus was decreased. (E) The percentage of later apoptotic and dead cells rate (Q1 + Q2) didn't change significantly. The values represent mean ± SD from 5 animals per group. Compared to the control group, $^{**}P < 0.01$. Compared to DM 24 h group, # $P < 0.05$.

percentage of DNA in the tail (% DNA_T) and olive tail moment (OTM) are the most useful measurements [17]. CASP software (Poland) was used to measure percentage of DNA in tail (% DNA_T) and Olive tail moment (OTM). The person who analyzed the slides was blinded to exposure status.

2.5. Flow cytometry analysis

Annexin-V-FITC/propidium iodide (AV-FITC/PI) assay was utilized to measure the level of apoptosis induced by DM with or without MG-132. The unicellular suspension of hippocampus was prepared according to the method of neuron primary culture with a little modification. Briefly, hippocampus were taken out rapidly, cut into small pieces and washed twice in PBS. Then the pieces were digested in 0.25% trypsin (Amresco, USA) for 20 min at 37 °C. The digested cells suspension was washed with PBS and the cell concentration was adjusted to $2 * 10^6$/mL. Finally, the cell pellet was double stained by AV-FITC and PI (Annexin-V-FITC/PI kit, Bender MedSystem, Austria) successively. The stained progress was performed in a dark place to avoid direct light exposure. After stained for 30 min samples were immediately analyzed using a Flow Cytometer (FACScan, BD Biosciences, Milano, Italy) with dedicated software.

2.6. Western blotting analysis

Hippocampal tissues were homogenized with lysing buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 0.5%NP-40, 1%TritonX-100, 1 mM PMSF and 1 μg/mL aprotinin). Protein concentrations were determined by Bradford [18] assay. Sixty microgram of protein from each sample was subjected to electrophoresis in 15% (w/v) SDS–polyacrylamide gel and transferred to 0.22 μm polyvinylidene difluoride (PVDF, Millipore, USA) membrane. Membranes were blocked with tris-buffered saline (TBS), 0.1% (v/v) Tween-20, and 5% (w/v) non-fat dry milk at room temperature for 1 h, followed by an overnight incubation with primary antibody (Bcl-2 1:400, GAPDH 1:2000, Santa Cruz Biotechnology, Dollas, USA; Caspase-3 antibody 1:1000, Cell Signal Technology, USA)

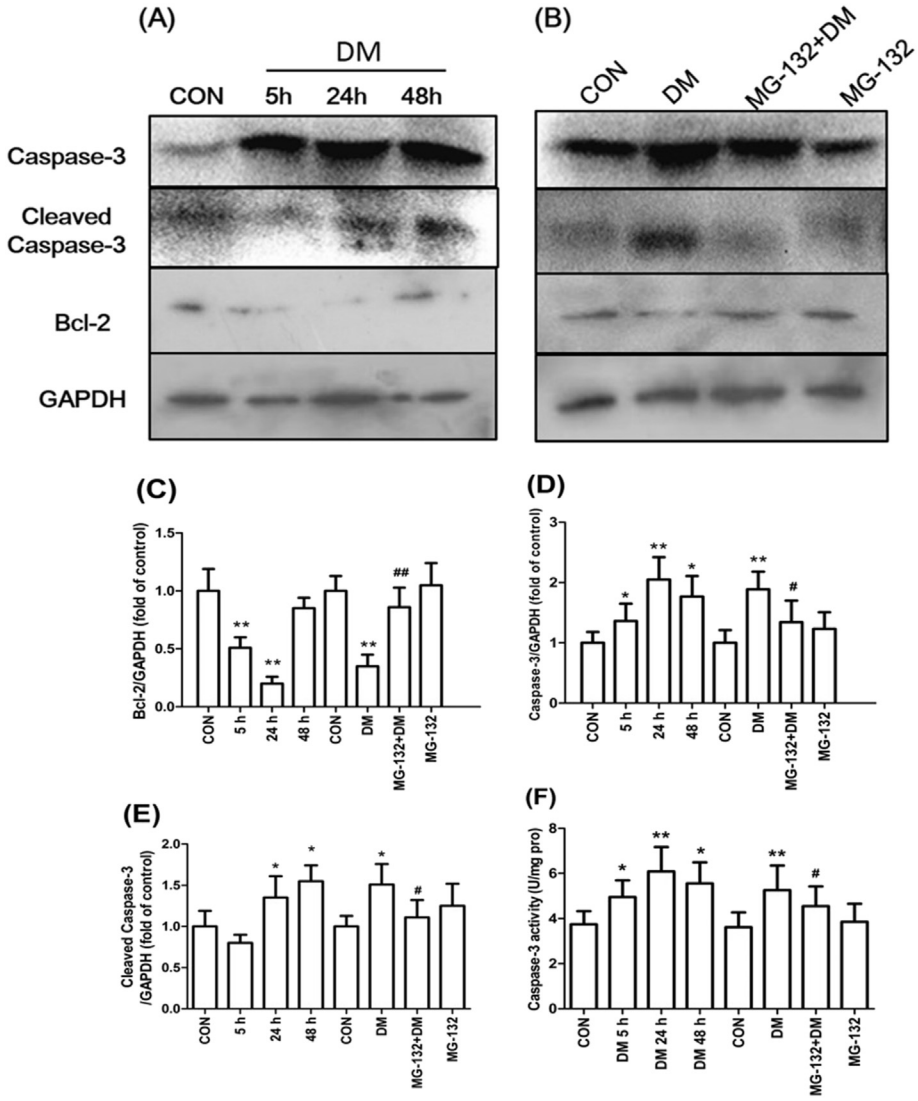

Fig. 4. Effects of DM treatment on apoptosis-regulatory proteins. Rats were treated with DM for 5 h, 24 h or 48 h. Hippocampal cell lysates were prepared and analyzed for the protein expressions of Bcl-2, Caspase-3 and Cleaved Caspase-3 expression, as well as the activity of caspase-3. Relative densities are presented in bar graphs and a representative blot is provided above the graph. GAPDH was used as a loading control. Caspase-3 activity was detected by assay kit using microplate reader. (A, C) Bcl-2 expression was significantly decreased at 5 h and 24 h but not 48 h. (A, D) Caspase-3 was increased at 5 h, 24 h and 48 h. (A, E) Cleaved Caspase-3 was increased at 24 h and 48 h but not 5 h. (B, C, D, E) When pretreated with MG-132, the expression of Bcl-2, Caspase-3 and Cleaved Caspase-3 in hippocampus were restored. (F) Caspase-3 activity showed the same tendency as the protein expression of Caspase-3 expression. The values represent mean ± SD from 5 animals per group. Compared to control group, $^{**}P < 0.01$, $^{*}P < 0.05$; Compared to DM 24 h group, $\#\#P < 0.01$, $\#P < 0.05$.

respectively dissolved in the diluted buffer (Beyotime Biotechnology, Shanghai, China) at 4 °C. After washing with 0.1% (v/v) Tween 20–TBS buffer, the membrane was incubated with peroxidase-conjugated secondary antibody (1:1000, Santa Cruz Biotechnology, Dollas, USA) at 37 °C for 1 h and then washed. Protein bands were detected with the enhanced chemiluminescent (ECL, Millipore, USA) detection system. Densitometric analysis of immunoblots was performed with the Gel pro 3.0 software.

2.7. Determination of Caspase-3 activation

The activity of caspase-3 was determined using the caspase-3 activity kit (Beyotime Biotechnology, Shanghai, China). To evaluate the activity of caspase-3, tissue samples were homogenized using lysis buffer containing 50 mM Tris-HCL (pH 7.4), 1 mM EDTA, 1% TritonX-100, 0.5% NP-40 and 1 mM PMSF and centrifuged at 10,000 ×*g* for 15 min at 4 °C. The supernatants containing 5 μg/μL proteins [18] were incubated with 2 mM Ac-DEVD-*p*NA (caspase-3 substrate) at 37 °C for 2 h. The products formed were measured using a plate reader (Molecular Devices, SpectraMax M2) at wavelength of 405 nm.

2.8. Statistical analysis

Statistical analyses were performed by using SPSS 16.0 software. The significance of the difference between control and experimental groups were examined by analysis of variance (ANOVA). Reported *P*-value < 0.05 were considered statistically significant. The data were expressed as Mean and standard deviation (SD) for each parameter.

3. Results

3.1. DM exposure induces the expressions of ubiquitinated proteins

To evaluate the effect of DM on UPS, we performed immunohistochemistry to detect the express of ubiquitinated proteins. In the hippocampus, neurons in CA1, CA2, CA3 and CA4 regions were strongly stained following the DM treatment for 5 h, 24 h and 48 h (Fig. 1A and B). The positive ubiquitinated proteins were visualized to reach the peak staining at 5 h then gradually decrease at 24 h and 48 h. But they were all increased significantly as compared to the control. Quantitative analysis revealed that the total numbers of cells with positive ubiqutinated proteins in all four target regions of hippocampus were increased by 58.8%, 41.1% and 17.6% at 5 h, 24 h and 48 h, respectively, as compared to the control group ($P < 0.01$). When rats were pretreated with MG-132 for 2 h followed by the 24 h-DM treatment, the positive ubiquitinated proteins were decreased by 36.4% as compared to the DM 24 h group ($P < 0.05$) (Fig. 1C). The treatment of MG-132 alone treatment had no effect on ubiquitinated proteins.

3.2. DM exposure induces DNA damage

The adverse effect of DM on DNA was evaluated by comet assay. The comet pictures were taken by microscopy whose brand is Olypus. In all DM treated groups, the number of comet cell was increased remarkably (Fig. 2A). When compared to the control group, DM increased the percentage of DNA in tail (% DNA_T) and olive tail moment (OTM) ($P < 0.01$). Interestingly, both increases in % DNA_T and OTM were peaked at 24 h but not 5 h post the DM treatment (Fig. 2B and C). When rats were pretreated with MG-132, increases in % DNA_T and OTM were attenuated by 37.7% and 30.4%, respectively ($P < 0.05$), as compared to the 24 h-DM group (Fig. 2D and E). The treatment of MG-132 alone showed no effect on DNA damage.

3.3. DM exposure induces apoptosis in hippocampus

Annexin V is one of the most sensitive indicator to detect early apoptosis, while PI could stain apoptotic nuclei in the middle and late stages. In this study, DM treatments for 5 h, 24 h and 48 h caused significantly increase in early apoptotic rate (Q4). Likewise, the peak rate was at 24 h not at 5 h, which was induced by 262.5% compared to the control group ($P < 0.01$) (Fig. 3A and B). However, the later apoptotic rate plus dead cells rate showed unconspicuous changes (Fig. 3C). When rats were pretreated with MG-132, the early apoptotic rate was decreased sharply by 37.3%, as compared to the 24 h DM-treated group ($P < 0.05$) (Fig. 3D). Though the later apoptotic rate plus dead cells rate had no changes in the presence of MG-132 (Fig. 3E). MG-132 alone treatment had no effect on apoptotic rate.

3.4. Alterations of apoptotic related proteins by DM exposure

To evaluate the mechanism of DM-induced apoptosis in hippocampus, the expression of apoptosis-related proteins, Bcl-2 and Caspase-3, were determined by western blotting. Meanwhile, caspase-3 activity was detected by using assay kit. The results showed that DM significantly decreased Bcl-2 expression in 5 h and 24 h but not 48 h with the lowest expression appeared at 24 h, which was reduced by 80% as compared to the control group ($P < 0.01$). When rats were pretreated with MG-132, the expression of Bcl-2 was induced by 145.7% as compared to DM 24 h group ($P < 0.01$) (Fig. 4A, B and C). In addition, DM significantly increased Caspase-3 expression in 5 h, 24 h and 48 h and the highest expression level was at 24 h, which was increased by 105% as compared to the control group ($P < 0.01$). Cleaved Caspase-3 expression increased in 24 h and 48 h but not 5 h ($P < 0.05$). When rats were pretreated with MG-132, the expression of Caspase-3 was reduced by 29.1% ($P < 0.05$). Cleaved Caspase-3 was attenuated as well ($P < 0.05$) (Fig. 4A, B, D and E). Similarly, caspase-3 activity showed the same tendency in comparison with Caspase-3 protein expression (Fig. 4D and E). MG-132 alone treatment had no effect on the expressions of Bcl-2 and Caspase-3.

4. Discussion

There is widespread use of pesticides, and this has raised questions about their potential health impact. Both epidemiological and laboratory evidence have suggested that exposure to pyrethroids may be associated with neurodegenerative disorders such as AD, PD and amyotrophic lateral sclerosis (ALS) [19]. Numerous evidence indicated that apoptosis and UPS play pivotal roles in the pathogeneses of neurodegeneration in many neurological diseases [20,21]. Previously, we reported that type II pyrethroid pesticide DM induced mitochondrial membrane permeability and decreased cytochrome C expression in rats' hippocampus [9]. In the present study, we investigated apoptosis and proteasome inhibition following a single i.p. dose of DM. Our results demonstrate that DM results in ubiquitination and apoptosis in hippocampus. Further, we demonstrate the ability of MG-132, a proteasome inhibitor, to reduce ubiquitination and apoptosis induced by DM exposure.

Intracellular proteolysis is a very strong mechanism that shapes the proteome following exposure to different stress circumstance [22]. The elimination of misfolded or damaged proteins is important for cells to recover from adverse conditions. In the cytosol and nucleus, the major proteolytic pathway used by eukaryotic cells to process misfolded or damaged proteins is the ubiquitin proteasome system (UPS) [23]. When the UPS function is damaged by many stimuli, for example $ZnCl_2$ and methamphetamine, it can result in the accumulation of ubiquitinated proteins leading to many neurodegenerative disorders [24,25].

Our present study, for the first time, illustrated that DM induced ubiquitinated proteins in all CA regions of hippocampus, suggesting that DM could affect the UPS function, which may contribute to DM-induced neurotoxicity. In addition, the increase of ubiquitinated proteins showed a time-dependent relationship, which was different from Huang's report [26]. They found that ubiquitinated proteins were increased in bilirubin-treated for 6 h, 12 h and 24 h in hippocampal neurons, and the increase expression was peak at 12 h. Other research showed that the expressions of ubiquitinated proteins were higher at prostaglandin J2 treated for 24 h than 4 h in hippocampal neurons [27]. Our results showed that the highest level of ubiquitinated proteins was at 5 h then went down in rat hippocampus. The discrepancy may due to different chemicals and experimental models. Furthermore, it was clearly that the pretreatment with MG-132 markedly reduced the DM-induced ubiquitinated proteins indicating that the disruption of UPS may contribute to the neurotoxicity induced by DM.

Apoptosis is a critical process to eliminate defective cells through an orderly process of cellular disintegration [28]. It was demonstrated that if cells fail to eliminate the high levels of ubiquitinated proteins, apoptosis went into effect by triggering caspase activation [29]. In our present study we used three kinds of techniques to evaluate how DM induces apoptosis in rat brain. First of all, the alkaline version of single cell gel electrophoresis assay (SCGE) was employed to determine the effect on DNA damage.

There are many parameters to analyze the outcome of the assay. In this study, percentage of DNA in tail and Olive tail moment were chosen, which are the most reliable measurements that truly reflect the extent of DNA damage. Following the induction of DNA damage, a predominant route of cell inactivation is apoptosis. During the last ten years, DM and other pyrethroids are confirmed to cause negative effects on insects and other living organisms which were reflected in genetic or molecular damages [30–32]. In our study, the neurotoxic effect of DM assessed by the comet assay revealed that DM significantly induced DNA damage manifested by the elevated percentage of DNA in tail (% DNA_T) and Olive tail moment (OTM) compared to control group, indicating that specific DNA lesions could trigger the apoptosis.

Secondly, flow cytometry analysis was utilized to observe the effect of DM on apoptotic rate. It was clear that DM of 12.5 mg/kg dosage in all time points caused a significant induction in early apoptotic rate, which was similar to previous *in vitro* findings [33]. However, late apoptotic rate remained unchanged.

Lastly, we focused on the expression of apoptotic related proteins such as Bcl-2 and Caspase-3. Bcl-2 is an anti-apoptotic protein can trigger apoptosis by releasing cytochrome *c* subsequently leading to the activation of a caspase cascade to destroy the cells [34]. The previous research in our lab showed that the Bcl-2 protein expression was increased by DM (12.5 mg/kg) treatment for 5 h but decreased for 24 and 48 h using immunohistochemical analysis [8]. Our present study showed that Bcl-2 protein expression was decreased at 5 h and 24 h by western blotting. Although there was a slight difference between these two outcomes which might be due to different methods, DM indeed decreased Bcl-2 expression in 24 h, indicating that proteasome impairment occurs earlier than activation of the apoptotic pathway in hippocampus.

Caspases are a family of cysteine proteases that are best known for

driving cell death. Their functions are closely associated with apoptosis in all metazoans, including *C. elegans*, zebra fish, rats, and humans. Caspase-3, -6, -7 that share a homogeneous short pro-domain, are defined as the executors of apoptosis [35]. There are many documents indicated that DM could cause activation of Caspase-3 *in vitro* and *in vivo*. Exposure to DM enhanced the activity of cleaved Caspase-3 in a concentration-dependent manner in PC12 cell [36]. In addition, exposure to DM (0.32 mg/kg) for 90 days increased the Caspase-3 activity in rat hippocampus and striatum [37]. In our study, we also confirmed that the activation of Caspase-3 in DM-exposed hippocampus cells.

Proteasome inhibitors were originally developed as agents with possible benefit in alleviating cancer-related cachexia due to the role of the UPS in protein turnover. During the past decade, proteasome inhibitors are mainly used as chemotherapeutics to treat different types of cancer and cardiovascular diseases [38,39]. Meanwhile, it was demonstrated that the treatment of MG-132 at the dose of $6.8 * 10^{-4}$ μg/g for 4 weeks under stereotaxic microinjection to substantia nigra could prevent nigral neurodegeneration in rats [40]. On the contrary, MG-132 at the dose of 0.5 μg/g for 8 weeks under intraperitoneal injection promoted the dopaminergic neuronal loss in rats [16]. In addition, when treated the cultured cerebellar granule cells with lactacystin, another kinds of proteasome inhibitors, it was demonstrated that cell cycle was interfered and entered in apoptosis [41]. Therefore, it is interesting that proteasome inhibitors might have opposite effects in neurons. Considering the potential protective effect of MG-132, in our study, we used MG-132 in the dose of 0.5 mg/kg referred to Wójcik [16]. Our results demonstrated that MG-132 protected against hippocampus cytotoxicity induced by DM. One potential reason is the exposure time of MG-132. In our experiment we administrated MG-132 for 2 h, which was much < 4 or 8 w. The other reason might depend on the dosage. Compared to $6.8 * 10^{-4}$ μg/g in Inden's experiment, our dosage of MG-132 was extremely high. However, considering the different exposure routes, the final concentration of MG-132 in hippocampus might not be so high following the i.p. injection. In short, we came up with an assumption that MG-132 with high dose and long-term exposure could induce neurodegeneration while low dose and short-term exposure may have protective effects against neurotoxicants.

At present, widespread and improper use of DM induces resistance, which has become a major obstacle for the insect-borne disease management. Fortunately, it's been domenstrated that proteasome inhibitors such as MG-132 or bortezomib could increase the susceptibility in DM-resistant cells and resistance larvae, indicating that they are suitable for use as a DM synergist for vector control [42,43].

In conclusion, the present study demonstrates that DM induces UPS dysfunction then leads to DNA damage and apoptosis. The proteasome inhibitor MG-132 attenuates DM-induced neurotoxicity, suggesting the possibility that UPS inhibition might be involved in DM associated neurodegenerative disorders. However, the current work only focuses on the protective effect of MG-132 against the acute toxicity induced by DM. The effects of MG-132 on chronic exposure of DM require further investigation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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