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The use of acute nicotine treatment to ameliorate the ultrastructural changes of neuron in the hippocampus CA1 region due to REM sleep deprivation

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Abstract

Rapid eye movement (REM) sleep deprivation is associated with learning and memory impairment accompanied by changes in the expression of the downstream regulatory antagonistic modulator (DREAM), cAMP response element-binding (CREB) and brain-derived neurotrophic factor (BDNF) proteins in the hippocampus. Acute nicotine treatment has been shown to attenuate this effect. This study was conducted to investigate the effects of acute nicotine treatment on changes of ultrastructural of neuron in the hippocampus CA1 region due to REM sleep deprivation. Sprague Dawley rats were subjected to a normal condition, REM sleep deprivation and control wide platform condition for 72 hours. During this procedure, saline or nicotine (1 mg/kg) was given subcutaneously twice a day. Then, the rats were sacrificed and the brain was harvested for Transmission Electron Microscopy (TEM) analysis. TEM analysis found that REM sleep deprivation for 72 hours significantly changes the ultrastructure of the neurons in the hippocampus CA1 region compared to the other groups. Treatment with acute nicotine significantly ameliorated these changes. This study suggests that acute nicotine treatment can prevent learning and memory impairment by ameliorating the ultrastructural changes in the neurons of the hippocampus CA1 region due to REM sleep deprivation.

Keywords: Hippocampus, REM sleep deprivation, Nicotine, Ultrastructural changes.

Introduction

Sleep is an important physiological process for all living organisms. It can be divided into two main stages: non-rapid eye movement (non-REM) and followed by a much shorter period of rapid eye movement (REM) sleep [1]. Sleep contributes significantly to the process of learning and memory [2-4]. The process of learning and memory in the brain consists of at least three stages: encoding, consolidation and retrieval of information [5]. Sleep is particularly beneficial to the consolidation stage of memory storage. Disruption of sleep during this stage will affect the consolidation of memory and impair the learning and memory process.

Studies have shown that REM sleep deprivation can impair the learning and memory processes [6] by reducing the level of brain-derived neurotrophic factor (BDNF) in the rat hippocampus [7]. A previous study has demonstrated that REM sleep deprivation impairs learning and memory processes through the modulation of downstream regulatory antagonistic modulators (DREAM), cAMP-response element binding (CREB) and BDNF

proteins expression in the rat hippocampus [8]. All these proteins have been found to be directly involved in learning and memory processes.

An acute nicotine treatment has been reported to prevent the impairment of learning and memory [9-10]. This finding is consistent with our previous study results [11]. However, the underlying mechanism of nicotine to prevent learning and memory impairment due to REM sleep deprivation is still elusive. Therefore, this study is conducted to investigate whether the effect of acute nicotine treatment prevents learning and memory impairment in REM sleep deprived rats and also whether it involves ultrastructural changes of neurons in the hippocampal CA1 region.

Materials and methods

Animal preparation

Thirty-six adult male Sprague Dawleys (230–280 g) were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia, with free access to food and water. They were placed in a room with 12 h light/dark (lights on at 7:00 am and turn off at 7.00 pm) cycle at 28˚C. There were six groups of rats in this study: Control (C, n=6), control treated with nicotine (CN, n=6), wide platform (W, n=6), wide platform treated with nicotine (WN, n=6), REM sleep deprivation (R, n=6), and REM sleep deprivation treated with nicotine (RN, n=6). The nicotine groups (CN, WN and RN) were treated with 1 mg/kg of nicotine (Sigma, St. Louis, MO) subcutaneously twice a day, for 72 hours. The non-treatment groups were treated with a subcutaneous saline injection. The nicotine dose in this study is based on a previous study which is known to produce nicotine blood levels similar to those of chronic smokers [12]. All experiments were approved by the Animal Care and Use Committee of Universiti Sains Malaysia [USM/Animal Ethics Approval/2012/ (81) (408)].

Induction of REM sleep deprivation

The modified inverted flowerpot method was used to selectively deprive animals of REM sleep for 72 hours based on our previous study method [8, 11]. This technique was effective in preventing REM sleep because the muscular atonia accompanying REM sleep causes the rat's body to come into contact with water, thus awakening the animal [11].

Transmission Electron Microscopy (TEM)

The rats were euthanized with an overdose of sodium pentobarbital (100 mg/kg; intraperitoneal) [13-14]. Then, the rats were perfused intracardially with a solution of 0.05 M phosphate buffered saline followed by 2.5 % of glutaraldehyde and 4 % of paraformaldehyde dissolved in a 0.1 M sodium cacodylate buffer (41°C and pH 7.4). The brains were removed and a region of hippocampus (CA1) was cut into 1 mm^3 slices. The samples were then soaked in the 4% buffered glutaraldehyde for at least 12 to 24 hours at 4°C to maintain the cell and tissue structure. Then, the samples were removed and washed with a 0.1 M sodium cacodylate buffer (Agar Scientific Ltd, UK) for three changes of 10 minutes each. The samples were then post-fixed in 1% buffered osmium tetroxide (Agar Scientific Ltd, UK) for two hours at 4°C and washed again with 0.1 M sodium cacodylate buffer for three times for 10 minutes each. The samples were dehydrated with a series of different concentrations of acetone: 35%, 50%, 75% and 95% for 10 minutes respectively, followed by dehydration in absolute acetone for 15 minutes with three changes. This step ensures that the water within the samples was completely flushed out for better fixation.

Infiltration, embedding and polymerization

The samples were infiltrated with a resin and acetone mixture in the ratio of 1:1 for one hour, 1:3 for two hours, 100% resin for overnight and 100% resin for two hours on the next day. Finally, the samples were placed into beam capsules and filled to the top with 100% resin and left for polymerisation in the oven at 60°C for 48 hours.

Thick or semi-thin sectioning

After 48 hours, the resin blocks were trimmed using a new clean Gillette Super Nacet blade to remove the resin around the sample. Tissue sectioning was then performed using a glass knife. The tissue block was trimmed until the surface area of the sample is seen. It is then cut into semi thin sections (70 nm). The sections were placed on glass slides and stained with 0.1% alkaline toluidine blue (Sigma-Aldrich). The glass slides were dried on the hot plate and the stains were gently rinsed away under tap water. The glass slides were examined with a light microscope (Leica, Germany) at 100x and 400x magnification to identify the morphological location and histological changes of the hippocampus neuron in the CA1 region. All of the procedures were done at the Electron Microscopy unit (EM unit), Faculty of medicine, Universiti Malaya (UM), Kuala Lumpur.

Ultrathin sectioning

After the areas of interest were identified, the block was re-trimmed and cut into ultrathin sections using a diamond knife (Diatome Ultra Diamond Knive, Agar Scientific Ltd, UK). The best sections were chosen based on their colour, which exhibited either silver or golden colour on the water level of the boat. The sections were placed on 200 mesh grids and allowed to dry on filter paper.

Staining for TEM

Grids on which the sections are mounted and dried were floated for 10 minutes on a drop of alcoholic uranyl acetate (Agar Scientific Ltd, UK) and then placed on the sheet of dental wax. The grids were then washed in two lots of 50% filtered alcohol drained and dried. Then the sections were stained with lead citrate (Agar Scientific Ltd, UK) in which the above stained grids were placed with the section side down on the drop of lead stain for 10 minutes and washed with continuous agitation in two lots of double-distilled water. The stained grids were drained dry and prepared for viewing through a TEM.

Semi quantitative histology examination

At the Electron Microscopy unit (EM unit), Faculty of medicine, Universiti Malaya (UM), Kuala Lumpur, TEM (Zeiss, Leo Libra 120, Germany) was used to view the ultrathin sections of hippocampal neuron at the CA1 region. Hippocampal neurons were examined and the changes seen on the cytoplasm, nucleus, mitochondria, rough endoplasmic reticulum (RER), lysosome and Golgi apparatus (GA) were scored as shown in Table 1.

The ultrastructures were scored with scores ranging from 1 to 4. Score 1 indicates that the organelles were structurally normal [15-21] (refer Table 1). However, scores 2 to 4 indicate that the organelles have undergone abnormal changes due to conditions such as REM sleep deprivation.

Statistical analysis

Statistical analysis was performed using the SPSS version 23. To score the ultrastructural changes, parametric one-way ANOVA test and post hoc Turkey Test were used to make comparisons between subject groups. Post hoc one-way ANOVA with Bonferroni correction was performed for comparisons of all data. 'p' value of less than 0.05

was considered significant. All values were presented as mean ± (Standard Error of the Mean (S.E.M)).

Table 1: Ultrastructural scoring system

Ultrastructure assessment	Score
Cytoplasm	
Intact with organelles	1
Disorganised	2
Dense	3
White or empty	4
Nucleus	
Normal	1
Irregular chromatin distribution	2
Increased heterochromatin	3
Degenerated nucleus	4
Mitochondria	
Normal	1
Prominent cristae	2
Swelling	3
Collection of amorphous material	4
Endoplasmic reticulum	
Normal	1
Dilatation	2
Vacuolization	3
Presence of focal breaks	4
Golgi Apparatus	
Normal	1
Mild dilatation	2
Moderate dilatation	3
Presence of focal break	4

Results

Ultrastructural changes and score of changes in the neurons of the hippocampus CA1 region

The ultrastructural changes in the organelles of the neuron of the hippocampal CA1 region in the C, CN, R, RN, W and WN groups are presented in Figure 1 respectively. From Figure 1, significant ultrastructural changes can be detected in the R group compared to the other groups. The ultrastructural changes detected was the cytoplasm of the neurons in the CA1 regions of the hippocampi of the R group appeared white and had empty cytoplasm. While there was increased heterochromatin in the nuclei of the hippocampal neurons of the R group, nuclear degeneration was not detected. Moreover, the mitochondria of the hippocampal neurons of these rats showed profound swelling with cristolysis and amorphous material collection. In addition, the RER and GA of the hippocampal neurons of these R group also displayed focal breaks (Figure 1). In this study, it is found that the ultrastructure of the neurons in the CA1 regions of the hippocampi of the RN group rats also showed disorganised cytoplasm, irregular chromatin distribution in the nuclei, prominent vacuolated cristae in the mitochondria, as well as RER and GA dilation (Figure 1). This indicates that the hippocampal neurons experienced early ultrastructural changes. However, this appearance was in better condition if compared to that in the R group, in which a profound swelling and cristolysis with the amorphous material collection are detected. Due to the effects of REM sleep deprivation, the neurons in the CA1 regions of the hippocampi of the R group showed the highest score of ultrastructure changes in the cytoplasm, nucleus, mitochondria, RER and GA compared with other groups ($p<0.001$) as shown in Figure 2. The administration of acute nicotine in the RN group significantly reduced the score of ultrastructure changes in the all organelles of the neuron in the hippocampal CA1 region when compared to the R group ($p<0.001$) (Figure 2). The ultrastructural neurons in the CA1 regions of the hippocampi for other groups (C, CN, W and WN) are found to be less affected and the scores of ultrastructure changes in their cytoplasm, nucleus, mitochondria, RER, lysosome and GA are not significantly different when compared between the groups (Figure 1 and 2).
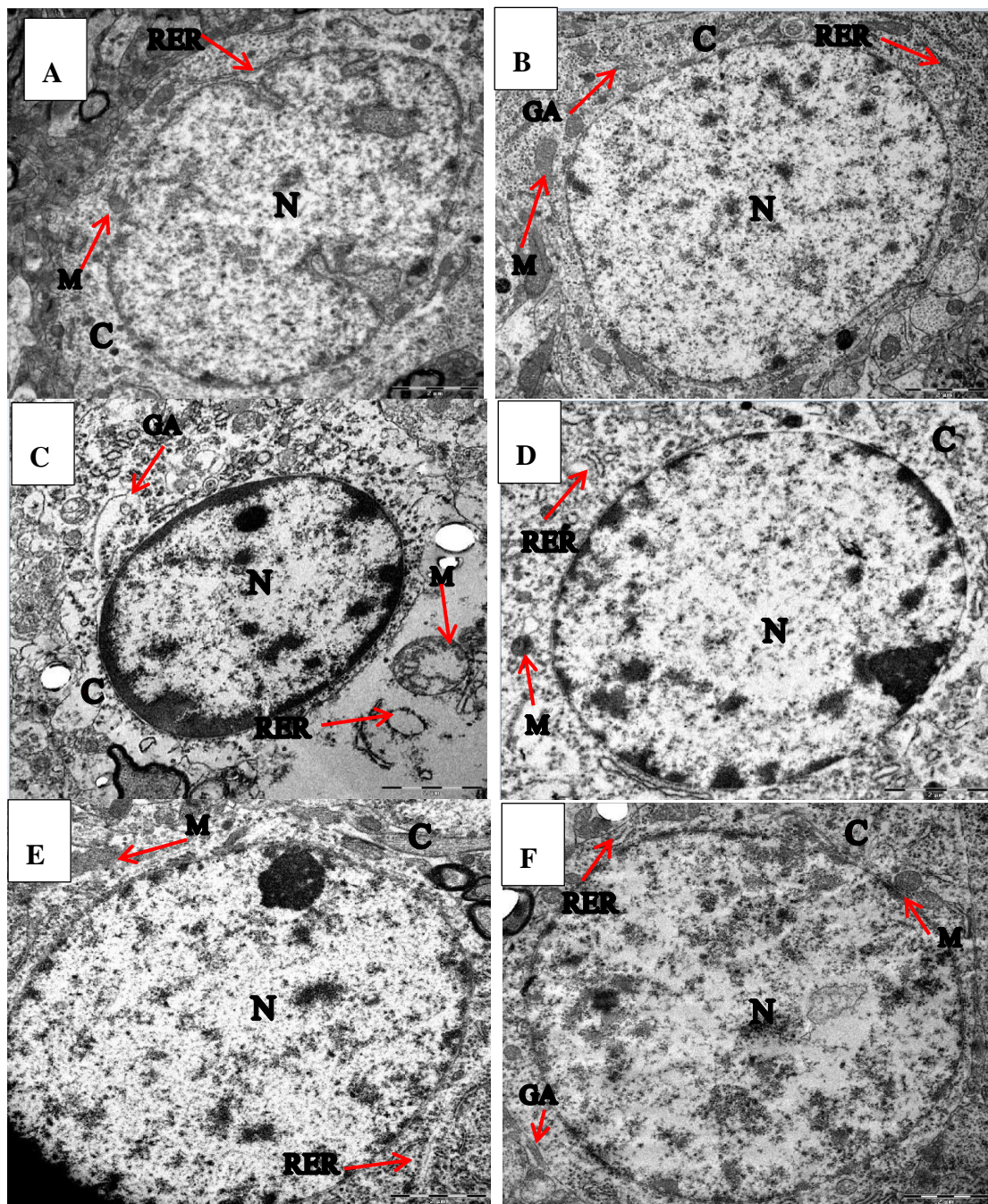
Figure 1: Ultrastructural changes in the CA1 hippocampal neuron of the C (A), CN (B), R (C), RN (D), W (E) and WN groups (F) at 6,000× magnification. Scale bar = 2 μm. (C (cytoplasm); N (nucleus); M (mitochondria); RER (rough endoplasmic reticulum) and GA (Golgi apparatus)).
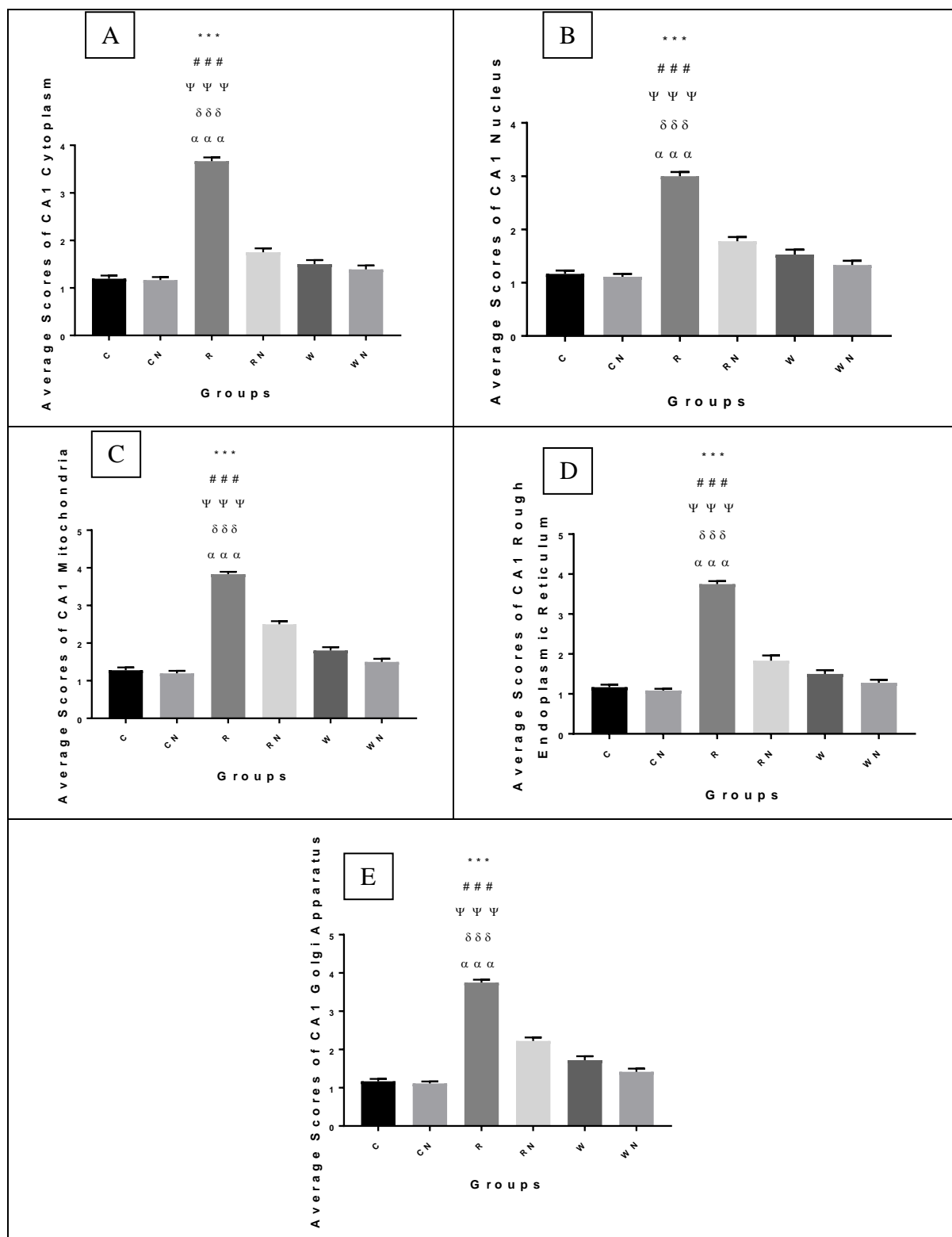
Figure 2: Mean scores for ultrastructure changes in the cytoplasm (A), nucleus (B), mitochondria (C), RER (D) and GA (E) of the hippocampal CA1 neurons between the groups. Each column represents mean ± S.E.M. n=6 for each group.

*** p<0.001, for comparison between R and C group
p<0.001, for comparison between R and CN group
ψψψ p<0.001, for comparison between R and RN group
δδδ p<0.001, for comparison between R and W group
ααα p<0.001, for comparison between R and WN group

Discussion

All ultrastructural changes in neurons in the CA1 regions of the hippocampi for R group in this study indicate that early damage of the hippocampal neurons occurs due to REM sleep deprivation. The white and empty cytoplasm denote neuronal swelling. The mechanism of the swelling could possibly be due to REM sleep deprivation which promotes noradrenaline (NA) activation and increases the Na-K-ATPase activity of α1-adrenoceptors. These in turn lead to neuronal depolarisation and an increase in sodium influx, hence increasing the intracellular osmotic pressure [22-23]. This mechanism stimulates water influx, resulting in neuronal swelling [23]. In this study, it was also found that the other organelles such as mitochondria, RER, and GA of the R group also appeared swollen or dilated. In this study, it was also established that the scores for the ultrastructure changes in the hippocampal CA1 neurons in the cytoplasm, nuclei, mitochondria, RER, and GA of the R group are significantly higher compared to the other groups. This indicates that the ultrastructure of the hippocampal neurons in the CA1 region of the R group was already affected.

REM sleep deprivation causes an increase in Deoxyribonucleic acid (DNA) damage due to an increase in reactive oxygen species (ROS) [24], which compromises the DNA repair mechanisms or gives rise to insufficient repair [25]. Insufficient antioxidant activity to balance oxidative stress results in cellular injuries [24]. This study revealed that the nuclei of the hippocampus neurons of the R group contain heterochromatin, indicating nuclear damage. However, nuclear degeneration did not yet occur because the duration of REM sleep deprivation was only 72 hours. Previous studies have shown that degenerated neurons can be detected after 6 days of REM sleep deprivation [26].

Although degenerated neurons cannot be detected earlier than the 6th day of REM sleep deprivation when the amino-cupric silver technique and light microscopy is used [26], 24-96 hours of REM sleep deprivation gave rise to increased ROS and lipid peroxidation in the hippocampal neurons [24]. These indicate that the neurons progressively undergo ultrastructural changes which can be detected using very high magnification (e.g. electron microscopy).

A mitochondrion is an organelle that is responsible for producing Adenosine triphosphate (ATP) through oxidative phosphorylation in the electron transport chain (ETC) [27]. REM sleep deprivation can impair the ETC, leading to mitochondrial dysfunction and increased ROS production [27]. When the ROS accumulates, it will overwhelm the mitochondrial and cytoplasmic antioxidant system, hence causing ultrastructural damage [28]. Therefore, in this study, the detected ultrastructural damages could possibly be due to the accumulation of ROS following the failure of the antioxidant system to counteract the effects.

It was suggested that sleep deprivation for 6 hours and above can induce stress on the endoplasmic reticulum (ER), leading to cell injury [29]. The ER responds to the stress by releasing proteins in a process known as unfolded protein response (UPR). This response is cytoprotective and helps the neurons overcome the adverse consequences of sleep deprivation [29]. However, UPR release is impaired in sleep deprivation, and this is detrimental to cellular survival [30]. Thus, in the hippocampal neurons of the R group, focal breaks in the ER causes injuries to the neurons.

The study also showed that administration of acute nicotine treatment (RN) group led to a significant reduction of cytoplasmic, nuclear, mitochondrial, RER, and GA scores compared to those in the R group (Figure 1 and 2). These indicated that the ultrastructure changes in the CA1 region of the hippocampi of the RN group rats were ameliorated compared to the R group. Nicotine has been regarded as an anti-apoptotic agent as it activates

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α7-nicotinic receptors that trigger the anti-apoptotic kinase (AKT) activity by stimulating the PI3-kinase through a src-family kinase [30]. In addition, α7-nAChRs have been suggested to be involved in the neuroprotective effect of nicotine [31]. Nicotine acts upon this by stimulating nAChRs and promoting Ca^{2+} influx via glutamate-NMDA activation [32], eventually reducing DREAM protein (a pro-apoptotic agent) expression. Thus, acute nicotine treatment can serve as a protective agent for the survival of the hippocampal neurons.

Apart from that, nicotine has been shown to exert beneficial and protective effects in a few neurodegenerative diseases [33]. *In vitro* studies on mitochondria obtained from male Wistar rat brain cells and human SH-SY5Y cultured cells demonstrate that nicotine can decrease ROS generation by interacting with the brain's mitochondrial respiratory chain and reduce mitochondrial swelling [33-34]. This effect is consistent with the findings in which the ultrastructure of the mitochondria in the RN group showed prominent vacuolated cristae. In addition, the reduction of ROS production by acute nicotine treatment ameliorated the REM sleep deprivation -induced ultrastructural damage in the hippocampal neurons. Thus it is suggested that, based on the hippocampal ultrastructure scores in the RN group, there exist significant improvements. In a cell culture study involving mouse neuro-2a neurons, nicotine is shown to suppress ER stress and promote UPR release [35]. This may explain the neuroprotective effect of nicotine in the RN group, in which the effects of REM sleep deprivation on the ultrastructure of neurons are ameliorated.

It can be concluded that REM sleep has vital roles in normal neuronal function and structure. Disturbance of REM sleep leads to intracellular changes that may interrupt normal structure and functions. Therefore, prolonged REM sleep deprivation can result in more damage to hippocampal cells. However, through acute treatment with nicotinic receptor agonist, nicotine, the neuronal cells' damage due to REM sleep deprivation can be reversible.

Author contributions
All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest
The authors have no disclosures to declare.

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