Original article

Melatonin attenuates cerebral hypoperfusion-induced hippocampal damage and memory deficits in rats by suppressing TRPM7 channels

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This study was conducted to examine if modulating transporters like transient receptor potential cation channels, subfamily M, member 7 (TRPM7) underlies the hippocampal neuroprotection afforded by melatonin (Mel) in rats exposed to cerebral hypoperfusion (CHP). Experimental groups included control, Mel-treated (1.87 g/kg), CHP, and CHP + Mel (1.87 g/kg)-treated rats. CHP was induced by the permanent bilateral occlusion of the common carotid arteries (2VO) method and treatments were conducted for 7 days, orally. Mel prevented the damage of the dental gyrus and memory loss in CHP rats and inhibited the hippocampal reactive oxygen species (ROS), lipid peroxidation levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1b), and prostaglandin E2 (PGE2). It also reduced the hippocampal transcription of the TRPM7 channels and lowered levels of calcium (Ca2+) and zinc (Zn2+). Mel also enhanced the levels of total glutathione (GSH) and superoxide dismutase (SOD) in the hippocampus of the control and CHP-treated rats. In conclusion, downregulation of TRPM7 seems to be one mechanism underlying the neuroprotective effect of Mel against global ischemia and is triggered by its antioxidant potential. © 2022 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Stroke is a major vascular disease that affects the brain cells due to a reduction in the blood and is associated with increased mortality and disability (Kuriakose and Xiao, 2020). Based on the etiology, stroke can be ischemic (80–87%) or hemorrhagic (<15%) (Deb et al., 2010). The ischemic results from the vascular occlusion of any of the cerebral arteries due to thrombosis, cardio-embolism, or atherosclerosis, and platelets plug (focal) or complete reduction in blood to the brain (e.g. in cardiac arrest) (global) (Kuriakose and Xiao, 2020). Typical clinical manifestations of stroke are motor, visual, and speech abnormalities including numbness, non-orthostatic vertigo; aphasia and altered speech, diplopia, and sudden unilateral weakness that is contralateral to the affected brain hemisphere (Hankey, 2017).

Cerebral hypoperfusion (CHP) due to cardiac arrest and other vascular abnormalities is the major trigger of ischemia global stroke (Auer, 2016). The increment in the intracellular Ca2+ levels within the neurons is the major mechanism that leads to neurodegeneration and cognitive deficits in patients or animals after CHP (Aoyagi et al., 2012; Auer, 2016). At the molecular levels, CHP increases glutamate accumulation which leads to sustained lethal levels of intracellular Ca2+ levels within the neurons (McBean and Kelly, 1998). Within this view, glutamate facilitates direct Ca2+ entry in the neurons through activating N-methyl-D-aspartate (NMDA) receptors and indirectly from the endoplasmic reticulum (ER) stored and through L-type-dependent Ca2+ channels (Lai et al., 2014; Singh et al., 2019). Consequently, glutamate-induced high Ca2+ levels activate various signaling pathways in the neural cortex, hippocampus, and other areas which leads to oxidative and inflammatory-induced apoptosis and...
cognitive impairment (McBean and Kelly, 1998; Lai et al., 2014; Lewerenz and Maher, 2015).

However, until now, there is a big debate if CHP-mediated neurodegeneration occurs only through the voltage-gated calcium channels (VGCCs) and findings regarding this matter are still controversial. Although many preclinical studies have shown the benefits of VGCCs and NMDA antagonists (Wu and Tymianski, 2018), results were disappointing in several clinical studies (Cataldi, 2013). This raised the question about the precise molecular mechanisms responsible for the neural damage after blood loss and led to a conclusion that other glutamate-independent mechanisms are involved (Cataldi, 2013; Annunziato et al., 2007). As a result, much research is now focusing on targeting non-glutamate-related Ca + 2 channels as a therapeutic protective strategy against CHP-induced brain damage and memory loss (Li et al., 2011).

Independently of glutamate, transporters like transient receptor potential cation channels, subfamily M, member 7 (TRPM7) were shown to have an indispensable role in the mechanism of neurons apoptosis during the early phases of brains ischemia (Lipski et al., 2006; Lin and Xiong, 2017). TRP channels include eight members (i.e. TRPM1-8) in which TRPM7 are widely found in the neonatal and adult brains, as well as other peripheral tissues including the heart, intestine, smooth muscles, kidney, and liver (Bae and Sun, 2011). In the brain, immunostaining of TRPM7 was detected in the plasma membranes, processes, and cell bodies of the hippocampal neurons, and cholinergic vesicles (Pepperberg and Xiong, 2017). Under normal physiological conditions, TRPM7 channels are important for cell proliferation, survival, and adhesion due to their high permeability of essential metals such as Zn2+, Mg2+, and Ca2+ (Lin and Xiong, 2017). However, several studies have confirmed the damaging and apoptotic roles of TRPM7 during brain ischemia (Visser et al., 2014). In this regard, accumulating data have shown that CHP-induced activation of TRPM7 channels is associated with higher neural levels of Ca2+ and Zn2+ (Lin and Xiong, 2017). Indeed, TRPM7 channels have been identified as a unique pathway for the entry of both Ca2+ and Zn2+ in the neurons with a preference to Zn2+ permeability (Monteill-Zoller et al., 2003; Clapham, 2003; Aarts et al., 2003). The inhibition or deletion of TRPM7 rescued neurons from ischemic injury by suppressing the Ca2+ and Zn2+ influx (Canzoniero et al., 1999; Aarts et al., 2003; Inoue et al., 2010; Bae and Sun, 2011; Chen et al., 2015). Therefore, targeting TRPM7 could be a novel mechanism to protect neural damage and memory deficits after cerebral hypoperfusion.

On the other hand, melatonin (Mel) is an indoleamine released mainly from the pineal gland to regulate circadian rhythms (Reiter et al., 2005). In addition, Mel has many neuroprotective properties and can alleviate the neural oxidative injury and improve cognitive function (Reiter et al., 2003; Reiter et al., 2010; Wang, 2009; Asayama et al., 2003; Jansen et al., 2006). Recently, our laboratory has revealed that Mel could protect against hippocampal damage and defects in memory function in CHP rats by downregulating the SK channels (Al Dera et al., 2019). Until now, it is still largely unknown if such protection also involves modulating the TRMP7 channels.

Therefore, in this study, we aimed to investigate if the protective effect of Mel against CPH-induced hippocampal damage and memory deficits in rats is mediated by suppressing/downregulating TRPM7.

2. Materials and methods

2.1. Animals

Adult male rats of the Wistar type (9 weeks/250 g) were used. All rats were provided by the animal unit at the College of Medi-
cine, Taif University, Saudi Arabia, and were maintained there during the whole period of the study. Housing conditions were always ambient (22 °C, 60–63% humidity). Normal chow and water were provided ad libetum. All animal protocols were approved by the institutional ethical board at Taif University (IRB #. HAO-02-T-105).

2.2. Induction of global ischemic (CHP)

The induction of the CHP was performed as per our established method using the 2-VO method (Al Dera et al., 2019). In brief, anesthesia was induced by ketamine hydrochloride/ Xylazin hydrochloride solution (50/5 mg/kg). Briefly, the neck of each animal was opened and both carotid arteries were permanently ligated. The incision was then sutured and closed in three-layer. During the surgery, blood temperature was continuously monitored using an anal probe and eye dryness was prevented by applying an ointment. A similar procedure without carotid arteries ligation was also followed in the Sham-operated rats.

2.2.1. Experimental groups

Four groups of rats were included in this study (n = 10 rats/each) as follows: (1) Control rats: sham-operated rats and treated with normal saline as a vehicle, (2) Mel-treated rats: sham-operated rats which were treated with Mel (1.87 g/kg) (Sigma Aldrich, MO, USA) (prepared in 96% ethanol which was then diluted with isotonic solution) at a final dose of 1.87 g/kg (Al Dera et al., 2019), (3) CHP-induced rats: rats with CHP and treated with normal saline, and (4) CHP + Mel-treated rats: rats with CHP and receive Mel (1.87 g/kg) 10 min before exposure to CHP and continue on Mel treatment (1.87 g/kg) for 7 days. All treatments were given by gavage, daily, and orally as 0.5 ml. The selected dose of Mel and treatment regimen were adopted from our previous study which has shown a potent ability of Mel at this dose to alleviate hippocampal degeneration and memory loss, 7 days after induction of CHP (Al Dera et al., 2019).

2.3. Memory function evaluation

The Morris water maze (MWM) test was conducted to evaluate the memory function in all tested rats (Bromley-Briggs et al., 2011). Briefly, each rat was trained to find a fixed rescue platform that is submerged in a large swimming container (60 cm m × 1.6 m). This training procedure was conducted daily (three trials/each of 90 s) for 4 days, each by releasing the rats from different 4 hypothetical quadrants with the hidden (submerged) rescue base is always fixed in the one quadrant (northern). The time required for each rat to localize and stand over the submerged rescue base (escape time) was recorded. Finally, a probe trial, with the removal of the escape platform was conducted on day 5 and the number of trials each rat crosses above this area was recorded.

2.4. Collection of the hippocampi and processing

After the behavioral analysis, all rats were anesthetized again as mentioned above and authenticated by the neck dislocation. The brains were removed and each hippocampus was isolated (Al Dera et al., 2019). Four hippocampi were preserved in 10% buffered formalin and the other 6 hippocampi were directly frozen at −80 °C. Levels of tumor necrosis factor-alpha (TNF-α),
prostaglandin-E2 (PGE2) malondialdehyde (MDA), manganese superoxide dismutase (MnSOD), total reduced glutathione, and interleukin-6 (IL-6) were measured in the homogenates using specific rat’s ELISA kit (# MBS2507393, MBS262150, # MBS268427, # MBS2881838, # MBS265966, # MBS175908 MyBio-sources, CA, USA). Homogenate levels of interleukin-1β (IL-1β) were measured by ELISA (# 100768, Abcam, MA, USA). The content of ROS was analyzed using a special fluorometric kit (#186027). The neural levels of Zn²⁺ and Ca²⁺ were assessed using special kits were measured by ELISA (102505, Caymen Chemical, MI, USA and # ab102507, Abcam, MA, USA). All protocols were conducted as instructed by each kit and for a total of 6 samples/group.

2.6. Real-time PCR (qPCR)

Primers were designed and purchased from ThermoFisher. The primer sequences for TRPM7 (acc. # NM_021450) were F: AACCCAA-CACCTCTGGAAGAGATCA R: TCAGTCAAGTTTTCTCCCACAC (128 bp) whereas the primer sequences for β-actin (acc. # NM_031144) were: F: ATCTGGCACCACACCTTC and R: AGCCAGGTCCAGACGCA (291 bp). Extraction of RNA and cDNA synthesis was achieved using commercially available kits and as per the provider’s instructions (# 12183018A and # K1621, ThermoFisher, Germany). qPCR was conducted in a CFX96 thermal cycler (BioRad, USA) using the Ssofast Evergreen Supermix (# 172-5200, BioRad, USA) in triplicates. The mRNA levels of TRPM7 were normalized against β-actin. cDNA was be removed in two samples/plate as control. The analysis was done in duplicate for n = 6 samples/group.

2.7. Western blot

A small fraction of each hippocampus (35 mg) was homogenized in the radioimmunoprecipitation (RIPA) buffer lysis buffer (# MBS842826, MyBioSource, CA, USA) plus protease inhibitors. The supernatants were isolated (11,200 x g/10 min/4 °C) and protein levels were determined using an assay reagent (# 23225, ThermoFisher, USA). Equal protein concentrations were separated using the SDS-PAGE. After successful transfer, the nitrocellulose membranes were incubated with the target antibodies (i.e TRPM7 and the loading control, β-actin) (# OST00031W, 240 kDa, 1:2000, ThermoFisher Scientific, USA and # 24042, 45 kDa, 1:2000, Cell Signaling technology, MI, USA, respectively). Membranes were incubated with the horseradish peroxidase (HPR)-corresponding antibodies. The ECL Plus Western Blotting Substrate (# 32106, ThermoFisher Scientific, USA) was used as a developing agent and all images were captured and analyzed using the C-Di Git scanner and its available software.

2.8. Histological analysis

Freshly collected hippocampi were in 10% buffered formalin overnight. Tissues were deparaffinized and rehydrated using xylen and descending alcohol concentrations (100%, 90%, and 70%) and then embedded in wax. Using a microtome, all tissues were cut (5 μM) and then stained with hematoxylin/glacial acetic acid solution. All tissues were then rinsed with deionized water, destained with 1:400 v/v HCL/70% ethanol solution. All tissues were then stained with Eosin, dehydrated with 95% and 100% ethanol. Slides were mounted using special media and covered with a coverslip. All images were photographed under a light microscope at a magnification of 200x.
Fig. 1. Melatonin improves spatial memory function in CHP-model rats. A and B: Represent the escape time intervals and their area under the curve to find the hidden base. C: Represents the average number of crossing times during the probe trial. All data are presented as means ± SD (n = 10 rats/group). ***: p < 0.0001 vs. sham-operated rats. ###: p < 0.0001 vs. Mel-treated rats. $$$: p < 0.0001 vs. rats with cerebral hypoperfusion (CHP).

Fig. 2. Melatonin (Mel) suppresses oxidative stress and enhances antioxidant levels in the hippocampi of CHP rats. All data are presented as means ± SD (n = 10 rats/group) *: p < 0.05, **: p < 0.01, and ***: p < 0.0001, respectively vs. sham-operated rats. ****: p < 0.01 and 0.0001, respectively vs. Mel-treated rats. $$$: p < 0.0001 vs. CHP-induced rats.
strongly and positively correlated with the hippocampal content of both ROS and Ca\(^{2+}\) (Fig. 5C&D).

### 3.5. Effect of Mel on the hippocampus structure

Melatonin administration didn’t affect the morphological features of the dental gyrus (DG) of the treated control rats and this area in both groups showed multiple layers of neural cells that contain intact nuclei (Fig. 6A&B). At the same time, no ultrastructural abnormalities of the DG area were seen between the sham and Mel-treated rats (Fig. 7A&B). An obvious reduction in the number of cells forming the glandular layer of the DG with an increased number of pyknotic cells was observed in CHP rats (Fig. 6C). At the ultrastructural levels, neurons of the DG of CHP-induced rats showed apoptotic cells with pyknotic nuclei, pleomorphic rough endoplasmic reticulum (RER), and damaged mitochondria (Fig. 7C). Normal morphological and ultrastructural features were seen in the DG of the CHP + Mel-treated rats (Fig. 6D& Fig. 7D).

### 4. Discussion

The exclusive results of this study confirm the role of TRPM7 channels in mediating the hippocampal oxidative and inflammatory damage, as well as memory impairment after CHP in rats which involves increasing Ca\(^{2+}\) and Zn\(^{2+}\) permeability. Besides, they clearly show that Mel is a novel drug that could prevent CHP-induced hippocampal damage by downregulating the expression of these channels mainly by decreasing the generation of ROS.

The brain, and particularly, the hippocampus is very rich in fatty acids which make it vulnerable to oxidative damage (Beaudoin-Chabot et al., 2019). After CHP, glucose, and energy (ATP) deprivation are the major mechanisms leading to neural apoptosis by promoting oxido-inflammatory damage, (Liu and Zhang, 2012), mechanisms which were well-explained in multiple reviews (Chen et al., 2011; Kim et al., 2015; Shooshtari et al., 2020). In this context, several authors have shown that ROS and inflammatory cytokines are cross-talked with each other and act in a vicious activation cycle. Within this context, ROS can directly damage the cellular macromolecules, induce lipid peroxidation, scavenge antioxidants, and activate the cellular inflammation by activating the transcription factor, NF-\(\kappa\)B (Liu and Zhang, 2012; Moghaddasi et al., 2014; Pirmoradi et al., 2019; Beaudoin-Chabot et al., 2019; Hafez and El-Kazaz, 2020). Besides, ROS can induce inflammation by upregulating and activating the PGE2 signal (Liu et al., 2019). Also, ROS and inflammatory cytokines promote mitochondria-mediated (intrinsic) cell apoptosis in the hippocampal neurons after CHP and brain ischemia through upregulating Bax and downregulating Bcl2 (Aboutaleb et al., 2015; Ferrucci et al., 2018; Zhao et al., 2018). Furthermore, neural-derived ROS can induce oxidative stress and impair the cerebral blood flow by interacting with nitric oxide (NO) and trigger the generation of peroxynitrite (ONOO\(^-\)), a vasoconstrictor and potent oxidant molecule (Liu and Zhang, 2012; Gryglewski et al., 1982; Shakil and Saleem, 2014; Shooshtari et al., 2020). In addition, ROS impair the cholinergic function by decreasing the cerebral and hippocampal levels of ACh and AChT (Choi et al., 2011; Qu et al., 2014).
Supporting these studies, and associated with the obvious decline in the spatial memory function and the damage of the dental gyrus, a significant increment in the hippocampal content of free radicals (ROS/RNS), lipid peroxides (MDA), and inflammatory mediators (PGE2, TNF-α, IL-1β, & IL-6) were seen in the CHP-model rats of this study. These data validated our animal model. Similar morphological alterations with reduced learning abilities were also reported in the hippocampi of CHP-induced rats in our previous studies, and effects that were attributed to increasing levels of ROS (Al Dera et al., 2019). However, antioxidant therapy protected against CHP-induced neural cell death and apoptosis (Gryglewski et al., 1982; Shirley et al., 2014; Shakil and Saleem, 2014). In this study, Mel attenuated all these damaging oxidative, inflammatory, and apoptotic effects, improved the dental gyrus structure, and enhanced spatial memory function in hypoperfused rats. We have also shown previously a similar protective effect in the same animal model, an effect that was also associated with increasing the hippocampal total antioxidant capacity (Al Dera et al., 2019). However, in this study, we are providing more mechanisms and showed the ability of Mel to also increase levels of intracellular antioxidants in the hippocampi of both the sham and CHP rats. However, since Mel didn’t modulate the hippocampal content of the above-mentioned inflammatory in the sham rats but attenuated them in CHP-rats, these data suggest that scavenging ROS and upregulation of antioxidants is a major mechanism underlying the anti-inflammatory neuroprotective effect of Mel in this animal model.

Similar to our data, Ozacmak et al. (2009) have also shown that chronic administration of Mel prevented ischemia-induced cerebral apoptosis in ovariectomized rats by decreasing MDA levels, restoring contents of GSH and SOD, and suppressing the expression of stress proteins. Many other studies have also reported a similar antioxidant neuroprotective potential of Mel in rodents after induction of ischemia (Pei et al., 2003; Ramos et al., 2017; Liu and Zhang, 2012). Also, the antioxidant protective effect of Mel was shown in other tissue of different animal models of tissue injury (Hardeland and Pandi-Perumal, 2005; Johns and Platts, 2014; Anwar et al., 2015; Tan et al., 2015; Vázquez et al., 2017). Interestingly, accumulating studies have shown the ability of Mel to cross the BBB and plasma membranes and directly scavenge ROS/RNS (up to 10/1 molecule) as compared to other known classic antioxidants (Anwar et al., 2015; Hácişevki and Baba, 2018). Besides, Mel can alleviate oxidative stress and apoptosis by stimulating GSH and antioxidant enzymes, reducing lipid peroxidation, improving mitochondria function, repairing the DNA after oxidation (Tan et al., 2015; Anwar et al., 2015; Hácişevki and Baba, 2018). The antioxidant potential of Mel was also shown to be largely mediated by generating several antioxidant metabolites like 4OHM; 6OHM, 7HM; and AFMK which all can also BBB and the plasma membranes (Johns and Platts, 2014; Hácişevki and Baba, 2018).

On the other hand, increased neural contents of Ca + 2 and Zn + 2 were seen in ischemic cortices and hippocampi and were suggested to be major damaging pathways after ischemia and major
mechanisms associated with cognitive impairment (Zhao et al., 2014; Suvanish Kumar et al., 2014; Ji et al., 2019). Blocking Ca\(^{2+}\) current or intracellular chelating of Zn\(^{2+}\) prevented neural cell death in the ischemic brain, cortices, hippocampi after an ischemic episode (Lukic-Panin et al., 2007; Suvanish Kumar et al., 2014). While Zn\(^{2+}\) can directly activate apoptotic pathways, intracellular Ca\(^{2+}\) overload induces neural injury and apoptosis by decreasing protein synthesis, promoting mitochondria and plasma membrane damage, stimulating ROS/RNS generation, and activating numerous apoptotic pathways (Kiedrowski et al., 1992; Morley et al., 1994; Suvanish Kumar et al., 2014).

However, glutamate excitotoxicity is a key player that mediates neural apoptosis during brain ischemia through increasing intracellular levels of Ca\(^{2+}\) and ROS (Belov Kirdajova et al., 2020). How-ever, the failure of anti-excitatory drugs to alleviate neural cell death in the ischemic brain, cortices, hippocampi after an ischemic episode (Lukic-Panin et al., 2007; Suvanish Kumar et al., 2014). While Zn\(^{2+}\) can directly activate apoptotic pathways, intracellular Ca\(^{2+}\) overload induces neural injury and apoptosis by decreasing protein synthesis, promoting mitochondria and plasma membrane damage, stimulating ROS/RNS generation, and activating numerous apoptotic pathways (Kiedrowski et al., 1992; Morley et al., 1994; Suvanish Kumar et al., 2014).

However, glutamate excitotoxicity is a key player that mediates neural apoptosis during brain ischemia through increasing intracellular levels of Ca\(^{2+}\) and ROS (Belov Kirdajova et al., 2020). However, the failure of anti-excitatory drugs to alleviate neural cell death in the ischemic brain, cortices, hippocampi after an ischemic episode (Davis, 2000; Li et al., 2011) Among all non-glutamate Ca\(^{2+}\) channels, particular interest was given to the TRPM7 in mediating global ischemia and neural apoptosis owing to their important roles as pathways for Zn\(^{2+}\) and Ca\(^{2+}\) (Sun et al., 2009; Bae and Sun, 2011; Sun, 2017). Indeed, in vivo and in vitro, hypoxia upregulated mRNA and protein levels and enhanced activities of TRPM7 which were coincided with higher intracellular levels of Zn\(^{2+}\) and Ca\(^{2+}\), increased neural and hippocampal cell apoptosis, and impaired memory and behavioral outcomes (Jiang et al., 2008; Bae and Sun, 2011; Chen et al., 2015; Turlova et al., 2016; Sun, 2017).

In the same line with these studies, a significant increment in the transcripts and protein levels of TRPM7 transcription was detected in the hippocampi of the CHP rats of this study. This was also concomitant with higher hippocampal levels of Ca\(^{2+}\) and Zn\(^{2+}\). Although such an increase in Ca\(^{2+}\) levels could be secondary to glutamate excitotoxicity (2010), the concomitant increase in the intracellular Zn\(^{2+}\) levels in the CHP-induced rats of this study supports our hypothesis from the involvement of TRPM7 in the obvious hypoxic damage of the hippocampus. Besides, a very strong positive correlation between mRNA levels of TRPM7 and ROS, as well as between Ca\(^{2+}\) and Zn\(^{2+}\) levels and ROS were seen in the hippocampi of rats of this study. This further confirms the role of TRPM7 in CHP-induced hippocampal damage and memory loss and suggests they are mainly triggered by ROS. Yet, the ability of Mel to downregulate mRNA and protein expression of TRPM7, in the hippocampi of CHP-induced rats, but not the control, suggests that the protective effect of Mel involves modulating the expression of these channels. This could be explained by the previously discussed ROS scavenging and antioxidant stimulatory effects of

Fig. 5. Melatonin (Mel) downregulates TRMP7 in the hippocampi of CHP-induced rats which are positively correlated with the intracellular levels of Zn\(^{2+}\) and Ca\(^{2+}\) levels. All data are presented as means ± SD (n = 10 rats/group). ***: p < 0.0001 vs. to sham-operated rats (lane 1). $$$: p < 0.0001, respectively vs. Mel-treated rats (lane 2). $$$: p < 0.0001 vs. CHP-induced rats (lane 3). Lane 4: CHP + Mel.
Interestingly, pharmacological suppression or knocking down TRPM7 prevented neural apoptosis in cultured cortical cells deprived of nutrients and oxygen (Jiang et al., 2008; Sun et al., 2017). It also improved the hippocampus structure and associated behavioral and motor outcomes (i.e. fear response, force, spatial memory, vestibular function, proprioceptive functions, grip test, maladaptive impulsive behavior) and attenuated Bax and caspase-3 in rodents after brain hypoxia (Aarts et al., 2003; Sun et al., 2009; Bae and Sun, 2011; Chen et al., 2015; Turlova et al., 2016; Sun et al., 2017).

These data indicate that Mel alleviates CHP-mediated hippocampal damage via downregulating TRPM7 and possibly through scavenging ROS levels and upregulating antioxidants. Although these data are novel on the expression of TRPM7 channels in the brain of CHP rats, some other existing data may support our findings. Indeed, Mel inhibited Ca\(^{2+}\) overload in the cerebrum of old mice (Molina-Carballo et al., 2007) and reduced protein levels of T-type VGCCs, TRPM-7, and NMDA receptors in rat's brain in epileptic models and exposure to radiation (Reuss et al., 2010; Davis, 2000).

Despite these data, some limitations still exist in this study. Importantly, these data remain observational results and may require more specific animal models and techniques to confirm it. In this context, the effect of Mel on Ca\(^{2+}\) and Zn\(^{2+}\) current should be targeted using more advanced techniques such as patch-clamp studies. Unfortunately, this is not unavailable in our laboratory. In addition, if Mel affords its effect using targeting these pathways could be further examined using transgenic animals or cells lacking TRPM7. In addition, ROS is a major product of glutamate excitotoxicity and given the well-known inhibitory effect of Mel on this pathway (Juan et al., 2014). It could be possible that Mel neuroprotection is also mediated by suppressing this pathway. This needs further future investigation to precisely reveal the proper mechanism of protection.

Overall, our data is still very interesting and show for the first time that Mel neuroprotection against CHP-mediated hippocampal damage involves at least, downregulating TRPM7 channels and subsequent reduction in the intracellular levels of Ca\(^{2+}\) and Zn\(^{2+}\). Mechanism of action involves, at least suppressing ROS and upregulating antioxidants.

![Fig. 6. Histological micrographs obtained from rat dental gyrus (DG) hippocampus (CA1 region) stained by H&E. 200X. A&B: represents control and Mel-treated rats, demonstrating a normal number of cell layers in the glandular layer of the DG with intact cells containing normally rounded nuclei (long arrow). C: represents CHP-induced rats and showed an obvious reduction in the number of cells (layers) forming the glandular layer of the DG hippocampal area (short arrow) with many pyknotic apoptotic nuclei (long arrow). D: represents CHP + Mel-treated rats and shows normal DG structure similar to the control rats (long arrow).](image-url)
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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