JOBIMB, 2023, Vol 11, No 2, 11-19
https://doi.org/10.54987/jobimb.v11i2.836

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index

JOBIMB VOL 11 NO 2 2023
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Signalling Mechanism in TRPM2-dependent Copper- induced HT22 Cell Death

Sharani Rathakrishnan[1], Iffa Nadhira Hazanol[1], Mohd Haziq Izzazuddin Dali[1], Muhammad Syahreel Azhad Sha'fie[1], Syahida Ahmad[1], Noorjahan Banu Alitheen[2] and Sharifah Alawieyah Syed Mortadza[1]*

[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[2]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

*Corresponding author:
Dr. Sharifah Alawieyah Syed Mortadza
Department of Biochemistry,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia,
43400 Serdang,
Selangor,
Malaysia.
Email: s_alawieyah@upm.edu.my

HISTORY

Received: 7th Nov 2023
Received in revised form: 23rd Dec 2023
Accepted: 25th Dec 2023

KEYWORDS

Copper toxicity
Oxidative stress
TRPM2 channel
Neuronal death
PARP

ABSTRACT

Copper (Cu) is one of the critical elements needed by the human body. However, this metal can cause cytotoxicity when present in excess amounts. In this study, we used HT22 hippocampus cells to examine the function of the Ca^{2+}-permeable transient receptor melastatin 2 (TRPM2) channel in Cu-induced neuronal cell death and the underlying mechanisms. Immunocytochemistry, single-cell imaging, acridine orange/propidium iodide (AO/PI) cell death assay and immunofluorescence microscopy were applied to interpret the mechanisms involved in Cu-induced HT22 cell death. Treatment of 30-300 μM Cu induced an increase in the $[Ca^{2+}]_i$ in HT22 cells. Further analysis indicates that Cu exposure induced substantial HT22 cell death. Such response on HT22 cells was significantly inhibited by 2-aminoethoxydiphenyl borate (2-APB) and N-(p-amylcinnamoyl)anthranilic acid (ACA), TRPM2 channel inhibitors. Furthermore, Cu-induced HT22 cell death was suppressed by pharmacologically inhibiting poly(ADPR) polymerase (PARP) using PJ-34 and DPQ. It is known that the activation of TRPM2 channel is via the increase of intracellular reactive oxygen species (ROS). A significant concentration-dependent increase in the generation of ROS was observed in HT22 following the treatment with 30-300 μM Cu. Additionally, Cu-induced HT22 cell death was ablated by inhibiting PKC using CTC, a PKC inhibitor and NADPH oxidase (NOX) using DPI, a NOX generic inhibitor and GKT137831, a NOX1/4-specific inhibitor. Overall, our present study provides evidence suggesting that PKC/NOX/ROS/PARP is an important signalling pathway in Cu-induced activation of the TRPM2 channel and increase in the $[Ca^{2+}]_i$ which eventually results in the toxicity of HT22 cells. These results provide new insights into the mechanisms of neurological-associated Cu-induced diseases.

INTRODUCTION

Copper (Cu) is one of the most prevalent metals in living beings [1]. It plays a primary role as a small cofactor that is required for the functionality of several key enzymes, including cytochrome c oxidase, ferroxidase, copper, zinc superoxide dismutase (CuZnSOD), and tyrosinase; engaged in a variety of functions such as energy metabolism, redox homeostasis, neurotransmitter metabolism, antioxidant defenses and mitochondrial respiration [2]. Despite its critical role in the physiological processes, this metal is toxic when present in excess and may result in adverse health effects. Therefore, the intracellular Cu content is tightly regulated and kept at a relatively low range to provide adequate supply and prevent the repercussions of Cu overload. The normal concentration of copper in plasma is approximately 100–150 μg/dL (1.0-1.5 mg/L) [3,4]. Cu overload cases are substantially more common than their deficiency in the human population. This is due to the high intake of copper obtained from food, drinking water, mineral and vitamin supplements. The Environmental Protection Agency (EPA) states that there is a 1.3 ppm maximum limit for the Cu^{2+} ion in drinking water [5,6]. In fact, Cu has been used a lot in agricultural, domestic and industrial processes [7]. Abnormal Cu homeostasis affects metabolism in multiple ways leading to severe metabolic and

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neurological disorders. Some of these conditions include genetically inherited Wilson and Menkes' diseases, Alzheimer's disease (AD), Huntington's disease, prion disease, amyotrophic lateral sclerosis (ALS), diabetes and cancer [2].

Dietary Cu is absorbed via enterocytes and transported from the liver by ceruloplasmin in the blood to the other organs, and this ion metal can be concentrated in the brain by passing through the blood-brain barrier [8]. In the brain, Cu is mostly found in the hippocampus, cerebellum and basal ganglia [9,10]. According to research conducted in 2019 by Liao et al., a significant increase in the Cu concentration was observed in the hypothalamus region of broilers following the increase in food intake [11]. The brain is a highly sensitive organ and therefore can be easily triggered by a high concentration of Cu [11-13]. Early studies have shown that excessive Cu with the presence of a high-fat diet can significantly accelerate cognitive decline in the elderly population [14]. Additionally, an overload concentration of Cu in the brain results in the impairment of locomotor and cognitive performance [1517]. It is, after all, commonly recognized that an excess of Cu in the brain region can lead to neurotoxicity and cell death [18].

Oxidative stress is one of the key mechanisms involved in the toxicity caused by heavy metals, particularly copper. According to earlier research, Cu excess can cause oxidative damage by releasing ROS, which can lead to cell death, DNA fragmentation, peptide bond breaking, and mitochondrial dysfunction [19,20]. ROS is a cell signalling molecule involved in normal cellular processes [21]. At the physiological condition, the level of ROS is maintained by antioxidants. Failure of the antioxidant defense mechanism to balance optimum ROS production in cells leads to oxidative stress [22]. *In vitro* studies using different cell types, including hepatocyte, endothelial and glioblastoma patient-derived cells, have demonstrated that Cu at a high concentration has the ability to induce oxidative stress [23,24,25]. In fact, the findings from *in vivo* studies using the zebrafish larval model have confirmed the fact that excessive Cu possess the capacity to induce oxidative stress [26]. There is a growing body of research on excessive Cu consumption, however, the mechanisms by which excessive Cu impacts the brain remain unclear.

Neurons, the primary brain residence, are essential for the metabolism of copper. Neurons express various cell surface ion channels, including transient receptor potential melastatin-related 2 (TRPM2) channel. This channel is known to regulate neuronal cell function. Previous studies have suggested that TRPM2 channel is crucial in regulating signalling mechanisms associated with degenerative processes [27,28,29]. Upon activation by the main agonist, which is ADP-ribose (ADPR), TRPM2 forms a pore to create a Ca^{2+}-permeable cationic channel. Other than ADPR, this channel is highly sensitive towards ROS. Such activation by ROS is mediated by ADPR-producing mechanisms [30]. TRPM2 channel is one of the most abundant proteins to be found in the brain [31]. The expression of this channel in hippocampal neurons has been well-known [32-35]. Previous studies have established the fact that Poly(ADPR) polymerase (PARP) plays a key function in mediating the production of ADPR which ultimately causes the activation of TRPM2 channel [27]. Indeed, the activation of PARP has been shown to mediate the cell death in HT22 [36,37,38]. However, whether TRPM2 channel is critical in mediating Cu-induced cell death, particularly hippocampal neuronal cell death, is yet to be revealed. In this study using HT22 neuronal cells, we provide evidence to suggest Cu induces neuronal cell death via the NOX/ROS/PARP/TRPM2 signalling pathway. Such findings provide new discoveries into the Cu-induced neurological diseases.

MATERIALS AND METHOD

Chemical and Reagents

In this study, we used analytical research grade chemicals and reagents acquired from Sigma-Aldrich unless otherwise indicated.

HT22 Cell Preparation

HT22 cells were grown in modified Eagle medium by Dulbecco (DMEM) consisting of 25 mM glucose, added with 10% of the heat-inactivated form of fetal bovine serum (FBS). Cells were cultivated in 25-cm^2 flasks and maintained at a body temperature of 37 °C in a humidified incubator with a constant supply of 5% CO_2. Sub-subcultures were carried out when the cells attained >80% confluency. To separate the cells from the bottom of the flask, 0.05% trypsin-EDTA (Invitrogen) was added into the cells and incubated for 5 min at 37 °C. Following the trypsinized step, the cell pellet was obtained through centrifugation for 5 min at 1000 revolutions per minute. Cells underwent resuspension by using fresh culture media and were then sub-cultured 25-cm^2 flasks. For experimentation purposes, the HT22 cells were plated in 96-microwell plates.

Immunofluorescent Staining

Thirteen mm glass coverslips were coated for 3 h using poly-L-lysine (PLL) and were left at room temperature for experimentation purposes. HT22 cells at 5×10^4 cells/mL were seeded on the coated coverslip and cultured in a humidified atmosphere incubator with 5% CO_2 at 37 °C for 24 hr. Following one day of incubation, HT22 cells were rinsed briefly using phosphate buffer saline (PBS). Cell fixation was then conducted using 4% paraformaldehyde (PFA) dissolved in deionized water. After fixing the cells on a coverslip at room temperature for 15 min, HT22 cells were treated using permeabilization buffer; 0.1% triton X-100 diluted in PBS and left at room temperature for 5 min. Following that, the cells were washed thoroughly with 0.5% Tween 20 diluted in PBS (PBST) 3 times at 3 min intervals for each rinsing. Then, the cells were blocked with 5% goat serum diluted in PBS and left at room temperature for 30 min. Following the removal of the blocking solution, cells were treated with a 1:1500 dilution of the primary rabbit anti-TRPM2 antibody (GeneTex) for an additional night at room temperature. After that, the cells were washed with PBST and incubated with a secondary antibody of FITC-conjugated anti-rabbit IgG (Cayman) at a ratio of 1:800 for one hour at room temperature. Coverslips were mounted using a fluorescent mounting solution containing 4, 6-diamidino-2-phenylindole (DAPI) following PBS washing and water rinsing. To prepare the negative controls, cells were treated with the secondary antibody alone. Stained cell images were taken through a Zeiss Axio Vert A1 inverted fluorescence microscope and Zen software (Zeiss).

Cell Death Assay

HT22 cells at a density of 7×10^3 cells/mL were added into a 96-microwell plate. The seeded cells were left at 37 °C for overnight prior to experimentation. Cu (using $CuSO_4$) was added into the cells at the stated concentrations and incubated at 37°C in a humidified incubator with 5% CO_2 for 8 or 24 h as specified in the Figure legends. A Cu stock solution was prepared by diluting the powder with distilled water and was then further diluted to the indicated concentrations using fresh media. Following the incubation with Cu, a final concentration of 1 µg/mL of acridine orange (AO) and 2 µg/mL of propidium iodide (PI) were added to the cells. The AO/PI-stained cells were left in the dark and

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incubated at a temperature of 37 °C for a further 30 min prior to imaging. Inhibitors were added into cells and incubated for 30 min at a temperature of 37 °C before and during Cu exposure. Nikon camera-equipped fluorescence microscope was used to image cells. At least 80 cells were inspected in each well. For each individual image, ImageJ was used to calculate the amount of red PI-stained dead cells and the sum of cells detected by AO-PI staining in three randomized sections. To illustrate cell death, the number of PI-stained cells is expressed as a proportion of AO-stained cells.

Single Cell Ca^{2+} Imaging

HT22 cells were added into the 96-microwell plate and maintained overnight at a temperature of 37 ºC. Different concentrations of Cu were prepared and treated in the cells for 24 hr. Following that, the culture medium was pipetted out, and 200 µl of phosphate saline buffer (PBS) was used to wash the cells twice. Next, at room temperature, the seeded cells were incubated with 5 µg/mL Fluo-4/AM (Life Technologies at 37 ºC for 20 min. The cells were washed with PBS to get rid of extra Fluo-4-AM dye before imaging. The fluorescent images were captured using fluorescent microscope (Nikon). Using ImageJ software, the intensity of Fluo-4 in each cell was counted, and at least 80 cells in each well were evaluated.

Detection of Reactive Oxygen Species (ROS)

Generation of intracellular ROS was accessed by using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining assay. Firstly, cells were seeded at a concentration of 15 x 10^3 on 96-well plate. Cu at indicated concentrations were added onto the cells. The cells were left incubated with Cu 8 hr. Inhibitors were added at 37 °C for 30 min before and during exposure to Cu. Following the washing with phosphate buffer saline (PBS), 20 µM DCFH-DA was pipetted into each well and the plate was left for 20 min at 37 ºC. Before imaging using fluorescent microscope, the cells underwent a single PBS wash in order to get rid of the excess DCFH-DA dye. The fluorescent images were captured using a fluorescent microscope (Nikon). Using ImageJ software, the intensity of Fluo-4 in each cell was counted, and at least 80 cells in each well were evaluated.

Data Acquisition, Presentation and Statistical Analysis

Every data set, derived from the mean value of individual independent cell preparations or experiments, is presented as mean ± standard error of the mean. When comparing two groups, the student's t-test was used, and when comparing multiple groups, one-way ANOVA was followed by a post hoc Tukey test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Expression of TRPM2 and Cu-Induced Increase in the $[Ca^{2+}]_i$

The expression of the TRPM2 channel in diverse cell types has been well-documented [30,39]. We began our study by verifying the TRPM2 channel's expression in HT22 cells using immunofluorescent microscopy. Based on the findings, TRPM2-immunoreactivity was positively observed in all HT22 cells labelled with an anti-TRPM2 antibody but not in the control cells, which can be identified by the nuclear staining DAPI (**Fig. 1a**). In fact, the immunoreactivity of the TRPM2 was observed to be intensely concentrated near or on the plasma membrane (**Fig. 1a**). An increase in the $[Ca^{2+}]_i$ has been known to be one of the main causes of cell death in various cell types [40]. In fact, earlier research has provided evidence to support one of the main functions of the TRPM2 channel in facilitating the influx of the extracellular Ca^{2+}, leading to an increase in the $[Ca^{2+}]_i$, which ultimately drives cell death [41,42]. To investigate the role of the TRPM2 channel in HT22 cell responses to Cu, we investigated whether Cu induces the TRPM2 channel activation by measuring the increase in the $[Ca^{2+}]_i$ in HT22 cells after exposure to Cu, using single-cell calcium imaging. The HT22 cells were exposed to different concentrations of Cu (30 – 300 µM) for 8 hr. There was a notable increase in the $[Ca^{2+}]_i$ after exposure to 100 – 300 µM Cu (**Fig. 2a** and **b**). However, Cu evoked a negligible increase in the $[Ca^{2+}]_i$ at a concentration of 30 µM, indicating that Cu induces the increase in the $[Ca^{2+}]_i$ in a concentration-dependent manner (**Fig. 2a** and **b**). Altogether, these findings strongly suggest that Cu can induce the increase in $[Ca^{2+}]_i$ in HT22 cells.

Cu-Induced HT22 Cell Death Is TRPM2 Channel-Dependent

Studies from previous literature have conducted an extensive investigation, using different cell types, on TRPM2-mediated ROS-induced cell death. Nonetheless, whether the TRPM2 channel has a similar role in Cu-induced HT22 neuronal cell death is still yet to be elucidated. Evidence regarding such matter is crucial for a deeper understanding of Cu-induced neuronal cell death in oxidative stress-related neurological diseases. Therefore, we further investigated whether HT22 neuronal cell death occurs upon exposure to Cu, using AO/PI staining. Application of 30 – 300 µM Cu for 24 h resulted in a concentration-dependent increase in HT22 neuronal cell death (**Fig. 3a** and **b**). In striking contrast with the robust increase in the HT22.

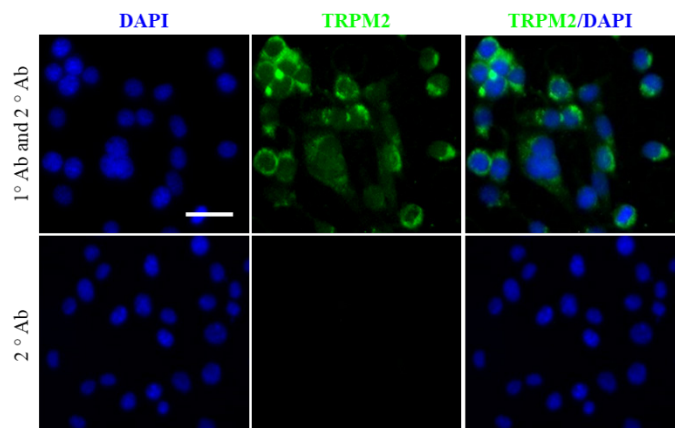

Fig. 1. Expression of TRPM2 in HT22 cells. (a) Representative images showing TRPM2 immunoreactivity in HT22 cells labelled with an anti-TRPM2 antibody. Cells were counter-stained with DAPI. Similar results were observed in three independent cell preparations. Scale bar, 50 µm.

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Application of 30 – 300 µM Cu for 24 h resulted in a concentration-dependent increase in HT22 neuronal cell death (**Fig. 3.a** and **b**). In striking contrast with the robust increase in the HT22 cell death, exposure to 300 µM Cu for 24 h induced a negligible increase in the HT22 cell death in the presence of 2-APB and ACA (**Fig. 3c** and **d**), TRPM2 channel inhibitors. Taken together, the results from pharmacological inhibition of the TRPM2 channel have provided evidence supporting the function of the TRPM2 channel in HT22 cell death induced by Cu.

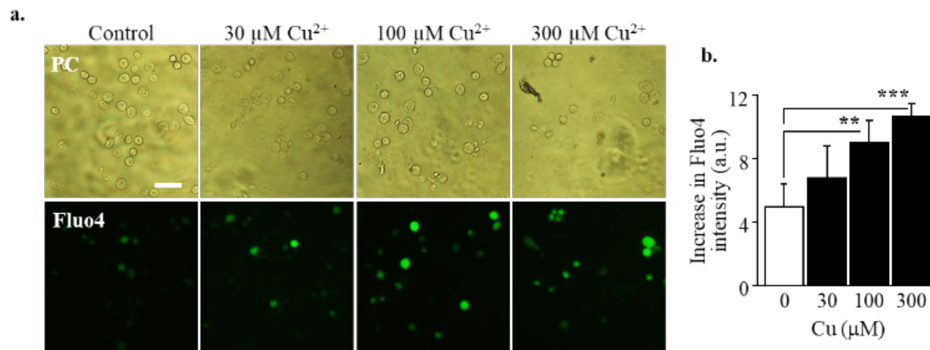

Fig. 2. Cu-induced increase in the $[Ca^{2+}]_i$ in HT22 cells. (a) Representative single-cell images showing Ca^{2+} responses in HT22 cells (top row: phase contrast; bottom row: Fluo-4 fluorescence). Cells were treated with different concentrations of Cu as indicated for 8 hr. (b) Mean summary of Cu-induced Ca^{2+} responses in HT22 cell under indicated conditions, from 3 independent experiments, using three wells of cells for each condition in each experiment. Scale bar, 40 µm. ***, $p < 0.005$ compared to control.

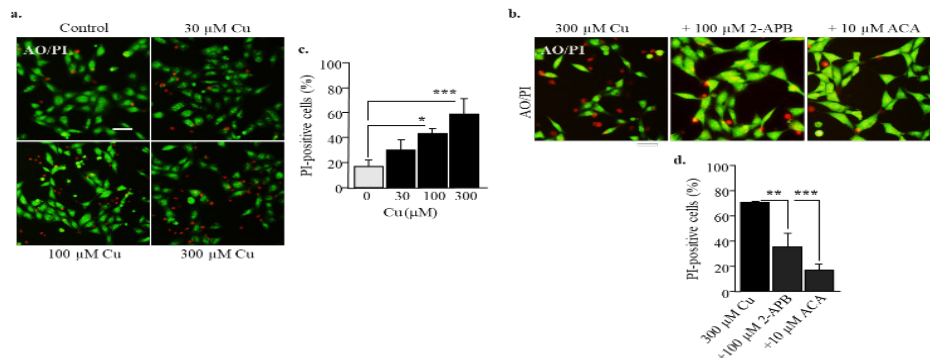

Fig. 3. Cu induces cell death in HT22 cells. (a) Representative images showing acridine AO/PI staining assay on HT22 cells without (control) and with exposure to indicated concentrations of Cu for 24 h. (b) Representative images showing AO/PI staining on HT22 cells exposed for 24 h to 300 µM Cu alone or together with 100 µM 2-APB or 10 µM ACA. (c, d) Mean summary of Cu-induced HT22 cell death under indicated conditions, from 3 independent experiments, using three wells of cells for each condition in each experiment. Cells were treated with 2-APB or ACA for 30 min prior to and during exposure to Cu. Scale bar, 40 µm. *, $p < 0.05$; ***, $p < 0.005$ compared to the indicated control group
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Exposure to Cu Induces TRPM2-Dependent HTT2 Cell Death via PARP Activation
To further acquire information regarding the signalling pathway of the TRPM2-mediated Cu-induced HT22 cell death, we then conducted an investigation on the mechanisms by which Cu activates the TRPM2 channel. PARP is a mitochondrial enzyme that catalyzes the production of ADPR. Therefore, it has been extensively shown by previous studies that ROS induces ADPR production via the activation of PARP [43]. Such ROS-dependent ADPR production subsequently results in the TRPM2 channel activation [43]. We therefore further our investigation by examining the effects of PARP inhibitors on the Cu-induced HT22 cell death, using AO/PI staining. The number of HT22 cell death induced by Cu was significantly reduced in the presence of 3 µM PJ-34 and 10 µM DPQ, PARP inhibitors (**Fig. 4a** and **b**). These findings provide clear evidence suggesting the crucial role of PARP in Cu-induced HT22 cell death (**Fig. 4a** and **b**).

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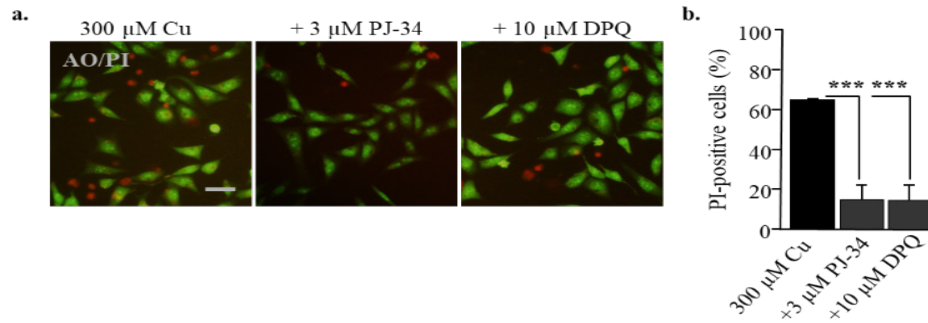

Fig. 4. PARP involves in Cu-induced HT22 cell death. (a) Representative images showing AO/PI staining on HT22 cells exposed for 24 h to 300 µM Cu alone or together with 3 µM PJ-34 or 10 µM DPQ. (b) Mean summary of Cu-induced HT22 cell death under indicated conditions, from 3 independent experiments, using three wells of cells for each condition in each experiment. Cells were treated with PJ-34 or DPQ for 30 min prior to and during exposure to Cu. Scale bar, 40 µm. ***, $p < 0.005$ compared to control.

PKC/NOX Signalling Pathway Is Critical in Cu-Induced HT22 Cell Death

One of the primary sources generating ROS in the central nervous system (CNS) is NADPH oxidases (NOX) and; therefore, NOX plays a critical role in neuropathology [44,45]. Hippocampal neuronal cells are shown to express NOX [46,47]. Previously, PKC and NOX have been suggested to induce microglial cell death following the treatment with Zn^{2+}, $A\beta_{42}$ and ethanol [27,43,48]. Therefore, we were curious to know more about the upstream signaling pathways by examining the PKC and NOX's involvement in Cu-induced HT22 cell death. Thus, we performed a single-cell imaging; using DCF, a fluorescent indicator for ROS generation, in order to access the ROS production in HT22 cells upon exposure to Cu. HT22 cells treated with Cu were observed to have an increase in the intracellular ROS level (**Fig. 5a** and **b**).

Exposure to Cu concentration-dependently induced ROS production. Intriguingly, previous studies have shown that PKC and NOX are crucial in the production of ROS [49-51]. Therefore, there is a possibility that these proteins play a role in facilitating Cu-induced TRPM2-mediated HT22 cell death.

We then performed AO/PI staining to determine the effects of PKC and NOX inhibitors on Cu-induced HT22 cell death. The number of HT22 cell death induced by Cu notably decreased following the treatment with PKC inhibitor, which is CTC (**Fig. 6a** and **b**). Similarly, Cu-induced cell death in HT22 cells was strongly suppressed by the treatment with NOX inhibitors, which are DPI and GKT (**Fig. 6c** and **d**). Taken together, these data consistently support the notion that suggests the role of the PKC and NOX in mediating the production of ROS which eventually results in PARP activation. PKC/NOX/PARP signalling mechanisms subsequently lead to the Cu-induced TRPM2-mediated HT22 cell death.

Exposure to Cu induces activation of protein kinase C (PKC) and NADPH oxidases (NOX), including NOX1/4 and NOX2, leading to the generation of ROS. ROS stimulate the activation of poly(ADP-ribose) polymerase-1 (PARP-1) in the nucleus to generate ADP-ribose (ADPR). ADPR binds to activate the TRPM2 channel, resulting in extracellular Ca^{2+} influx to increase the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). A high concentration of the $[Ca^{2+}]_i$ ultimately drives HT22 neuronal death.

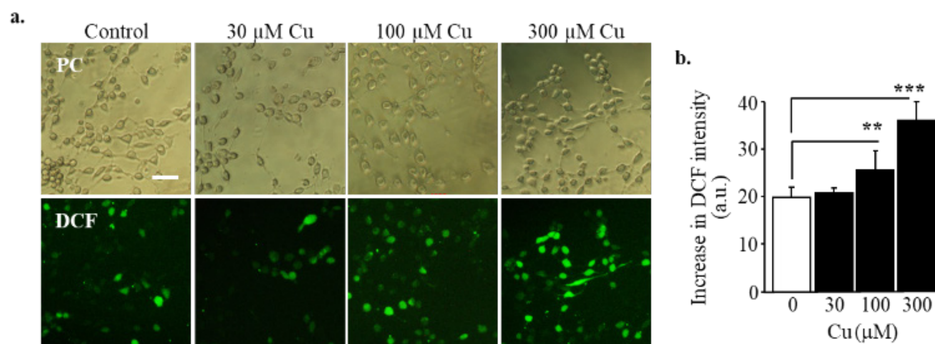

Fig. 5. Cu-induced ROS production in HT22 cells. (a). Representative images showing cellular ROS levels in HT22 cells (top row: phase contrast; bottom row: DCF fluorescence). Cells were treated with different concentrations of Cu as indicated for 8 hr. (b) Mean summary of Cu-induced ROS production in HT22 cell under indicated conditions, from 3 independent experiments, using three wells of cells for each condition in each experiment. Scale bar, 40 µm. **, $p < 0.01$; ***, $p < 0.005$ compared to control.

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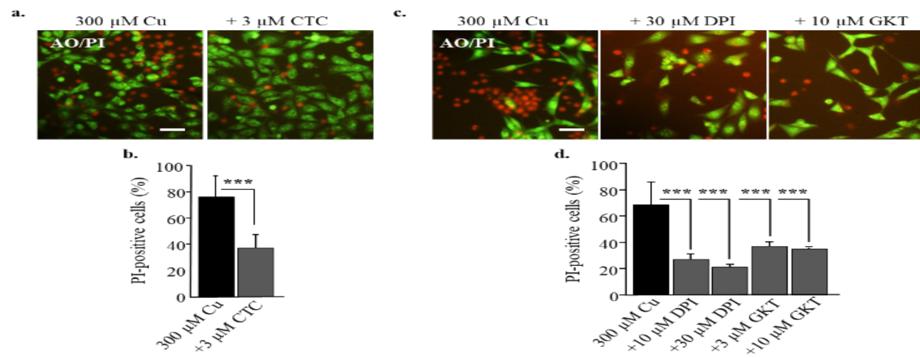

Fig. 6. PKC/NOX signalling involves in Cu-induced HT22 cell death. (a, c) Representative images showing AO/PI staining on HT22 cells exposed for 24 h to 300 µM Cu alone or together with 3 µM CTC (a) or DPI and GKT (c) at indicated concentrations. (b, d) Mean summary of Cu-induced HT22 cell death under indicated conditions, from 3 independent experiments, using three wells of cells for each condition in each experiment. Cells were treated with CTC or DPI or GKT for 30 min prior to and during exposure to Cu. Scale bar, 40 µm. ***, $p < 0.005$ compared to control

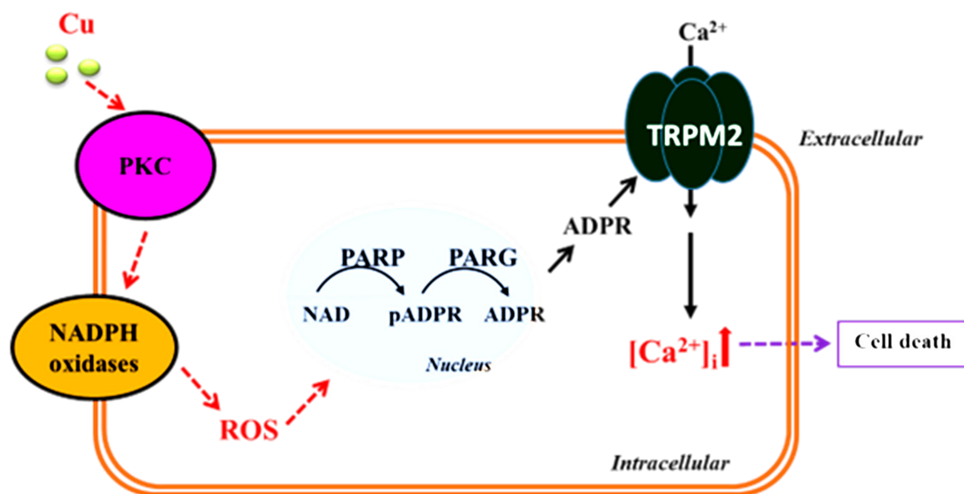

Fig. 7. Schematic summary of the signalling mechanisms mediating Cu-induced TRPM2-mediated HT22 neuronal death.

DISCUSSION

Cu is known to be one of the main transition metals with multiple functions in different biological activities; however, an excessive amount of copper can cause detrimental effects on the brain [52,53]. How exactly copper affects the brain remains poorly elucidated. As introduced above, there is increasing evidence suggesting neurons as the main target for copper toxicity in the brain pathologies [54-56]. However, there is still no clear understanding of the mechanisms that drive the progression of neuronal cell-associated Cu-induced brain pathologies.

The involvement of TRPM2 channel in causing cell death in several cell types, such as neuronal cells, following exposure to pathogenic stimuli known to increase ROS generation has been extensively described [57-60]. However, it is yet unknown if the TRPM2 channel plays a crucial role in the Cu-induced death of neural cells. In the current work, we offer pharmacological proof that the TRPM2 channel is involved in mediating HT22 cell death in response to Cu treatment. Additionally, we discovered that the NOX/ROS/PARP/TRPM2 signalling pathway is responsible for Cu-induced neuronal cell death (**Fig. 7**).

In the current study, we first, using the immunofluorescent microscopy technique, confirmed that the HT22 neuronal cells express the TRPM2 channel. All HT22 cells, which can be identified by the nuclear staining DAPI, were positively labelled with anti-TRPM2 antibodies (**Fig. 1a**). Such positive immunostaining was not observed in control cells. Additionally, it was noted that the area around the plasma membrane had a higher intensity of green fluorescence (**Fig. 1a**). This finding implies that the HT22 cell membrane is the primary site of TRPM2 channel expression. Overproduction of Ca^{2+} can mediate neuronal death and such result ultimately contributes to the pathogenesis of many neurological diseases including, Parkinson's disease (PD) and Alzheimer's disease (AD) [61-63].

We postulated that the activation of the TRPM2 channel in response to Cu leads to the increase of $[Ca^{2+}]_i$, which in turn results in the HT22 cell death. We, therefore, investigated the possible involvement of the TRPM2 channel in mediating Ca^{2+} responses following the treatment with Cu, by monitoring the increase of $[Ca^{2+}]_i$ in HT22 cells.

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We demonstrated that the application of 100–300 µM of Cu evoked a significant increase in the $[Ca^{2+}]_i$, but exposure to 30 µM Cu failed to induce any significant increase in the $[Ca^{2+}]_i$ (**Fig. 2a** and **b**). Cu-induced Ca^{2+} responses were strongly dependent on the concentration, which occurred at a significant level only at a high concentration of Cu. These results are consistent with what has been reported in primary neuronal cells [64].

Excessive Cu is highly cytotoxic, particularly to neuronal cells [65-67]. It is commonly known that the TRPM2 channel plays a crucial role in mediating cytotoxicity in a variety of cell types. Nevertheless, this channel's function in Cu-induced cytotoxicity has not yet been explored in HT22 cells. In the current investigation, we demonstrated that 30–300 µM Cu caused considerable HT22 cell death, as compared to the control group where only a few dead cells were observed (**Fig. 3a** and **c**). Interestingly, such an increase in cell death induced by 300 µM Cu was strongly ablated in the presence of two structurally different TRPM2 channel inhibitors, 2-APB and ACA (**Fig. 3b** and **d**).

The attenuation of cell death by 2-APB was shown to be at a lesser extent compared to ACA. Collectively, these results strongly provide evidence suggesting that the TRPM2 channel plays a critical role in triggering Cu-induced HT22 cell death. It is worth mentioning that both 2-APB and ACA, are not TRPM2-specific inhibitors. However, intensive investigations have been done and the findings showed that these compounds were able to induce inactivating current through the TRPM2 channel [68-72], therefore suggesting that 2-APB and ACA are powerful and effective in inhibiting this channel. The exposure of Cu leading to the occurrence of oxidative stress and an increase in the PARP activity represents a major mechanism by which oxidative stress induces ADPR generation and subsequent TRPM2 channel activation [73].

Consistently with our previous study demonstrating the microglial cell death induced by ROS or Zn^{2+} or ethanol [27,43,48], here we observed that the Cu–induced HT22 cell death was remarkably abolished by application of PJ-34 and DPQ, PARP inhibitors (**Fig. 4a** and **b**). These findings provide credence to the idea that PARP plays a significant part in the TRPM2-mediated Cu–induced HT22 cell death.

We then made an effort to look into the upstream signalling pathways, especially those that produce ROS and activate PARP. Cu has been shown to cause the generation of ROS [65,74,75]. Additionally, prior research demonstrated the critical roles that PKC and NOX play in modulating the generation of ROS [76,77]. Here, we consistently demonstrated that Cu caused a notable rise in the generation of ROS (**Fig.** 5.a and b). Furthermore, the mortality of HT22 cells produced by Cu was significantly decreased by blocking PKC and NOX with CTC, DPI and GKT inhibitors, respectively (**Fig. 6a-d**). The idea that PKC/NOX functions as an upstream signalling route in promoting Cu-induced PARP and TRPM2 channel activation, which eventually leads to HT22 neuronal cell death, is amply supported by these data.

The results of the current investigations collectively have strongly implied that the Cu-induced HT22 cell death is related to the PKC/NOX/ROS/PARP/TRPM2 signalling pathway. Such convincing data provides fascinating and new insights into the mechanisms underlying copper-induced brain diseases, as shown in **Fig. 7**.

CONCLUSIONS

In summary, the study provides evidence that the TRPM2 channel plays a critical role in the mechanism mediating copper-induced Ca^{2+} signalling and cell death in neuronal HT22 cells. We further hypothesize that activation of the PKC/NOX/ROS pathway is an important mechanism in copper-induced stimulation of PARP-1, activation of the TRPM2 channel, and the increase in $[Ca^{2+}]_i$ that ultimately leads to HT22 neuronal cell death. It is worthwhile to investigate the findings reported in this study on copper-induced brain damage in vivo. These new findings offer the possibility of developing new therapeutic interventions to prevent neurological diseases.

ACKNOWLEDGMENTS

This research was supported by the Ministry of Education (MOE) through the Fundamental Research Grant Scheme (FRGS/1/2020/SKK06/UPM/02/18)

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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