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Original Article

Regeneration of mechanical sciatic nerve injury is affected by cold and heat exposure: involvements of the TRPM2 and TRPM8 channels

Fuat Uslusoy[1], Mustafa Nazıroğlu[2,3], Kemal Ertilav[4]

[1]Department of Plastic Reconstructive and Aesthetic Surgery, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey; [2]Neuroscience Research Center, Suleyman Demirel University, Isparta, Turkey; [3]Drug Discovery Unit, BSN Health, Analyses, Innovation, Consultancy, Organization, Agriculture and Industry Ltd, Göller Bölgesi Teknokenti, Isparta, Turkey; [4]Departmant of Neurosurgery, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey

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Abstract: Background: Mechanical sciatic nerve (MSN) injury has a high rate within trauma cases. Heat and cold exposure in the treatments of MSN injuries have been clinically used in human. The MSN injury results in apoptosis, overload Ca^{2+} influx, and reactive oxygen species (ROS) generation in the sciatic nerve. TRPM2 and TRPM8 cation channels are activated by ROS. TRPM2 is activated by warmth (36-38°C) and heat (45-47°C), although TRPM8 is activated by cold (0-25°C). Heat or cold exposure may aid recovery MSN injury through modulation of TRPM2 and TRPM8 in sciatic nerve. Objective: The protective roles of cold and heat treatments via modulation of TRPM2 and TRPM8 were evaluated on MSN injury-induced neurotoxicity in *in vitro* models of mouse and the SH-SY5Y cell line. Method: The mice sciatic nerves and SH-SY5Y cells were divided into control (37°C), cold (10°C), and moderate heat (40°C) groups. Results: Our data identified a decrease in injury diameter in the neurons following heat exposure, but not cold exposure. In addition, the results of laser confocal microscopy analyses were indicative of a protective role of TRPM8 antagonist (ACA) against cold-induced increases in Ca^{2+} influx in the sciatic nerve and TRPM8 expressing SH-SY5Y cells. The results of the automatic plate reader and laser confocal microscope assays indicated a protective role of heat treatment against MSN injury-induced increases in apoptosis, mitochondrial ROS, cytosolic ROS, caspase -3, and -9 in the neurons. Conclusions: The heat treatment via possible modulation of TRPM2 channel and heat shock proteins induced protective actions against injury-mediated increases of oxidative stress, excitotoxicity, and apoptosis in the sciatic nerve and SH-SY5Y cells.

Keywords: Apoptosis, heat treatment, cold treatment, sciatic nerve injury, TRPM2 and TRPM8 channels

Introduction

Several factors, including traffic accidents and surgical operations can induce peripheral nerve injury [1]. In the regeneration of mechanical sciatic nerve (MSN) injury, the peripheral nervous system is potentially more active than in the central nervous system, but suitable treatment for the MSN injury needs an accurate diagnosis with regard to the degree of injury. Heat and cold exposures are present in the current treatment approaches, although conflicting reports are present on the effects of such exposures [2, 3]. Therefore, suitable non-invasive treatment methods are needed for the MSN injury-induced neurodegeneration.

Axons of peripheral nerve systems, including sciatic nerve have regenerate capacity after injury. After injury in the axons of sciatic nerve, several molecular repair and injury pathways are activated. It is well-known that an accumulation of cytosolic calcium ion (Ca^{2+}) serves several repair and injury functions in neurons [4-6]. Transient receptor potential (TRP) superfamily was discovered within last decades [4-6]. Two members of the TRP superfamily are TRP melastatin 2 (TRPM2) and TRP melastatin 8 (TRPM8) channels. The TRPM8 channel is activated by several stimuli, including cold (25°C ≤) and oxidative stress [7-9]. Expression levels of TRPM2 and TRPM8 channels are high in peripheral nerves [10-12]. In addition to the

ADP-ribose and oxidative stress, TRPM2 channel is activated by warm temperature [13-16]. Direct activation of TRPM2 by heat alone needs high temperatures (threshold > 47°C) [17, 18], although exposure to the warm temperature (35-38°C) activates the TRPM2 via potentiation of ADP-ribose currents [15]. Hence, cold or heat exposure treatment methods via stimulation of TRPM2 and TRPM8 may be useful for the treatment of peripheral nerve injuries.

It has been shown that moderate Ca^{2+} influxes through activation of TRPM2 and TRPM8 cause sciatic nerve recovery, although excessive Ca^{2+} influxes via activation of TRPM2 and TRPM8 result in apoptosis in neuronal cells and sciatic nerve [8, 19-21]. In addition to Ca^{2+} influx-induced nerve injury, several studies suggest that mitochondrial oxidative stress is also one of the main reasons that cause peripheral neuropathy and neurotoxicity through activation of TRPM2 and TRPM8 channels [9, 11, 22]. In addition to the apoptotic and oxidant effects of Ca^{2+} influx, the heat-induced Ca^{2+} influx has anti-apoptotic and antioxidant effects by activating several molecular mechanisms such as production of heat shock proteins and inhibition of extracellular-signal-regulated kinase [5, 23, 24]. Involvement of TRPM2 and TRPM8 in the induction of heat/cold hyperalgesia and sciatic nerve injury were recently reported [20, 21, 25]. However, the effects of heat or cold exposure on the TRPM2 and TRPM8 channel activations in the recovery process of MSN injury has not been clarified yet.

In order to obtain further insight into the molecular pathways of action of cold and heat treatments, we investigated the effect of cold and heat applications on MSN injury recovery in a sciatic nerve-injured mouse model. We further explored the influence of cold and heat applications on apoptosis in vitro and its possible effects on Ca^{2+} signaling via TRPM2 and TRPM8 activations. The results of our study may provide further explanation for the effect of cold and heat applications on promoting peripheral nerve regeneration.

Material and methods

Animals and crush injury

We used 24 male C57BL/6j black mice (aged 6-8 weeks old) in the current study. The mice were kept on a 12-h light/dark schedule with free access to food and water. After purchasing the mice from the Burdur Mehmet Akif University (BMAU), Turkey, the mice were transported to BSN Health, Analyses, Innovation, Consultancy, Organization, Agriculture and Industry Ltd, Isparta, Turkey for the heat and cold exposure treatments. Approve of the experiment was taken from Local Experimental Animal Research Ethical Committee of BMAU (Permit Numbers: 388. Date: 19.07.2018).

A crush injury at the proximal-thigh level of the sciatic nerve under the ketamine and xylazine anesthesia was induced by a 3-0 silk ligation [26]. After 5 min of the ligation, the suture was carefully released to produce a regenerating axonotmesis model. The sciatic nerve samples were obtained from left and right legs. In each experiment, the control sciatic nerve was obtained from the right leg of the mouse, although sciatic nerve of cold or heat groups was obtained from the left leg of same mouse.

Study groups in the sciatic nerve of mice

Cold and heat exposures were applied to the neurons as described by physicians in the Department of Plastic Reconstructive and Aesthetic Surgery, Faculty of Medicine, SDU, Isparta, Turkey. After inducing MSN injury, the isolated sciatic nerve samples were divided into three groups as follows.

Control (37°C) group (n=8): The right sciatic nerve was used as control and kept at 37°C in the cell culture conditions for 3 days.

Cold (10°C) group (n=8): The left sciatic nerve sample of the same animal was kept cold (10 ± 3°C) for 15 min of each hour (8 times in a day). In the remaining times (45 minutes of the 8 h period and for the remaining fraction of the daily 24 h), it was kept in the cell culture conditions (37°C, 5% CO_2 and 65% humidity).

Heat (40°C) group (n=8): The left sciatic nerve in the heat treatment group was simultaneously kept at 40°C in the cell culture conditions for 3 days, although the right sciatic nerve with MSN injury of the animal was used as control (at 37°C).

DNA transfection procedure of SH-SY5Y cells

According to our knowledge, there is no report on the presence of TRPM8 channel in the human (h) SH-SY5Y neuronal cells. Hence, we

performed transient transfections in the hSH-SY5Y cell lines by using the 2 μg cDNAs of hTRPM8 as described by the manufacturer (B'SYS GmbH, Witterswil, Switzerland). The hTRPM8 was obtained from Dr. Simon Hebeisen (B'SYS GmbH). In the control samples, we used wild type TRPM8 empty vector hTRPM8 plasmid (2 μg) (OriGene Technologies, Istanbul, Turkey) and Lipofectamine 2000 (Invitrogen; Istanbul, Turkey). The transfection procedure was confirmed in a fluorescent camera (Axiocam 702 mono, Zeiss, Ankara, Turkey) by presence of EGFP [8].

Cell culture and study groups of normal and transfected SH-SY5Y neuronal cells

The un-transfected (control) and transfected SH-SY5Y cells were cultured as previously described in the cell culture conditions (5% CO_2 plus humidified air at 37°C) [27]. In brief, the SH-SY5Y cells were maintained in DMEM with low glucose (1 g/l)/Ham's F12 (50:50) medium mixture, 10% fetal calf serum, 2 mM glutamine, 100 IU penicillin, and 100 μg/ml streptomycin (Thermo Fischer Scientific Inc., Istanbul, Turkey). For laser confocal microscopy (LSM800, Zeiss, Ankara Turkey) analyses, we waited 24 h for the attachment of SH-SY5Y cells on the glass bottom dishes (Mattek Corporation Inc., Ashland, MA, USA). Then, the cells were divided into three groups as follows.

Control (37°C) group: After the line injury in the bottom dishes, the SH-SY5Y cells were kept at 37°C in the cell culture conditions for 24 hours.

Cold (10°C) group: The SH-SY5Y cells were kept cold at 10°C in the cell culture conditions for 15 min each hour after the line injury in the glass bottom dishes. The 15 min cold exposure period was repeated 8 times. For the remaining times of 24 h, the cells were kept in the cell culture conditions.

Heat (40°C) group: The SH-SY5Y cells were kept at 40°C in the cell culture conditions for 24 h after the line injury in the glass bottom dishes.

Preparation of in vitro mechanical injury cell culture model

It is well known that the SH-SY5Y cells have a structure similar to neurons such as axons and dendrites. We used SH-SY5Y cells as a model for neurons to perform this study, because the structure of SH-SY5Y cells was destroyed just like the loss of axons and dendrites and the breakage of perikaryon seen after mechanical injury [28, 29]. The SH-SY5Y cells were seeded in 35 mm glass bottom dishes. At the beginning of cold and heat experiments, the cells from control, cold and heat groups were scratched with a sterile 10 μl white pipette tip (**Figure 3**). We did not change the culture medium after wounding to keep the medium conditioned with cell debris and factors released from the detached cells. The supernatant was removed for the assays of bright field diameter and free cytosolic Ca^{2+} concentration.

Bright field (black/white) imaging records

By the Zeiss Company (Ankara, Turkey), the Axiocam 702 mono camera was adapted to an AxioVert A1 microscope and estimations of the diameter (μm) of injuries in bright field images of the control, cold, and heat groups. The diameters of the injuries in the sciatic nerve and SH-SY5Y cells were measured by using a ZEN program (Zeiss). We accepted 100% of the control (0 day) wounds. Then, we divided the wound diameter values (experiment/control). The mean values in the sciatic nerve and SH-SY5Y cells were expressed as % of 0 day and 0 h, respectively.

Preparation of sciatic nerve cell culture samples

Details of induction of sciatic nerve injury in the isolated sciatic nerve were given in previous studies [20, 21]. Briefly, the sciatic nerve samples were removed from the mice under anesthesia. For laser confocal microscopy, the neurons were seeded in 35 mm glass bottom dishes for at least 6 h at 37°C with 5% CO_2/95% O_2 in 1 ml DMEM.

Measurement of intracellular Ca^{2+} fluorescence intensity induced by TRPM2 and TRPM8 activation

We investigated heat-induced TRPM2 channel activation in the sciatic nerve neuron culture and SH-SY5Y samples in the laser confocal microscope (LSM800) with a 40× oil objective by using with 1 μM florescent dye Fluo-3 (Calbiochem, Darmstadt, Germany) as described in recent studies [8, 20]. The Fluo-3 dye in the cells was excited by a 488 nm argon laser. The

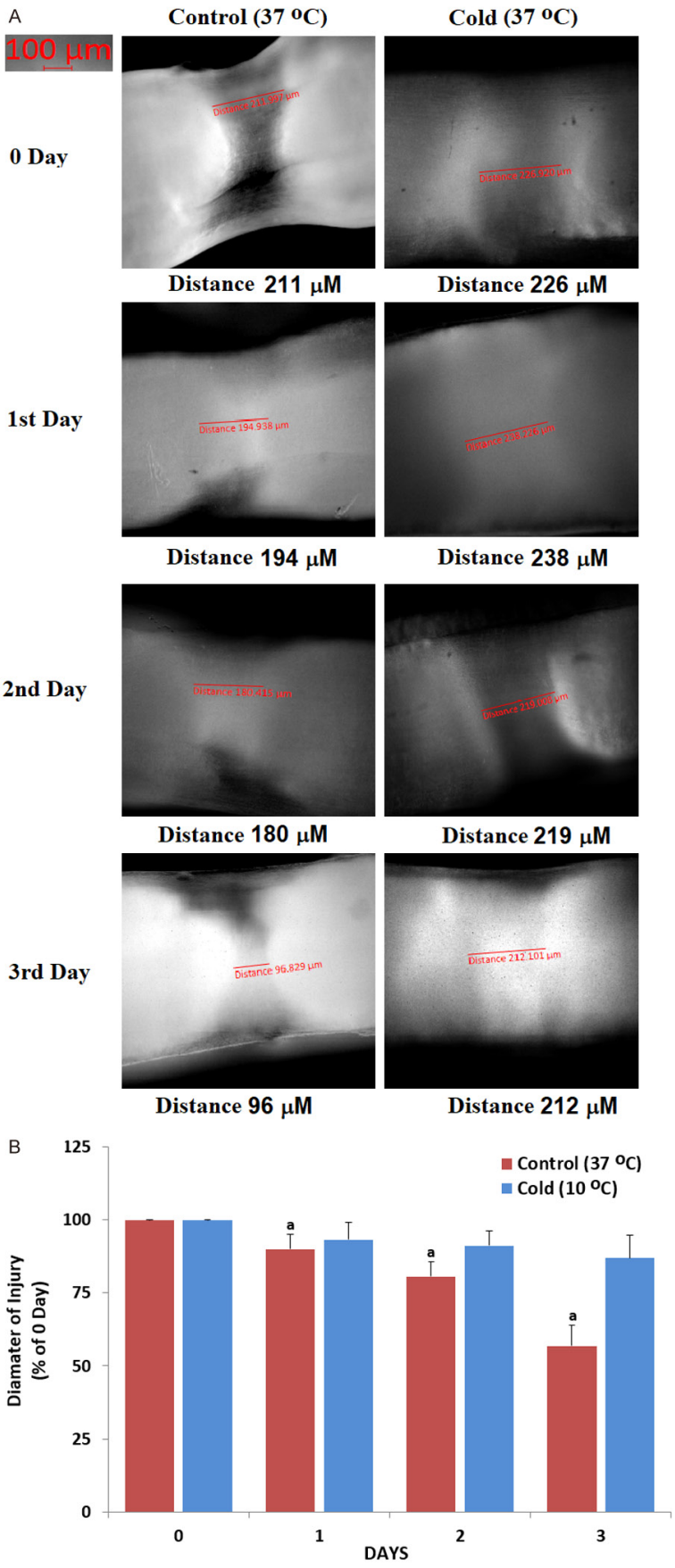

Figure 1. Cold exposure (10°C) accelerated the MSN injury in the sciatic nerve of mice. (n=8 and mean ± SD). A. Bright field (BF) images of injuries in the control and cold groups were taken in the microscope (AxioVert A1) (objective: 10×) by using the high-performance camera (Axiocam 702 mono). The samples were analyzed by the microscope. B. Numbers in the column indicate mean values. ($^{a}P \leq 0.001$ *vs.* 0 day).

sciatic nerve neurons and SH-SY5Y cells were treated with N-(p-amylcinnamoyl) anthranilic acid (ACA and 25 µM) to inhibit Ca^{2+} entry after stimulation of H_2O_2 (1 mM) and menthol (100 µM) [8, 21, 30]. The fluorescence intensity results of Fluo-3 in the cytosol of cells and neurons were expressed as arbitrary units (a.u.) per cell by using the ZEN program (Zeiss).

Apoptosis, cell viability, caspase -3 and -9 analyses

The induction of apoptosis in the membranes of apoptotic MSN injury was determined in a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) by using a commercial kit (Biocolor Ltd., Northern Ireland) as described in a previous study [20]. We used cell viability analyses as MTT in the neurons as described elsewhere [31] and absorbance changes of MTT were recorded in the spectrophotometer (UV-1800) at 490 nm before and after cold/heat exposures.

To determine caspase -3 and -9 activity, the neurons in the control and cold and heat-treated groups were incubated with fluorogenic substrate solutions (AC-DEVD-AMC for caspase -3 and ACDEVD-AMC for caspase -9) for 1 h at 37°C as previously described [32]. Cle-

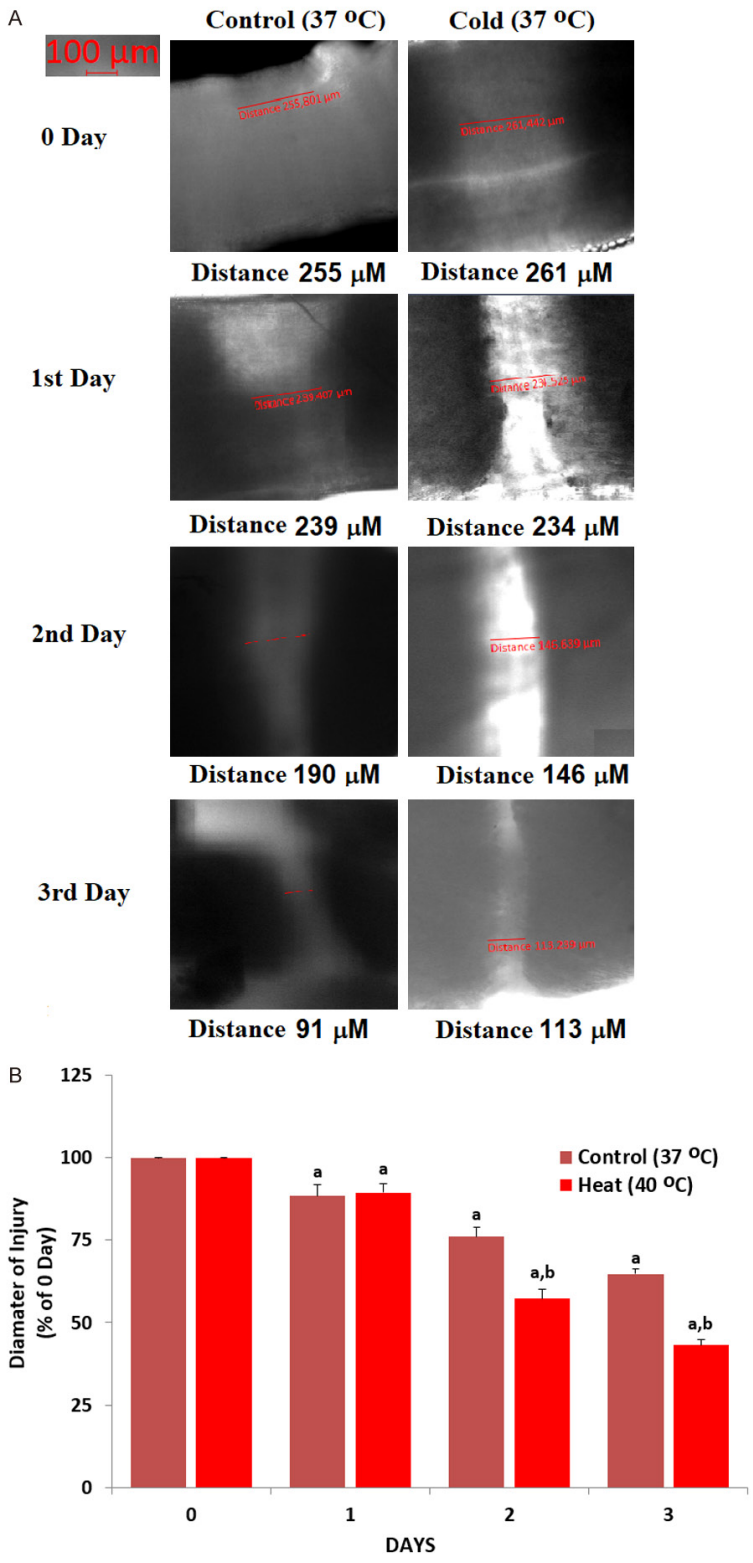

Figure 2. Heat exposure (40 °C) accelerated the recovery of MSN injury in the sciatic nerve of mice. (n=8 and mean ± SD). (A) Bright field (BF) images of injuries in the control and cold groups were taken in the AxioVert A1 microscope (objective: 10×) by using the high-performance camera (Axiocam 702 mono). Changes in the diameter of sciatic nerve injury were shown by columns (B). ([a]$P \leq 0.001$ *vs.* 0 day. [b]$P \leq 0.001$ *vs.* control (37 °C) group).

avage of the substrate was measured with a microplate reader (Infinite pro200; Tecan Austria GmbH, Groedig, Austria). Wavelengths of excitation and emission were kept as 360 nm and 460 nm, respectively. Analyses of mitochondrial membrane depolarisation (MMP) level and intracellular ROS production in the plate reader were measured in the plate reader (Infinite pro200) by using dyes (JC1 and DHR-123) as described in previous studies [33, 34]. Data of apoptosis, cell viability, caspase -3, caspase -9, MMP, and ROS were calculated as fluorescence units/mg protein and presented as % of control.

Imaging ROS generation in the mitochondria and cytosol of SH-SY5Y using laser confocal microscopy

Mitochondrial ROS (MitoROS) generation visualized under laser confocal microscopy (LSM800) was assayed using MitoTracker Red CM-H2Xros fluorescent dye (Life Technologies) following the manufacturer's instructions. Cytosolic ROS generation was monitored by using DCFH-DA stain [35]. After washing the cells with 1xPBS prior to imaging, MitoTracker Red CM-H2Xros was excited with a diode laser at 561 nm, although the DCFH-DA were excited with a diode laser at 488 nm. The Plan-Apochromat 10× oil objective was used in the analyses of mitochondrial and cytosolic ROS. The fluorescence intensity data were analyzed by using the ZEN program.

Live (Hoechst)/death (propidium iodide) analyses

Imaging of live and dead cell rate in the laser confocal micr-

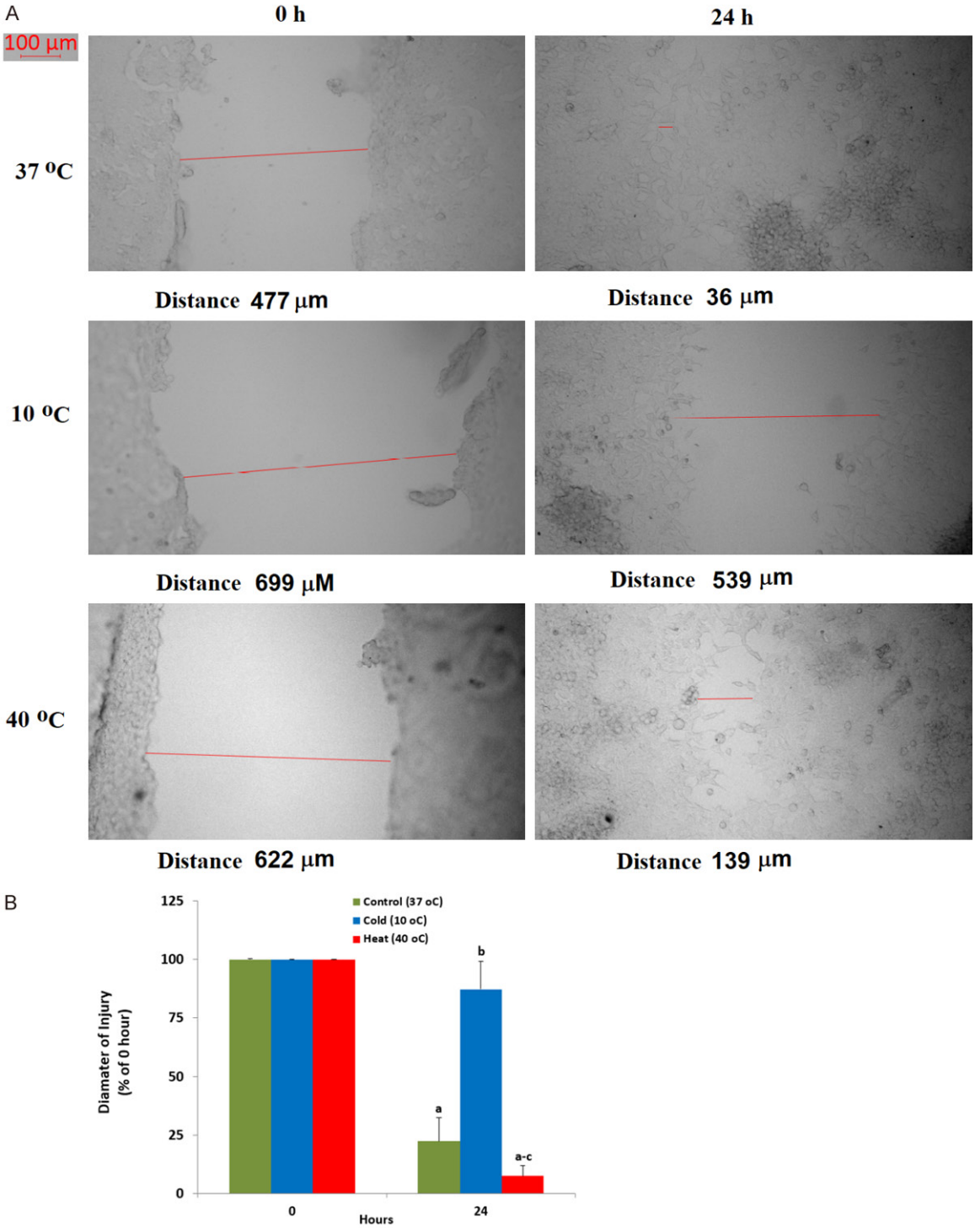

Figure 3. Cold exposure (10 °C) accelerated the injury in SH-SY5Y neuronal cells. (n=8 and mean ± SD). (A) Bright field (BF) images of injuries in the control, cold and heat groups were taken in the microscope (AxioVert A1) with a 10× objective by using the high-performance camera (Axiocam 702 mono). Changes in the diameter of injury in the SH-SY5Y cells were shown by columns (B). Numbers in the column indicate mean values. ($^{a}P \leq 0.001$ *vs.* 0 h. $^{b}P \leq 0.001$ *vs.* 37 °C. $^{c}P \leq 0.001$ *vs.* 10 °C).

oscope (LSM 800) with a 20× objective was investigated by using Hoechst 33342 and propidium iodide (PI) staining, respectively [35]. Images in the cells and neurons were captured by using a ZEN program. The death cell rates were expressed as %.

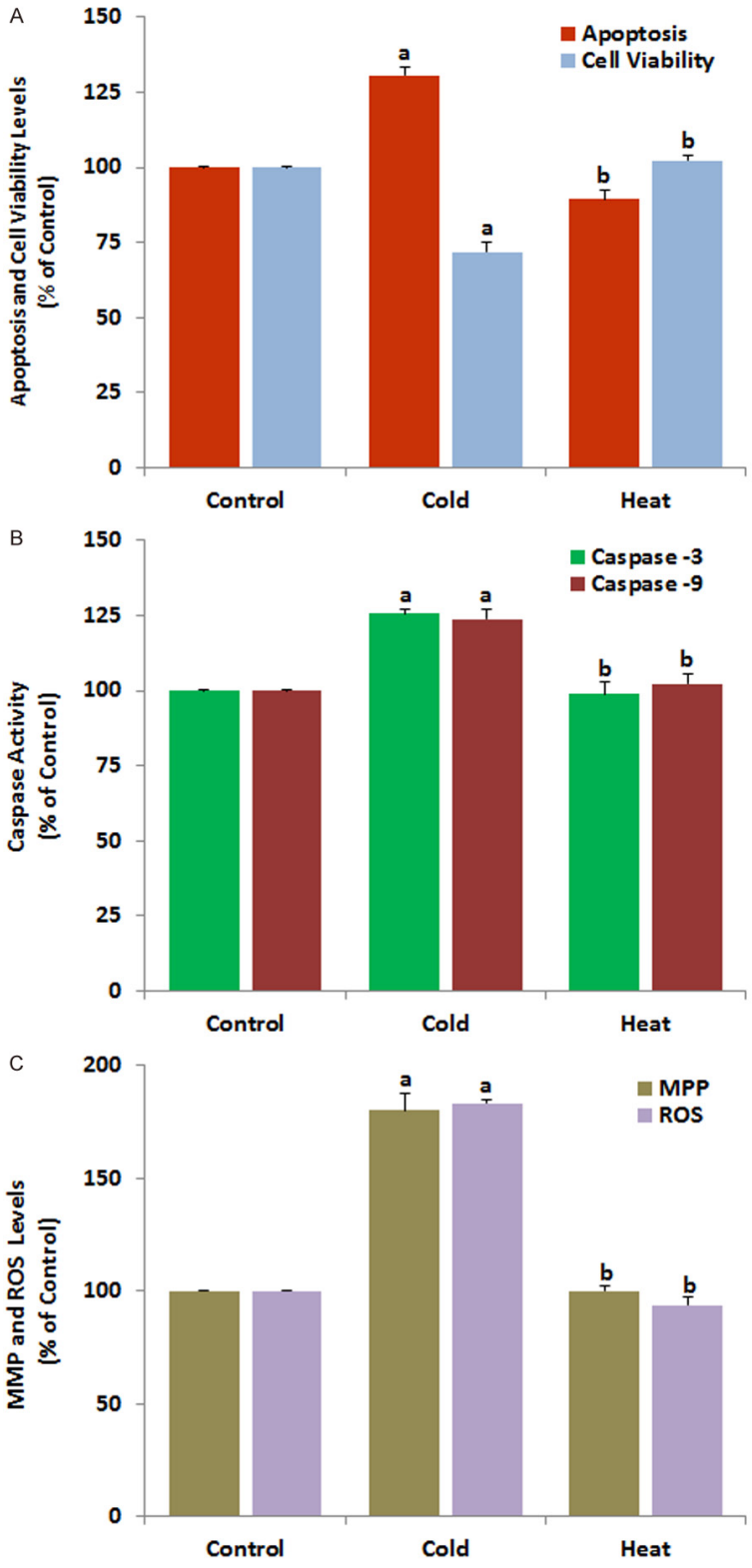

Figure 4. Heat exposure decreased MSN injury-induced apoptosis (A), cell viability (A), caspase -3, caspase -9 activities (B), mitochondrial membrane depolarization (MMP), and intracellular ROS production (C) in the sciatic nerve of mice. (Mean ± SD and n=8). The level of apoptosis was measured in the plate reader (Infinite pro200) by using commercially available kits. ($^{a}P \leq 0.001$ *vs.* control. $^{b}P \leq 0.001$ *vs.* cold group).

Statistical analysis

Statistical analysis was performed using the statistical software SPSS20.0. All data were presented as mean ± standard deviation (mean ± SD). N numbers of the groups were calculated by power analyses. In the ANOVA test, the mean data wasn't normally distributed. Hence, presences of statistical significance in the data were analyzed using *post hoc* least significant difference (LSD) test. For significant *P* values, a non-parametric (Kruskal-Wallis) test was utilized. Statistically significant values were those with a *P* value ≤ 0.05.

Results

Imaging morphological changes by laser confocal microscopy

For investigating MSN injury-induced apoptotic status under cold and heat treatments, morphological examinations in the injured-sciatic nerve and SH-SY5Y cells were performed by using bright field imaging. As shown in **Figures 1A**, **1B** and **3A**, **3B**, the cold treatment did not induce significant recovery effects in the injuries of MSN neurons and SH-SY5Y cells. There was no difference on the injury diameter in MSN injury of cold group between 0 day and 3rd day ($P \geq 0.05$). However, the injuries in the sciatic nerve at 1st, 2nd, and 3rd days (**Figure 2A**, **2B**) and SH-SY5Y cells at 24 h (**Figure 3A**, **3B**) were dramatically recovered by treating with heat. The injury levels were significantly lower in the heat groups than in cold group at 2nd and 3rd days or at 24 h ($P \leq 0.001$).

MSN injury-induced apoptosis, neuron viability, neuron death, caspase activity, intracellular ROS production, and mitochondrial membrane potential (MMP) levels in the MSN injury were decreased by heat treatment, but not cold treatment

After observing recovery changes following heat exposure, we decided to investigate changes in the mitochondrial ROS, cell death (PI/Hoechst), and apoptosis pathways by using established fluorescent dyes. The levels of apoptosis (**Figure 4A**), caspase -3, caspase -9 (**Figure 4B**), MMP (JC-1), ROS (DHR123) (**Figure 4C**), and neuron (PI/Hoechst rate) death (**Figure 5A**, **5B**) were increased in the MSN injury by cold exposure ($P \leq 0.001$); however, neuron viability (MTT) levels (**Figure 4A**) were decreased in the neurons by the cold treatment ($P \leq 0.001$). The treatment of neurons with heat diminished MSN injury-induced mitochondrial oxidative cytotoxicity (apoptosis, JC-1, ROS, caspase -3, and caspase -9) and cell death (PI/Hoechst) rate. These results implied that MSN injury-induced mitochondrial oxidative neurotoxicity and apoptosis might be attributed to cold-dependent activated TRPM8 activation.

MSN injury-induced increases of mitochondrial and cytosolic ROS imaging in the neuron were diminished by the heat treatment

It is well known that the sensitivity of laser confocal microscope analyses is higher than in the plate reader analyses. In addition to the cytosolic ROS analyses in the plate reader, we supplemented these results with examination by laser confocal microscopy. Fluorescence intensities and mean values of MitoROS and cytosolic ROS (DCFH-DA) in the sciatic nerve are shown in **Figure 6A**, **6B**. The fluorescence intensities of cytosolic ROS and MitoROS levels in the SH-SY5Y cells were higher in the cold group than in the control and heat-treated groups ($P \leq 0.001$). However, cytosolic ROS and MitoROS levels were diminished in the heat group by the heat treatment ($P < 0.001$).

Intracellular Ca^{2+} concentration in the control, cold and heat group of sciatic nerve and SH-SY5Y cells

We investigated changes on $[Ca^{2+}]_c$ in the sciatic nerve neurons with MSN injury. The $[Ca^{2+}]_c$ was higher in the heat group than in the cold and control group at 3rd days ($P \leq 0.001$) (**Figure 7A**, **7B**). The $[Ca^{2+}]_c$ was further increased in the heat groups but not in the cold group by the TRPM2 activator (H_2O_2) treatment. ACA as a cell-permeable and nonselective TRPM2 and TRPM8 inhibitor completely blocks responses to stimulation with icilin, menthol and cold (13°C) [7, 30]. Inhibitions of TRPM8 through ACA treatments have been shown to be effective in the inhibiting H_2O_2-induced $[Ca^{2+}]_c$, cell death, and ROS production [8, 21]. Hence, the $[Ca^{2+}]_c$ was decreased in the neurons of MSN injury by ACA treatment and their concentrations were significantly lower in the heat groups than in the cold group ($P \leq 0.001$).

The $[Ca^{2+}]_c$ in the control group of SH-SY5Y cells was increased by the H_2O_2 treatment, although it was decreased in the control+H_2O_2+ACA group by the ACA treatment ($P \leq 0.001$). However, there was no difference on $[Ca^{2+}]_c$ in the absence of TRPM8 channel in the heat and cold groups after H_2O_2 stimulation. Hence, we observed TRPM2 channel blocker action of heat treatment in the mechanical injured-SH-SY5Y cells (**Figure 8A**, **8B**). In addition, we investigated effects of cold and ACA treatments on the $[Ca^{2+}]_c$ in the absence and presence of TRPM8 channel in the SH-SY5Y cells. We observed increase of $[Ca^{2+}]_c$ in the TRPM8 transfected SH-SY5Y cells, but not in the normal SH-SH5Y cells. The cold treatment-induced increase of $[Ca^{2+}]_c$ was higher in the TRPM8 transfected SH-SY5Y cells than in the normal SH-SH5Y cells (**Figure 8A**, **8C**). In addition, the increase of $[Ca^{2+}]_c$ via H_2O_2 stimulation in the TRPM8 transfected SH-SY5Y cells was decreased by the ACA treatment ($P \leq 0.001$).

TRPM8 channel antagonism decreased cold-induced increase of $[Ca^{2+}]_c$ in the TRPM8 expressing neurons

After demonstrating the protective role of ACA on SH-SY5Y neuronal injury, we investigated the involvement of TRPM8 channel-induced Ca^{2+} influx in the SH-SY5Y cells. To clarify the effects of cold on the TRPM8 channel activation in the normal and TRPM8 transfected SH-SY5Y cells, the cells were further gated by TRPM8 agonist menthol (100 μM). Stimulations with menthol did not induce changes on the $[Ca^{2+}]_c$ in the normal SH-SY5Y cells (in the absence of TRPM8) (**Figure 9A-C**) ($P \geq 0.05$).

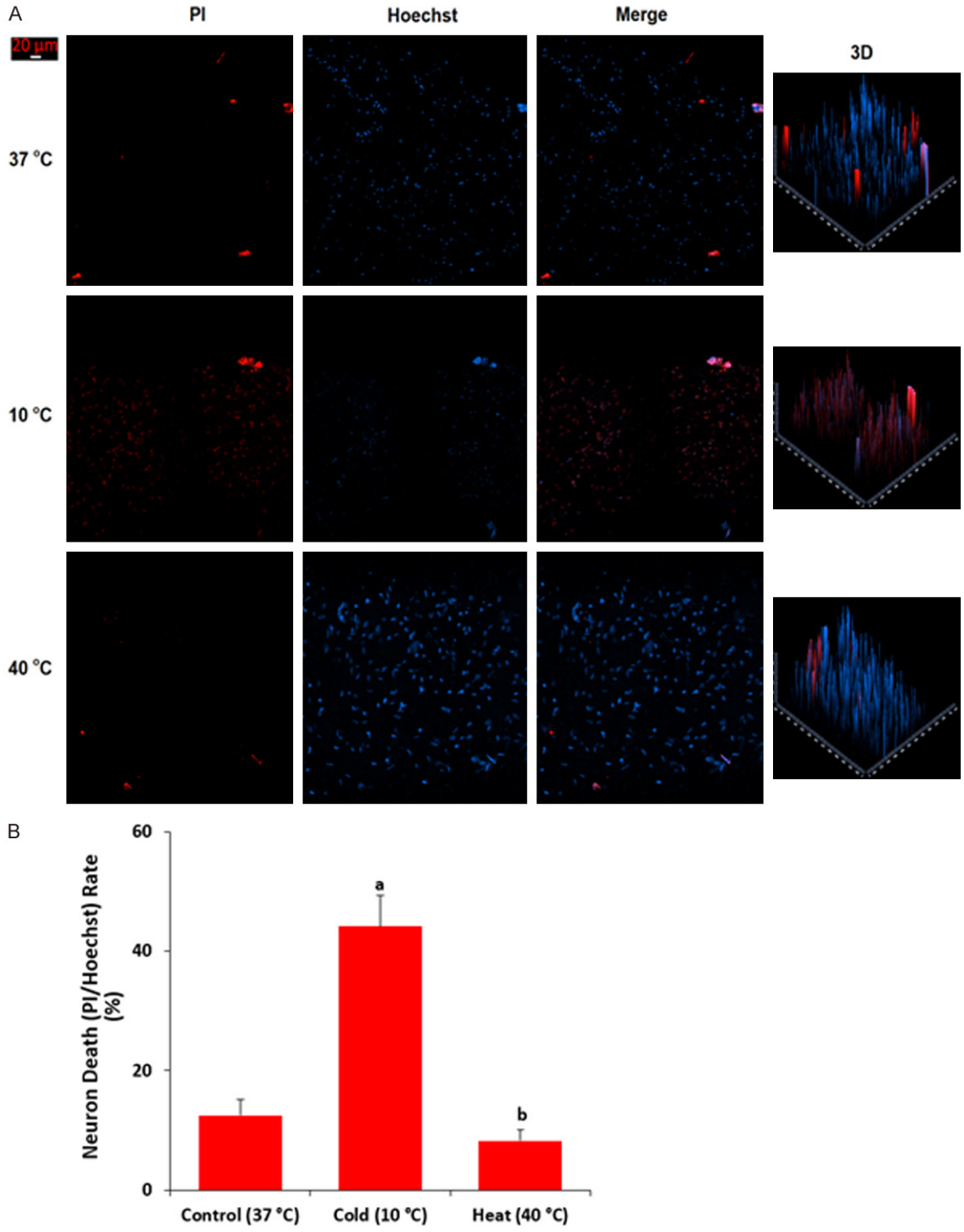

Figure 5. Cold exposure (10 °C)-induced sciatic nerve death was inhibited by heat exposure (40 °C) (mean ± SD). A. Is representative normal and 3D images showing death (propidium iodide, PI) and live (Hoechst 33342) staining of sciatic nerve neurons under control conditions or after exposure to cold and heat. B. The red bars represent the percentage of neuronal death. ($^{a}P \leq 0.001$ *vs.* control. $^{b}P \leq 0.001$ *vs.* cold group).

Stimulations with menthol induced a significant increase on $[Ca^{2+}]_c$ in the TRPM8 transfected SH-SY5Y cells treated with cold (**Figure 10A-C**), which was attributed to the activation of Ca^{2+}-permeable TRPM8. As shown in **Figure 10A-C**, despite the higher concentration of $[Ca^{2+}]_c$ in

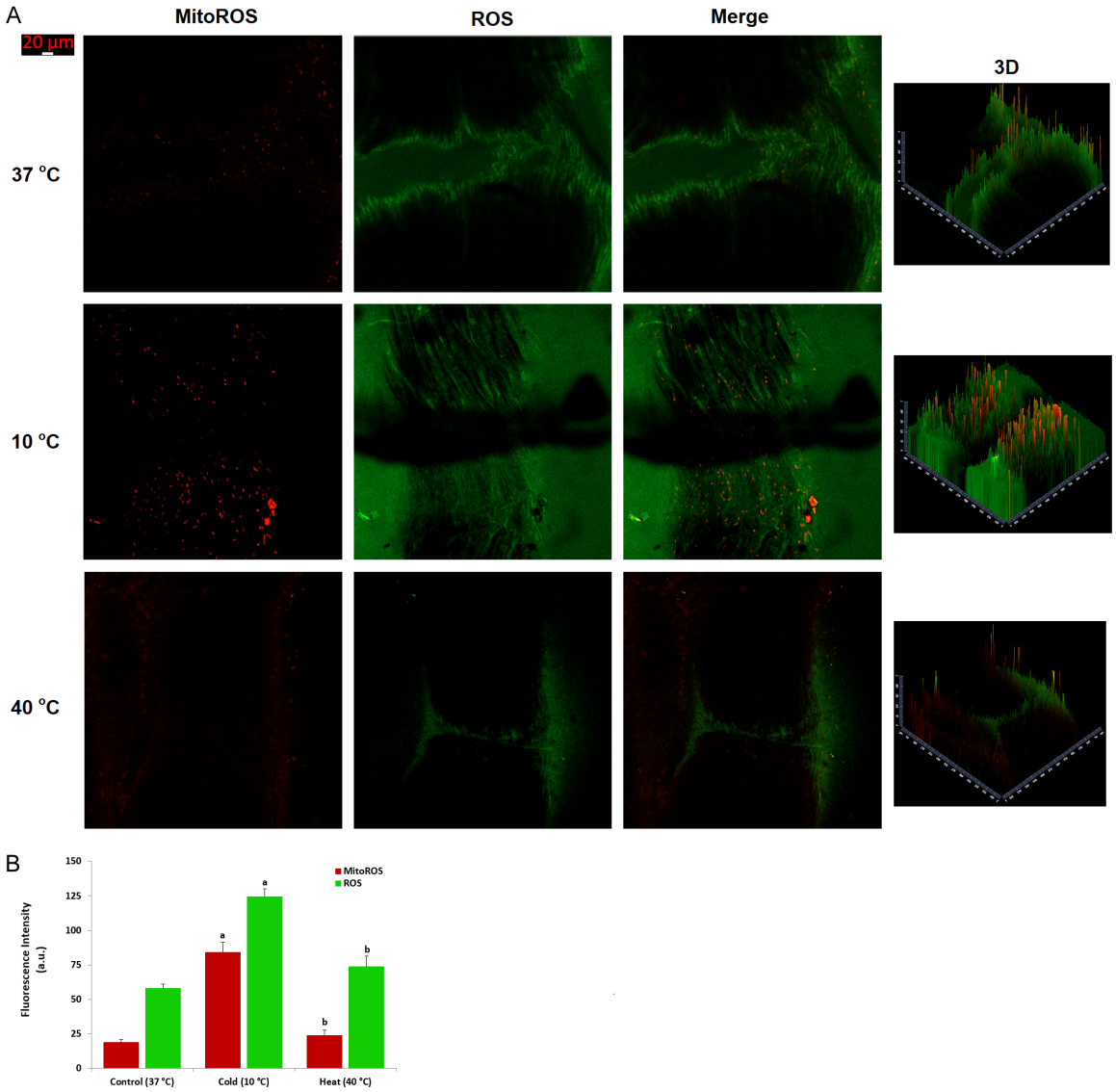

Figure 6. Cold (10°C) treatment-induced mitochondrial (MitoROS) and cytosolic ROS in the MSN injury-induced sciatic nerve of mice were attenuated via inhibition of TRPM8 by heat (40°C) treatment (mean ± SD and n=8). A. The injury-induced sciatic nerve samples were treated by control (37°C), heat (40°C) and cold (10°C) temperatures for 72 hours. Then they were stained with 0.1 μM MitoROS (MitoTracker Red CM-H2Xros) and 1 μM cytosolic ROS (DCFH-DA) dyes for 30 min before analyzing in the laser confocal microscope with 10× objective. B. The mean percentage of MitoROS and DCFHA-DA-positive sciatic neurons. ($^{a}P \leq 0.001$ vs. control. $^{b}P \leq 0.001$ vs. cold group).

the cold+menthol group than in the cold+control group, the TRPM8 antagonist (ACA) could effectively decrease the concentration of $[Ca^{2+}]_c$, which had been increased by the TRPM8 blocker treatment ($P \leq 0.001$). Therefore, TRPM8 channel blocker could diminish the cold-induced increase of $[Ca^{2+}]_c$ by regulating TRPM8 in the neuron line.

Discussion

The results of the present data indicate that the TRPM2 channel may be involved in MSN injury induced-nerve recovery. In addition, cold treatment did not provide protective action via stimulation of TRPM8 activation, mitochondrial ROS release, and apoptosis in MSN injury. However, in our current data, the levels of mitochondrial oxidative stress, cell death, and apoptosis were reduced by heat and TRPM8/TRPM2 blocker (ACA) treatment. To our knowledge, this is the first study to use laser confocal microscopy and microplate plate reader assays to investigate novel mechanisms for MSN injury-induced apoptosis and oxidative neurotoxici-

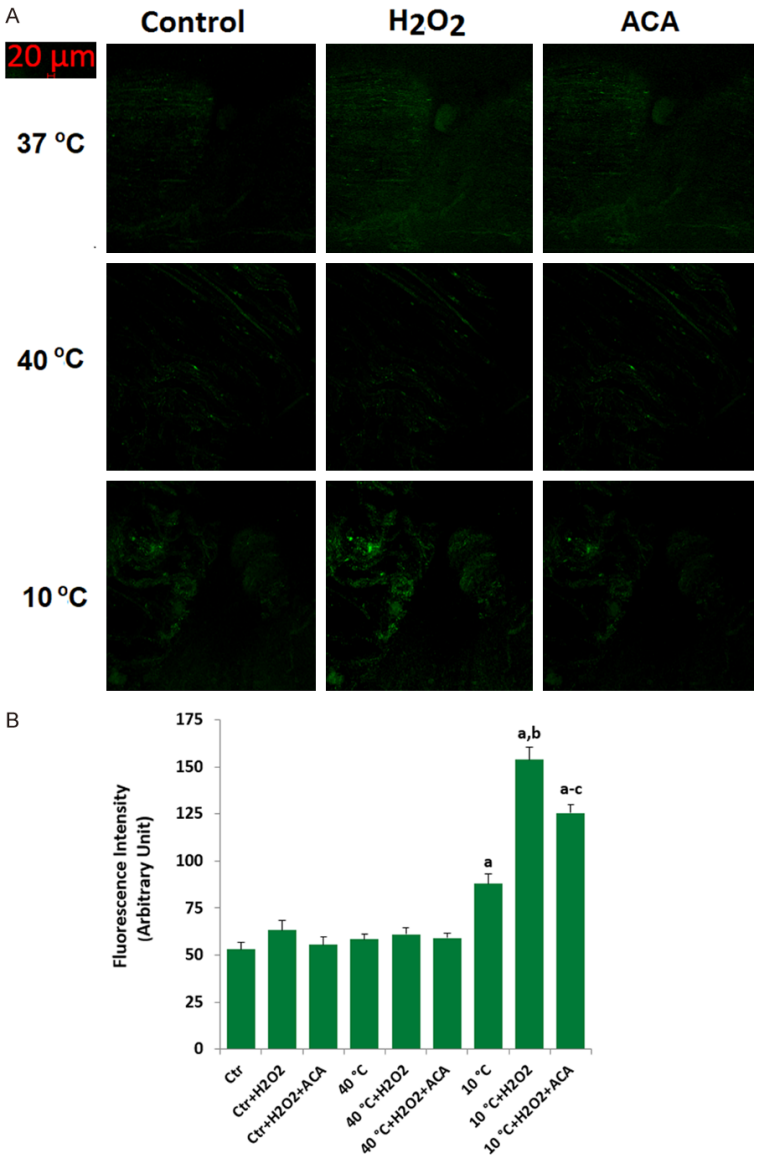

Figure 7. Cold induced increase of $[Ca^{2+}]_c$ was decreased in the MSN injury-induced whole (axon) sciatic nerve by heat (40°C) and ACA treatments (via inhibition of TRPM8 channel) (n=25). The neurons of 3rd days were stained with Fluo-3 calcium dye and mean ± SD of fluorescence in the neuron as arbitrary unit (a.u.) are presented. The cells were extracellularly stimulated by H_2O_2 (10 mM for 10 min), but they were inhibited by extracellular ACA (25 µM for 10 min). The images of samples were analyzed by laser confocal microscopy fitted with a 10× (A). Changes in the fluorescence intensity in the sciatic nerve samples were shown by columns (B). ($^aP \leq 0.001$ vs. control and 10°C group. $^bP \leq 0.001$ vs. 40°C group. $^cP \leq 0.001$ vs. 40°C+H_2O_2 group).

ty pathophysiological processes and implicating heat treatment on TRPM2 activation in the recovery of the sciatic nerve.

In addition to the endogenous activators such as ROS and ADP-Ribose, TRPM2 activation is also affected by environmental temperature, making it one member of the TRP super family channels. However, it seems that activation of TRPM2 is a cell specific issue, because the current literature data contain conflicting opinions. The heat treatment dependent activation of TRPM2 in the insulin secretion of β-cells needs high temperatures (threshold > 47°C) [17]. In the hypothalamus of mice, warm-sensitive dependent TRPM2 activation was reported [16]. In the peripheral nervous system, TRPM2 is involved in perception of non-noxious heat and thermotaxis [15]. In the absence of TRPM2, it was reported that there was no significant increase of TRPM2 activation in the HEK293 cells by the increase of temperature from room temperature to 37°C. However, activation and heat sensitivity actions of TRPM2 in the TRPM2 transfected HEK293 cells were increased by temperature changes (from 42°C to 47°C) [36]. In the current study, we observed a modulator role of heat (40°C) on the TRPM2 activation in the neurons. It seems that cell specific action of heat (40°C) on the TRPM2 activation occurs in the sciatic nerve and SH-SY5Y cells.

A group of conserved stress proteins is heat shock proteins and they are abundantly found in neurons. Heat shock proteins are encoded by heat shock genes and their expression was increased by heat stress or other stress conditions [37, 38]. Hence, heat shock protein chaperones modulate apoptosis, cell division, and other stress pathways, resulting in cytoprotection from various environmental stresses, including heat [39]. Heat shock proteins are regulated Ca^{2+} homeostasis and oxidative stress in PC12 cells and appear to have a strong anti-apoptotic function [40]. Two recent studies reported that overexpression of heat shock protein protected PC12 cells

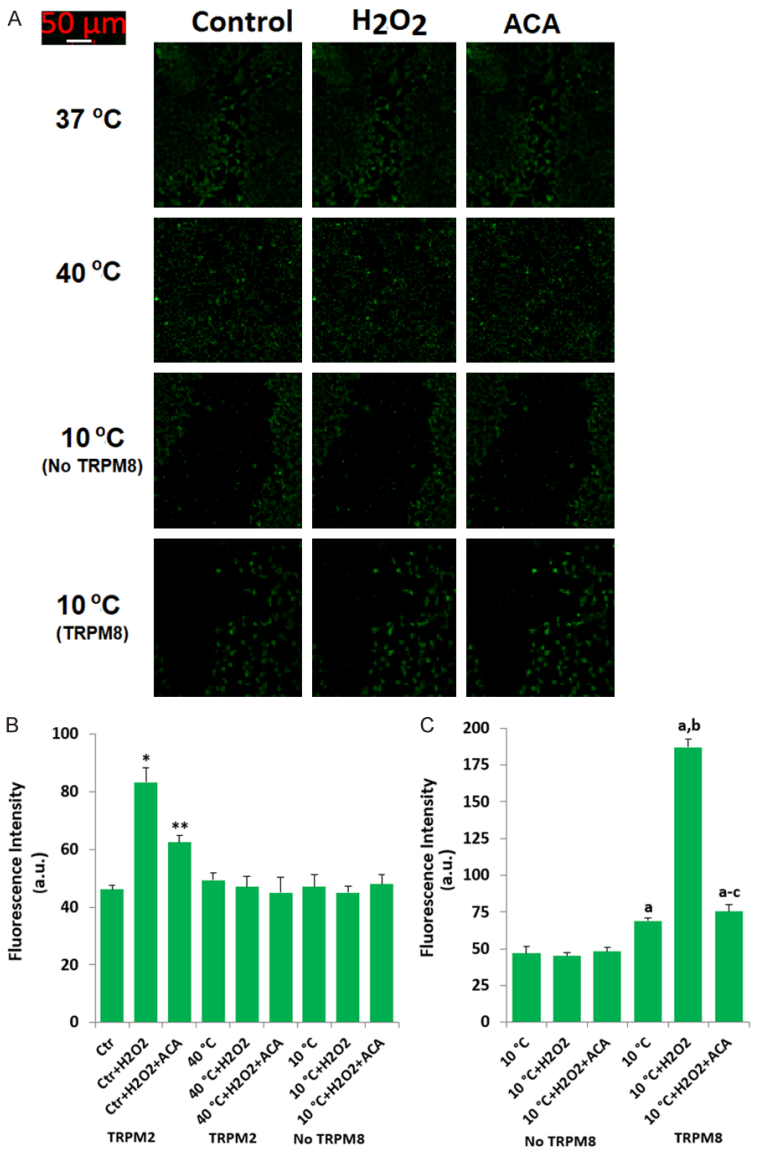

Figure 8. Heat treatment induced TRPM2 blocker action and cold-induced increase of $[Ca^{2+}]_c$ was diminished in the presence of TRPM8 channel in the SH-SY5Y cells by heat (40 °C) and ACA treatments. (n=25 and mean ± SD). The mean fluorescence of Fluo-3 at 0 and 24 hours in the SH-SY5Y cells are presented as arbitrary unit (a.u.). The cells were stimulated by H_2O_2 (1 mM for 10 min), but they were inhibited by ACA (25 µM for 10 min). The images of samples were analyzed by laser confocal microscope (Objective: 40× oil) (A). Changes in the fluorescence intensity in the absence of TRPM8 and presence of TRPM2 were shown by columns (B). Changes in the fluorescence intensity in the TRPM8 transfected SH-SY5Y cells were shown by columns (C). (*P ≤ 0.001 vs. control. **P ≤ 0.001 vs. control+H_2O_2. 10 °C, 10 °C+H_2O_2 and 10 °C+H_2O_2+ACA groups in the absence of TRPM8. [b]P ≤ 0.001 vs. 10 °C and [c]P ≤ 0.001 vs. 10 °C+H_2O_2 groups in the presence of TRPM8).

against oxidative stress injury and apoptosis by regulating the Ca^{2+} entry [5, 41]. Likewise, our current results also demonstrate that heat treatment regulates Ca^{2+} entry through inhibition of TRPM2 channels in the neurons and that its absence from these neurons can stimulate Ca^{2+} entry. Our results also demonstrate that perturbed Ca^{2+} homeostasis plays an important pathophysiological role in neurons. Similarly, the result of a recent study has shown that Ca^{2+} through TRPM2 channel activation plays a key role in MSN injury [20]. The expression levels of several heat shock protein in epithelial cells were increased by TRPV1 antagonist treatments (capsazepine and AMG-9810) [42].

An increase in the $[Ca^{2+}]_c$ via stimulation of TRPM8 results in increases of MMP [6]. In turn, the increase in $[Ca^{2+}]_c$ induces an increase of cytosolic and mitochondrial ROS production [43]. Contrary, inhibition of $[Ca^{2+}]_c$ via modulation of TRPM2 induces decrease of cytosolic and mitochondrial ROS production in several cells and neurons [5, 31, 35]. MSN injury induction and cold treatment exhibited a significant decrease via stimulation of TRPM8 in cell viability and nerve recovery, as well as increased levels of cytosolic and mitochondrial ROS and $[Ca^{2+}]_c$. Heat treatment attenuated the decline in cell viability and TRPM2 activity, but reduced MMP and ROS levels, thereby decreasing $[Ca^{2+}]_c$ concentrations and cell death after sciatic nerve recovery. These results suggest that heat treatment improves the injury-induced perturbation in Ca^{2+} homeostasis in neuronal cells.

The observed increases in MMP through excessive Ca^{2+} influx induces increases in both caspase -3 and -9 activation [6, 44]. In turn, the increase in caspase activation results in higher levels of apoptosis and neuron death [43]. This study

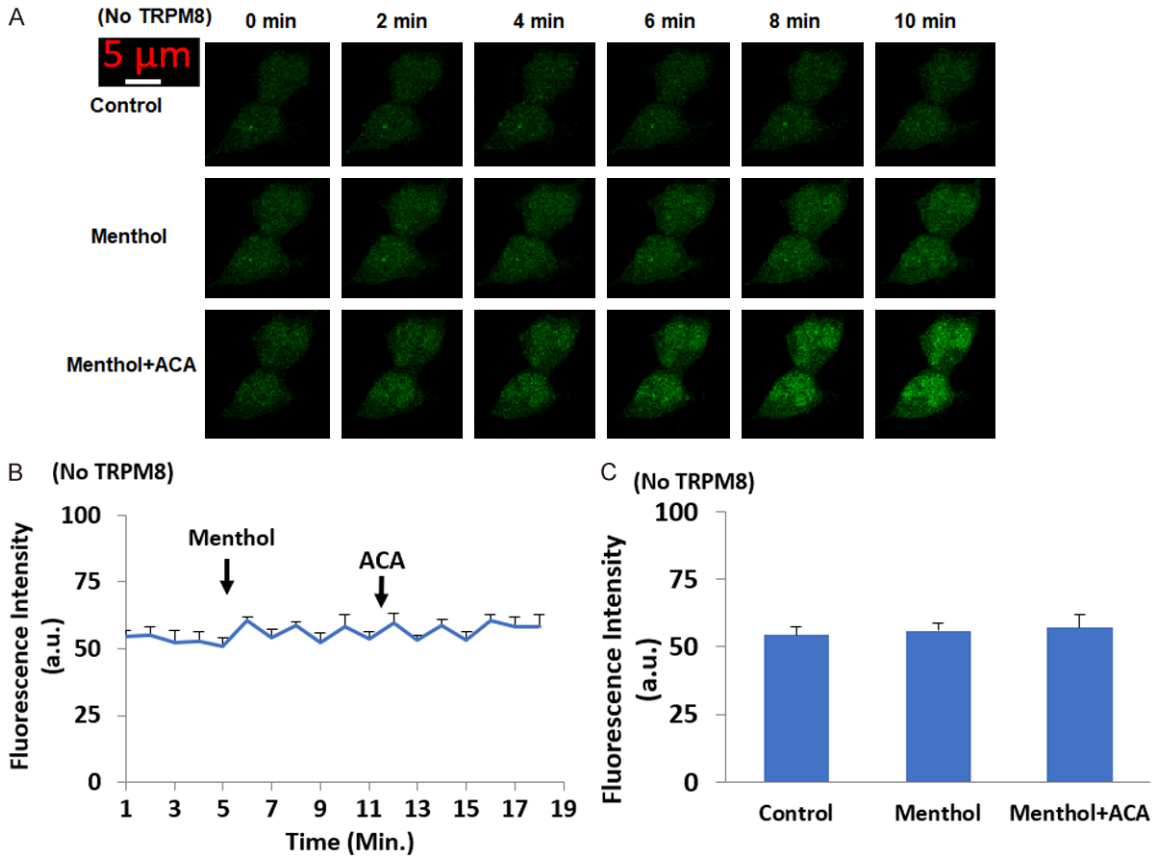

Figure 9. There is no effect of cold on the $[Ca^{2+}]_c$ in the absence of TRPM8 channel in the SH-SY5Y cells. (n=25 and mean ± SD). The SH-SY5Y cells were stained with Fluo-3 calcium dye and the fluorescence in the cells are presented as arbitrary unit (a.u.). The cells were stimulated by menthol (100 μM for 10 min), but they were inhibited by ACA (25 μM for 10 min). The samples were analyzed by the laser confocal microscopy (Objective: 40× oil). A. Images and mean value lines of the Ca^{2+} fluorescence intensity in the three groups of cold groups. B. Images and mean value lines of the Ca^{2+} fluorescence intensity in the cold groups. C. Changes of the Ca^{2+} fluorescence intensity in the three groups of cold groups were also shown by columns.

further investigated the influence of cold and heat applications on apoptosis, neuron death and caspase values in the MSN injury of mice. The apoptosis and neuron death assay demonstrated that heat application could inhibit the apoptosis and caspase activation in neurons, although cold application via activation of TRPM8 increased apoptosis and caspase activation in neurons. Taken together, these results suggest that cold treatment-induced neuronal apoptosis and death contributes to the progression of MSN neuronal injury and then results in the reduction of injury recovery in the sciatic nerve. However, heat treatment attenuated apoptosis, and caspase -3 and -9 activity in the neurons. Similarly, it was reported that the survival of Schwann cells was increased through inhibition of oxidative stress, apoptosis level, caspase -3 and -9 activities by heat shock protein incubation [45].

Conclusions

In summary, sciatic nerve injury activates Ca^{2+} influx through activation of TRPM8 and mitochondrial ROS release, followed by activation of caspase -3 and -9, subsequently leading to the apoptosis and neuron death of the sciatic nerve (**Figure 11**). However, heat treatment has a significant beneficial effect on reducing wound formation in sciatic nerve-injured mice by reducing sciatic nerve apoptosis and TRPM2 activation. Further research is needed to verify at the effect of heat exposure on other calcium channels and heat shock proteins in the process of sciatic nerve recover in the clinic.

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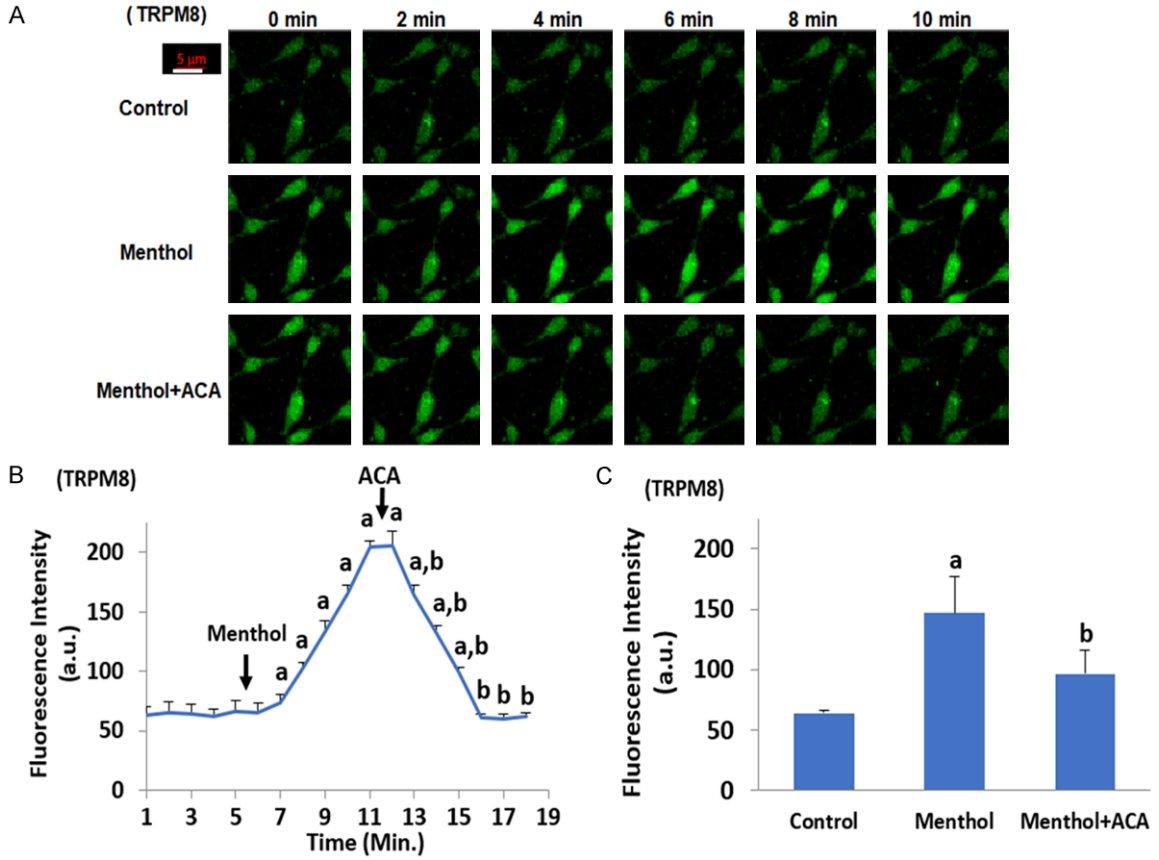

Figure 10. Cold-induced increase of the $[Ca^{2+}]_c$ was diminished in the presence of TRPM8 channel in the SH-SY5Y cells. (n=25 and mean ± SD). After staining with Fluo-3 calcium dye, the fluorescence intensity in the SH-SY5Y cells are presented as arbitrary unit (a.u.). The cells were stimulated by menthol (100 μM for 10 min), although they were inhibited by ACA (25 μM for 10 min). The samples were analyzed by the laser confocal microscopy (Objective: 40× oil). A. Images and mean value lines of the Ca^{2+} fluorescence intensity in the three cold groups (control, menthol, and mentho+ACA). B. Mean value changes of the Ca^{2+} fluorescence intensity in the cold groups were indicated by line figures. C. Changes of the Ca^{2+} fluorescence intensity in the groups were also shown by column figures. ($^aP \leq 0.001$ vs. control group. $^bP \leq 0.001$ vs. menthol group).

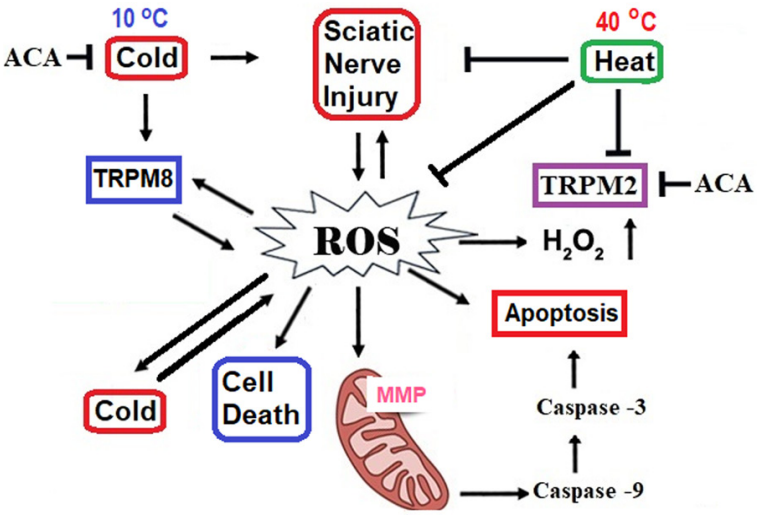

Figure 11. Summary of pathways involved in cold exposure (10°C)-induced apoptosis and mitochondria reactive oxygen species (ROS) production via activation of TRPM8 channel in the sciatic nerve injury of mice and clearance by heat exposure (40°C) and ACA treatment. The cold treatment leads to the excessive productions of several ROS. The ROS induce excessive Ca^{2+} influx through the TRPM8 activation in the cells. Main mechanism in the anti-apoptotic effect of heat is mediated in the sciatic nerve injury of mice by inhibition of ROS-mediated caspase -3 and -9 activations. Increase of mitochondrial membrane potential (MMP) activates apoptosis factors and ROS from the mitochondria. In response, heat exposure in the injured sciatic nerve initiates an antioxidant response and TRPM2 blocker action that facilitates the neutralization of ROS into less harmful products.

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Disclosure of conflict of interest

None.

Abbreviations

$[Ca^{2+}]_c$, intracellular free Ca^{2+} concentration; ACA, N-(p-amylcinnamoyl) anthranilic acid; MMP, mitochondrial membrane depolarization; MSN, the mechanical sciatic nerve; PI, propidium iodide; ROS, reactive oxygen species; TRPM2, transient receptor potential melastatin 2; TRPM8, transient receptor potential melastatin 8; TRPV1, transient receptor vanilloid 1.

Address correspondence to: Dr. Mustafa Nazıroğlu, Department of Neuroscience, Neuroscience Research Center (NOROBAM), Suleyman Demirel University, TR-32260, Isparta, Turkey. Tel: +90246211-3641; Fax: +902462371165; E-mail: mustafanaziroglu@sdu.edu.tr

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