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Curcumin Reduces High-dose Morphine-Induced Apoptosis and Oxidative Neurotoxicity via TRPV4 Cation Channel Suppression

Kurkumin Yüksek Doz Morfinin Neden Olduğu Apoptozis ve Oksidatif Nörotoksisiteyi TRPV4 Katyon Kanalını Düzenleyerek Azaltır

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ABSTRACT

Objective: Long-term and high-dose morphine (H-MRP) treatments for neuropathic pain cause the body to become extremely susceptible to morphine tolerance, which increases the amount of toxic reactive oxygen species (ROS), apoptosis, and calcium (Ca^{2+}) entering the neuron. It has been known that curcumin (CRC) decreased these increases in ROS-damaged SH-SY5Y cells by blocking the TRPV4 cation channel. It has not been studied whether CRC can also suppress the high levels of ROS and apoptosis caused by H-MRP in SH-SY5Y cells by affecting TRPV4. So, the study was carried out to investigate whether CRC can suppress the high level of mitochondrial ROS and apoptosis.

Materials and Methods: In the SH-SY5Y, four primary groups were induced as control, normal morphine (N-MRP) (50 µM for 24h), H-MRP (500 µM for 24h), H-MRP + CRC (5 µM for 24h).

Results: While the incubations of TRPV4 antagonist (ruthenium red) and CRC decreased the H-MRP-induced increases of apoptosis, caspase-3, caspase-8, caspase-9, ROS, mitochondrial dysfunction, debris number, and lipid peroxidation levels, the TRPV4 agonist (GSK1016790A) stimulation further increased these levels. The CRC increased glutathione, glutathione peroxidase, live cell number, and cell viability percentage, all of which were decreased by H-MRP.

Conclusions: The levels of H-MRP-induced neuronal death and mitochondrial oxidative stress were reduced by CRC treatment through TRPV4 inhibition. For H-MRP-induced mitochondrial oxidative neuronal injury, CRC is a potential treatment option.

Keywords: Curcumin, neuronal injury, mitochondrial oxidative stress, morphine, TRPV4 channel

ÖZ

Amaç: Nöropatik ağrıyı tedavi etmek için yüksek doz (H-MRP) ve uzun süreli morfin uygulanması kullanılır. Bununla birlikte, H-MRP tedavisi, morfin toleransı, aşırı reaktif oksijen türleri (ROS) üretimi, apoptozis ve Ca^{+2} akışının artmasına neden olur. Sinir hasarı nedeniyle zarar gören SH-SY5Y sinir hücrelerinde, kurkumin (CRC) TRPV4 katyon kanalını inhibe ederek ROS'un neden olduğu apoptozis artışını azaltır. H-MRP, SH-SY5Y hücrelerinde TRPV4 katyon kanalını inhibe ederek aşırı ROS ve apoptozis oluşumunu önlemek için CRC etkisi henüz araştırılmamıştır. Bu çalışmada, CRC tedavisinin TRPV4 kanalını düzenleyerek mitokondriyal ROS üretimini ve apoptozis oluşumunu H-MRP inkübasyonunu nasıl etkilediğini SH-SY5Y hücrelerinde araştırıldı.

Materyal ve Metot: SH-SY5Y hücrelerinde dört ana grup oluşturuldu. Bunlar; kontrol, normal morfin (N-MRP) (50 µM ve 24 saat), H-MRP (500 µM ve 24 saat) ve H-MRP + CRC (5 µM ve 24 saat).

Bulgular: TRPV4 agonisti (GSK1016790A) ile inkübasyon, ROS, mitokondriyal fonksiyon bozukluğu, debris (ölü hücre artığı), apoptosis, kaspaz -3, kaspaz -8, kaspaz -9 ve lipit peroksidasyon düzeylerini artırdı. Bununla birlikte, TRPV4 antagonisti (rutenyum kırmızısı) ve CRC inkübasyonları bu artışları azalttı. Rutenyum kırmızısı ve CRC inkübasyonları, H-MRP inkübasyonunun neden olduğu hücre canlılığı yüzdesi, canlı hücre sayısı, glutatyon ve glutatyon peroksidaz düzeyleri azalışlarını artırdı.

Sonuç: CRC tedavisi, TRPV4 kanalını baskılayarak H-MRP'nin mitokondriyal oksidan ve sinir hücre ölümü etkilerini azalttı. H-MRP neden olduğu mitokondriyal oksidatif stres ve sinir hücre harabiyetini önlemek için CRC tedavisi alternatif bir kaynak tedavi olarak gözükmektedir.

Anahtar Kelimeler: Kurkumin, sinir hasarı, mitokondriyal oksidatif stres, morfin, TRPV4 kanalı

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INTRODUCTION

Present chronic pain management methods are typically ineffective and insufficient. Neuropathic pain is commonly treated with opioids, such as morphine.[1] Conversely, prolonged exposure to large doses of morphine causes analgesic tolerance.[1] Morphine analgesic tolerance lowers endogenous antioxidant levels and induces oxidative stress in a dose-dependent manner.[2] So, morphine has antiapoptotic and antioxidant properties at normal dosages, but at high doses (H-MRP), it can increase Ca^{2+} influx and cause cell death and damage in neuronal cell lines and experimental animal models due to mitochondrial reactive oxygen species (ROS).[3,4,5]

As a natural taste and polyphenol with numerous neuroprotective and cognitive-increasing features, curcumin (CRC) is one of the therapeutic plants that has a significant impact on opioid dependence in cells.[6,7] There is evidence that CRC reduces morphine dependency in the hippocampus via having anti-apoptotic and antioxidant properties.[8] Furthermore, CRC has been demonstrated to prevent opioid tolerance and dependence by inhibiting Ca^{2+} influx through the modification of Ca^{2+}/calmodulin-dependent protein kinase II.[9] In an animal model of neurodegenerative disorders, CRC has also been shown to inhibit neuronal cells.[10]

Numerous biological processes, including as morphine-induced oxidative stress and death, have been shown to need the TRP, a non-selective cation channel superfamily.[7,11] In the TRP superfamily, TRP vanilloid 4 (TRPV4) is a member. GSK1016790A (GSK) and H_2O_2 are two examples of pharmaceutical substances that activate TRPV4.[12] Prior research showed that non-selective TRPV4 inhibitors, such as ruthenium red (RuR), reduced oxidant and apoptotic indicators.[12,13,14] Moreover, TRPV4 has been demonstrated to be highly expressed in SH-SY5Y neuronal cells,[15] and it has been reported that TRPV4 inhibition in SH-SY5Y cells results in oxidative and apoptotic effects via stimulating GSK.[16]

CRC improves mitochondrial activity, scavenges free radicals, including ROS and lipid peroxidation (LPO), and reduces tissue oxidative damage in SH-SY5Y neural cells.[13,14] CRC also inhibits ROS-sensitive TRP channels, including TRPV4 and TRP melastatin 2 (TRPM2).[12,13,14,16, 17] The injection of resveratrol in diabetes mellitus reduced the increases caused by morphine-mediated stimulation, which increased ROS, caspases, and apoptosis due to an excessive Ca^{2+} influx.[5,11,16]

The suppression of oxidative stress-dependent TRP channel inhibition thereby influenced the degree of morphine-induced oxidative damage and apoptosis. CRC has not yet been investigated for its ability to suppress the disruptive effects of H-MRP on neuronal cells via modulating TRPV4. Thus, this investigation was realized to determine the protective effect of CRC on H-MRP-induced mitochondrial oxidative stress and apoptosis in SH-SY5Y neuronal cells.

MATERIALS AND METHODS

Ethics Committee Approval: The ethics committee accepted the study, which used cells cultivated using commercial cell culture. Ethics committee approval of this project is not required.

Cells: A common cell line used in TRPV4 research, including morphine addiction and numerous neurological disorders, is SH-SY5Y.[12,13,18] For this reason, the cells (ATCC, VA, USA) were employed in the current investigation as a model for neural cell culture. The SH-SY5Y was cultivated in a cell culture setting, as documented in earlier research.[12,14,17] 90% DMEM/Ham's F12 equal mixture, 10% fetal bovine serum, and 1% antibiotic mixture comprised the medium mixture.[12, 17]

Experimental Groups: Four groups—control (CON), N-MRP, H-MRP, and H-MRP + CRC—were each given a 25 cm^2 sterile flask with $1x10^7$ SH-SY5Y cells. In the plate reader experiments, the TRPV4 channel in the cells of four groups was activated with 100 nM GSK despite being blocked by 1 μM RuR incubation.[13,17]

In the incubator, the SH-SY5Y cells in the CON group were maintained for twenty-four hours. The cells in the N-MRP and H-MRP groups were cultured for 24h after being treated with 50 μM and 500 μM of MRP, respectively.[23] 500 μM morphine and 5 μM CRC (Sigma–Aldrich Inc., St. Louis, MO, USA) were administered to the cells of the H-MRP and CRC combination group for a duration of 24h.[13,17]

N-MRP doses were reported in different cells between 10 μM and 50 μM, although the H-MRP dose in the cells was reported between 500 μM and 1000 μM.[5,19,20] Hence, the N-MRP dose was used as 50 μM, although the H-MRP dose was used as 500 μM in the SH-SY5Y cells of the current study.

Stock solutions of CRC, GSK, and RuR were prepared in dimethyl sulfoxide. Then their appropriate concentrations were added to the cell culture medium.

***Cell Viability, Number and Debris Counts*:** Debris (organic waste left over after cell death) and cells were counted using a Casy Modell TT automatic cell counter (Roche, Reutlingen, Germany).[13,17] The percentage changes were used to display the cell viability. But for viable cell counts, $x10^7$ per milliliter was employed, and for debris numbers, $x10^6$ per milliliter.

Analyses for Apoptosis, Cell Viability, Caspase-3, -8, and -9: A commercial APOPercentage kit

(Biocolor Ltd., Co Antrim, UK) was utilised to measure the apoptosis of SH-SY5Y. By employing the Infinite PRO 200 microplate reader (Tecan Austria GmbH, Groedig, Austria), changes in apoptosis were identified in absorbance at 550 nm.[16,17]

The CASY cell viability electronic count analysis was performed in conjunction with MTT for the cell viability experiments. Each white well received 100 microliters of MTT (five mg per ml) in 1xPBS, and the cells were then incubated for 3–4 hours at 37 °C. Five hundred microliters of dimethyl sulfoxide were employed to dissolve the formazan crystals. Following a minute of shaking the 96 white well plates, a microplate reader (Infinite 200 PRO) was used to measure the absorbance at 492 nm.[16,17]

The Infinite PRO 200 microplate reader was used to measure the cleavage of the substrates for caspase-3 (Ac-DEVD-AMC), caspase-8 (Ac-VETD-AMC), and caspase-9 (Ac-LEHD-AFC) (Bachem, Heidelberg, Germany). Under the Infinite PRO 200, the excitation (380 nm) and emission (460 nm) wavelengths were kept constant. The alterations of the caspases were captured by the fluorescence units.[16,17]

These ApoPercentage and substrate loadings were followed by 30-minute cell stimulation with 100 nM GSK, either with or without a TRPV4 inhibitor (1 μM RuR), in order to quantify the TRPV4-dependent apoptosis induction and caspase releases in the cells of four groups. Once the fluorescence units were determined, the results related to caspases and apoptosis were shown as percentage changes from the control (fold increase).

Analyses for Measuring ROS Generation and Mitochondrial Membrane Dysfunction: The ROS probe (DCFH-DA) (Cat # D399, Thermo Fisher Scientific) and JC-1 dye (2 μg/ml; Cat # T3168, Thermo Fisher Scientific) were used to incubate the cells in order to analyze ROS generation and mitochondrial membrane malfunction. Following that, the cells were incubated for 30 minutes at 37 °C in the dark. After that, the changes of DCFH-DA and JC-1-stained cells in fluorescence intensity were recorded using an Infinite Pro 200 plate reader. Following the measurement of fluorescence intensity, the results were displayed as a percentage of the control (experiment/control).

The cells of four groups were stimulated with 100 nM GSK for 30 minutes, either with or without a TRPV4 inhibitor (1 μM RuR), after these DCFH-DA and JC-1 loadings, in order to quantify the TRPV4-dependent ROS and mitochondrial dysfunction. Following the calculation of fluorescence units, the ROS and mitochondrial dysfunction data were shown as percentage changes from the control (fold increase).

Glutathione (GSH), Lipid Peroxidation (LPO) and Glutathione Peroxidase (GSH-Px) Analysis: In the frozen SH-SY5Y samples, the total protein content and the optic density (absorbance) values of LPO (532 nm), GSH (412 nm), and GSH-Px (412 nm) were measured using a spectrophotometer (Shimadzu-UV1800, Kyoto, Japan). The concentrations of GSH and LPO in SH-SY5Y were expressed in micromoles per gram of protein. IU per g of protein is the measure of GSH-Px activity in the SH-SY5Y cells.

Analysis of the Data: The data is presented as mean standard deviation (SD). The comparison was done using one-way analysis of variance followed by Tukey's post hoc test to compare between groups (SPSS, Inc., Chicago, IL, USA), where p-values less than 0.05 were identified.

RESULTS

The cell viability percentage (Figure 1A) and viable cell number (Figure 1B) of H-MRP groups were lower than those of the control (CON) and N-MRP groups, but they were higher in the H-MRP + CRC group than in the H-MRP group ($p < 0.05$). The H-MRP group had a higher debris number (Figure 1C) CON and N-MRP groups, whereas the supplementation of CRC in the H-MRP + CRC group had a lower debris number than the H-MRP group ($p < 0.05$).

Compared to the CON, H-MRP, and H-MRP + CRC, the CON + GSK, H-MRP + GSK, and H-MRP + CRC + GSK groups showed higher percentages of apoptosis (Figure 2), caspase-3 (Figure 3A), caspase -8 (Figure 3B), and caspase-9 (Figure 3C) ($p < 0.05$). For the CON + GSK + RuR, H-MRP + GSK + RuR, and H-MRP + CRC + GSK + RuR groups, however, the treatments of RuR and CRC reduced their percentages ($p < 0.05$).

In the CON + GSK, H-MRP + GSK, and H-MRP + CRC + GSK groups, JC-1 (Figure 4A) and DCFH-DA (Figure 4B) were higher than in the CON, H-MRP, and H-MRP + CRC, respectively ($p < 0.05$). For the CON + GSK + RuR, H-MRP + GSK + RuR, and H-MRP + CRC + GSK + RuR groups, however, the treatments of RuR and CRC reduced their percentages ($p < 0.05$).

In comparison to the H-MRP + CRC group, the GSH level (Figure 5A) and GSH-Px activity (Figure 5B) of the H-MRP groups were lower ($p < 0.05$) but higher than those of the CON and N-MRP groups. The LPO level was lower in the H-MRP + CRC group than in the H-MRP group ($p < 0.05$), whereas the LPO level was greater in the H-MRP group (Figure 5C) than in the control (CON) and N-MRP groups.

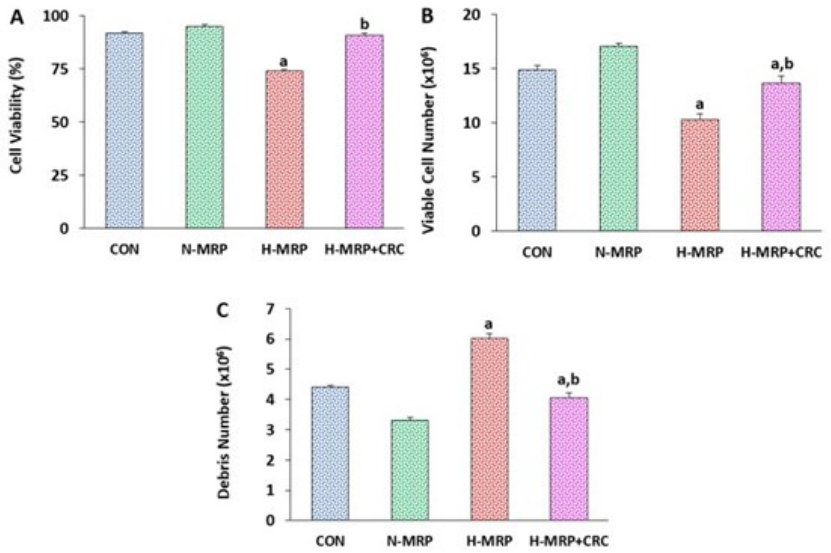

Figure 1. The cell viability percentage, viable cell count, and debris number of SH-SY5Y cells changed when CRC (5 µM) was incubated with H-MRP (500 µM). (Mean ± SD). **A**. cell viability percentage. **B.** Viable cell number. **C**. Debris number. ([a]p < 0.05 versus (*vrs.)* CON and N-MRP. [b]p < 0.05 *vrs.* H-MRP).

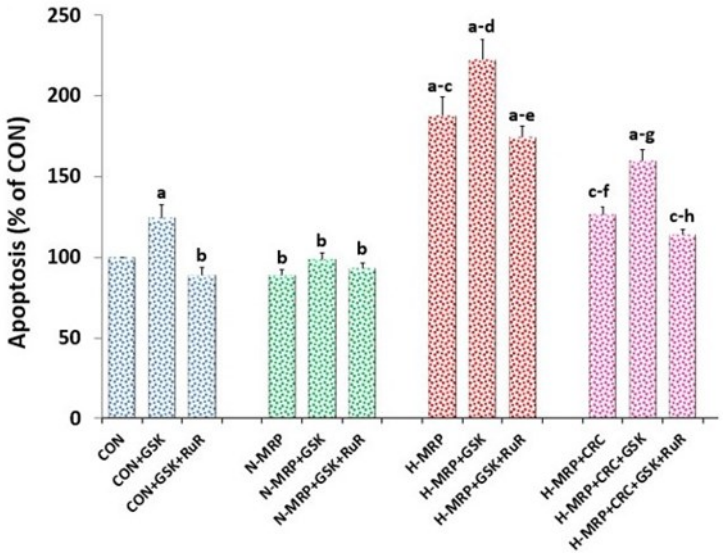

Figure 2. The incubation of CRC (5 µM) modulated H-MRP (500 µM), causing an increase in apoptosis in the SH-SY5Y. (Mean ± SD and n=3). Apoptosis percentage. The TRPV4 channel in the cells was stimulated by 100 nM GSK, although it was inhibited by 1 µM RuR. ([a]p < 0.05 versus (*vrs.)* control (CON) and N-MRP. [b]p < 0.05 *vrs.* CON+GSK. [c]p < 0.05 *vrs.* N-MRP, N-MRP + GSK, and N-MRP + GSK + RuR. [d]p < 0.05 *vrs.* H-MRP. [e]p < 0.05 *vrs.* H-MRP + GSK. [f]p < 0.05 *vrs.* H-MRP + GSK + RuR. [g]p < 0.05 *vrs.* H-MRP + CRC. [h]p < 0.05 *vrs.* H-MRP + CRC + GSK).

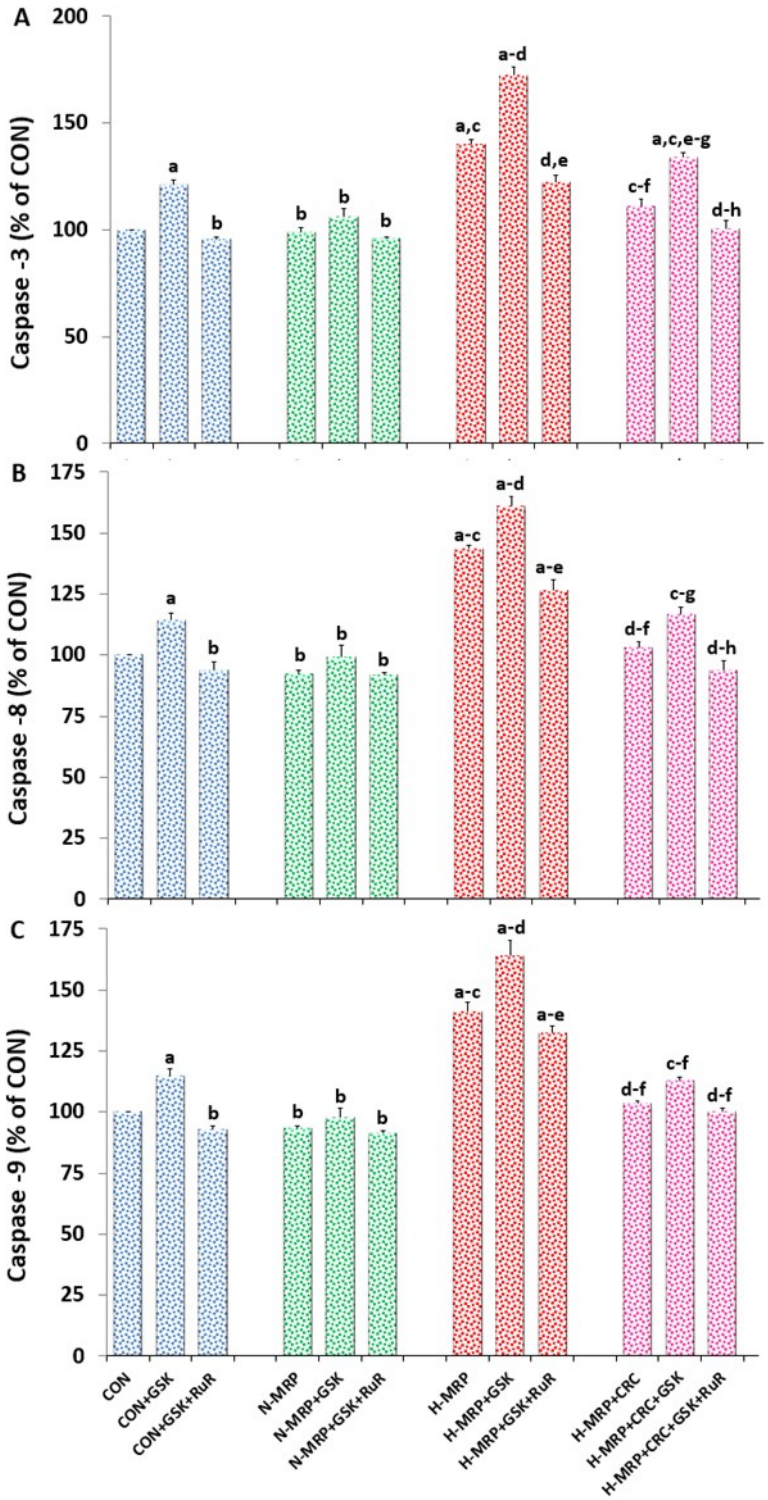

Figure 3. The incubation of CRC (5 µM) modulated H-MRP (500 µM), causing an increase in caspase percentages in the SH-SY5Y. (Mean ± SD and n=3). **A.** Caspase-3 percentage. **B**. Caspase -8 percentage. **C**. Caspase -9 percentage. TRPV4 channel in the cells was stimulated by 100 nM GSK, although it was inhibited by 1 µM RuR. ($^{a}p < 0.05$ versus (*vrs.)* control (CON) and N-MRP. $^{b}p < 0.05$ *vrs.* CON+GSK. $^{c}p < 0.05$ *vrs.* N-MRP, N-MRP + GSK, and N-MRP + GSK + RuR. $^{d}p < 0.05$ *vrs.* H-MRP. $^{e}p < 0.05$ *vrs.* H-MRP + GSK. $^{f}p < 0.05$ *vrs.* H-MRP + GSK + RuR. $^{g}p < 0.05$ *vrs.* H-MRP + CRC. $^{h}p < 0.05$ *vrs.* H-MRP + CRC + GSK).

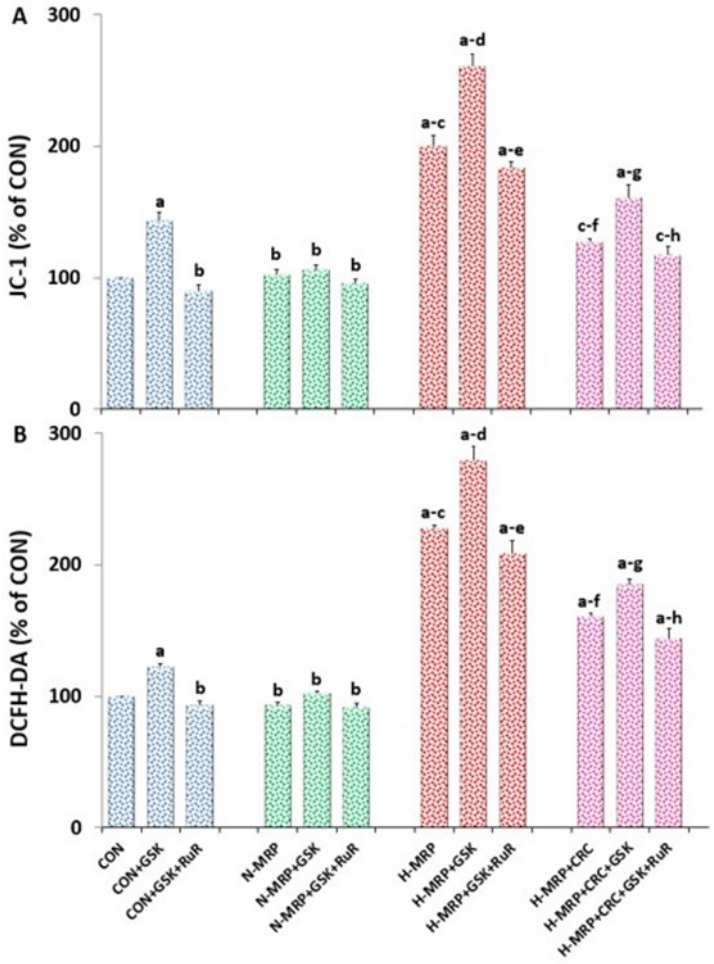

Figure 4. The incubation of CRC (5 µM) modulated H-MRP (500 µM), causing an increase in mitochondrial dysfunction (JC-1) and ROS (DCFH-DA) percentages in the SH-SY5Y**.** (Mean ± SD and n=3). A. JC-1 percentage. B. DCFH-DA percentage. TRPV4 channel in the cells was stimulated by 100 nM GSK, although it was inhibited by 1 µM RuR. ($^{a}p < 0.05$ versus (*vrs.)* control (CON) and N-MRP. $^{b}p < 0.05$ *vrs.* CON+GSK. $^{c}p < 0.05$ *vrs.* N-MRP, N-MRP + GSK, and N-MRP + GSK + RuR. $^{d}p < 0.05$ *vrs.* H-MRP. $^{e}p < 0.05$ *vrs.* H-MRP + GSK. $^{f}p < 0.05$ *vrs.* H-MRP + GSK + RuR. $^{g}p < 0.05$ *vrs.* H-MRP + CRC. $^{h}p < 0.05$ *vrs.* H-MRP + CRC + GSK).

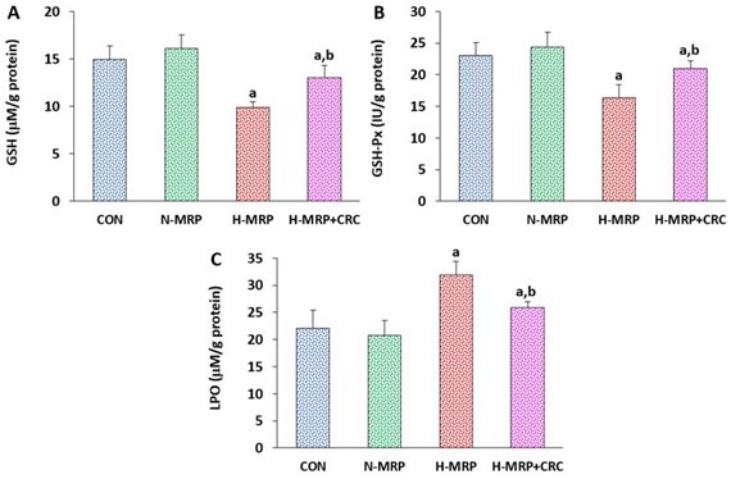

Figure 5. The incubation of CRC (5 µM) modulated H-MRP (500 µM), causing changes in glutathione (GSH), glutathione peroxidase (GSH-Px), and lipid peroxidation (LPO) in the SH-SY5Y cells. (Mean ± SD). A. GSH. B. GSH-Px. C. LPO. ($^{a}p < 0.05$ versus (*vrs.)* CON and N-MRP. $^{b}p < 0.05$ *vrs.* H-MRP.

DISCUSSION AND CONCLUSION

I found that giving CRC to the neuronal cells reduced the H-MRP-mediated increase in oxidative neurotoxicity and apoptosis in the current study by inhibiting the TRPV4 cation channel and increasing GSH and GSH-Px.

Morphine affects intracellular Ca^{2+} levels in two ways. According to certain research, administering H-MRP reduced the level of Ca^{2+} in many brain regions.[4] Increased binding sites to voltage-gated calcium channel blockers (nimodipine) are prevented when morphine and Ca^{2+} channel blockers are administered at the same time.[4] TRPM2 channel blockers may increase ATP generation in SH-SY5Y and experimental animal neurons while reducing mitochondrial oxidative damage.[12–16] TRPM2 cation channel stimulation enhanced the oxidant and apoptotic effects of morphine, while its inhibition reduced these effects in the mouse and rat dorsal root ganglion.[16,22] The results in the dorsal root ganglion showed a comparable protective effect from TRPV1 channel blockage.[23] To the best of my knowledge, no studies have examined how TRPV4 affects the oxidative and apoptotic characteristics of H-MRP in brain cells. While the TRPV4 antagonist (RuR) decreased the apoptotic and oxidant activities of H-MRP in the SH-SY5Y cells, TRPV4 activation in the current study further increased them. It seems that while oxidative damage and neuronal death were decreased when TRPV4 stimulation-mediated Ca^{2+} influx was decreased, both events were increased when TRPV4 stimulation-mediated Ca^{2+} influx was increased.

TRPV4 stimulation results in increased mitochondrial Ca^{2+} uptake in the kidney, dorsal root ganglion, and SH-SY5Y cells. This leads to an increase in ROS production and cell death indicators (apoptosis, caspase-3, caspase-8, and caspase-9) that are caused by dysfunction of the mitochondrial membrane.[12,13,16] The effects of morphine on the rat hippocampal TRPM2 stimulator have been reported.[7] The oxidant (ROS and LPO) and apoptotic (caspase-3, caspase-8, and caspase-9) indicators are thus enhanced as a result of TRPV4 stimulations and increased mitochondrial membrane failure.[4] Conversely, the percentage of oxidant and apoptotic indicators, such as SH-SY5Y, in neural cells is decreased when TRPV4 is suppressed.[12,13,16] The information that is now available indicates that H-MRP-induced TRPV4 activation was the source of the increased mitochondrial membrane dysfunction in SH-SY5Y. In turn, this resulted in a decrease in cell viability but an increase in caspase-3, -8, and -9, ROS, LPO, and apoptosis. TRPV4 blocker (RuR) and CRC treatment influenced the changes. According to the current findings, CRC incubation decreased Parkinsonism-induced increases in mitochondrial dysfunction, ROS, LPO, caspases, and apoptosis in SH-SY5Y cells by inhibiting TRPV4.[13] In mice, morphine therapy altered the anti-hyperalgesic effects of neurotoxin GsMTx4-based 17-residue peptide, which inhibits TRPV4.[24] By raising GSH-Px activity and GSH levels in the rat hippocampal region, the CRC therapy enhanced H-MRP-induced elevations in LPO, caspase-3, and caspase-9 activities.[8] The TRPV4 channel was stimulated in human embryonic kidney 293 and mesenteric artery endothelial cells by CRC, which is contrary to the findings.[25] The CRC treatment reduced the rise in nitric oxide radicals and H-MRP-induced memory impairment in the brain of rats.[7]

The observed reductions in GSH-Px activity and GSH level suggest that components of the thiol redox antioxidant system contribute to regulating the oxidative imbalance induced by H-MRP. After incubation with CRC or TRPV4 suppression with RuR, the oxidative effects of H-MRP decreased, as shown by lower levels of ROS and LPO, while GSH levels and GSH-Px activity increased. These findings imply that RuR and CRC increase GSH levels and GSH-Px activity while decreasing LPO and ROS. It is well-known that GSH-Px transforms H_2O_2 into water. GSH is used as a substrate by GSH-Px during the process. The antioxidant property of CRC is responsible for its strong scavenging of a range of oxidants, such as superoxide radicals, hydroxyl radicals, and H_2O_2.[26,27,28] Consistent with the findings, CRC therapy has been shown to restore GSH level and GSH-Px activity in SH-SY5Y cells while lowering LPO concentration.[13] The LPO caused by H-MRP was also reduced by the CRC treatment by raising GSH-Px activity and GSH levels in the rat hippocampal region.[8] Present results are in line with these observations.[8,13]

In conclusion, TRPV4 attenuation during CRC incubation protected SH-SY5Y cells from H-MRP-mediated apoptotic and oxidative mediators because TRPV4 inhibition downregulated neuronal damage. Even while CRC therapy reduces H-MRP-induced oxidative neurotoxicity and apoptosis, it may still trigger TRPV4-mediated caspases, ROS, mitochondrial dysfunction, and LPO, which in turn may produce H-MRP-induced oxidative damage and apoptosis.

Ethics Committee Approval: The ethics committee accepted the study, which used cells cultivated using commercial cell culture. Ethics committee approval of this project is not required.

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept – HÖO; Materials – HÖO; Data Collection and/or Processing – HÖO; Analysis and/or Interpretation – HÖO; Writing –

HÖO.

Peer-review: Externally peer-reviewed.

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