OTJHS
Online Turkish Journal of Health Sciences

e-ISSN: 2459-1467

OTSBD
Online Türk Sağlık Bilimleri Dergisi

Online Turkish Journal of Health Sciences 2025;10(4):382-389 *Online Türk Sağlık Bilimleri Dergisi 2025;10(4):382-389*

Resveratrol Reduces Hypoxia-Caused Increases of Apoptosis and Oxidative Neurotoxicity via TRPA1 Cation Channel Suppression in Glioblastoma Cells

Glioblastoma Hücrelerinde Resveratrol Hipoksinin Neden Olduğu Apoptozis ve Oksidatif Nörotoksisite Artışlarını TRPA1 Katyon Kanalını Baskılayarak Azaltır

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ABSTRACT

Objective: Hypoxia (HPX) increases the amount of Ca^{2+} influx, apoptosis, and harmful free reactive oxygen species (ROS) in the brain and neurons. Resveratrol (RES) has been shown to reduce these increases in ROS-damaged neuronal cells by inhibiting voltage-gated Ca^{2+} channels. The aim of the study was to ascertain whether RES could also inhibit the elevated ROS and apoptosis induced by HPX in SH-SY5Y glioblastoma cells via inhibiting TRPA1.

Materials and Methods: In the SH-SY5Y, four primary groups were induced as control, RES (50 µM for 24h), HPX (200 µM $CoCl_2$ for 24h), and HPX + RES.

Results: While the incubations of the TRPA1 antagonist (AP-18) and RES decreased the HPX-mediated upregulations of apoptotic (caspase -3, -8, and -9) and oxidants (ROS, mitochondrial dysfunction, and lipid peroxidation) concentrations, the TRPA1 agonist (cinnamaldehyde) stimulation further increased these concentrations. The RES increased viable cell percentage, glutathione concentration, and glutathione peroxidase activity, all of which were diminished by HPX.

Conclusions: The concentrations of HPX-induced neuronal apoptosis and mitochondrial oxidative stress were reduced by RES treatment through TRPA1 inhibition. It seems that RES is a potential treatment option for HPX-induced mitochondrial oxidative neuronal injury.

Keywords: Apoptosis, hypoxia, mitochondrial oxidative stress, resveratrol, TRPA1 channel

ÖZ

Amaç: Hipoksi (HPX), beyin ve sinir hücrelerinde Ca^{+2} girişi, apoptozis ve toksik reaktif oksijen türleri (ROS) oluşumunu artırır. Resveratrol (RES) voltaja duyarlı Ca^{+2} kanallarını bloke ederek ROS'un neden olduğu sinir hücre ölümünü azaltır. Bu çalışmanın amacı, HPX nedeniyle zarar gören SH-SY5Y sinir hücrelerinde, RES TRPA1 katyon kanalını inhibe ederek apoptozis ve ROS artışını nasıl etkilediğini SH-SY5Y glioblastoma sinir hücrelerinde araştırmaktır.

Materyal ve Metot: SH-SY5Y hücreleri kontrol, RES (50 µM ve 24 saat), HPX (200 µM $CoCl_2$ ve 24 saat) ve HPX + RES olacak şekilde dört gruba ayrıldı.

Bulgular: $CoCl_2$ ile inkübasyon, apoptotik (kaspaz-3, -8, ve -9) ve oksidan (ROS, mitokondriyal fonksiyon bozukluğu ve lipit peroksidasyon) düzeylerini artırdı. TRPA1 agonist (sinnamaldehit) inkübasyonu bu değerleri daha da artırdı. Bununla birlikte, TRPA1 antagonisti (AP-18) ve RES inkübasyonları bu artışları azalttı. AP-18 ve RES inkübasyonları, HPX inkübasyonunun neden olduğu hücre canlılığı yüzdesi, glutatyon miktarı ve glutatyon peroksidaz aktivitesi azalışlarını artırdı.

Sonuç: RES tedavisi, TRPA1 kanalını baskılayarak HPX'nin mitokondriyal oksidan ve sinir hücre apoptotik etkilerini azalttı. HPX neden olduğu mitokondriyal oksidatif stres ve sinir hücre harabiyetini önlemek için RES tedavisi potansiyel bir kaynak tedavi olarak gözükmektedir.

Anahtar Kelimeler: Apoptozis, hipoksi, mitokondriyal oksidatif stres, resveratrol, TRPA1 kanalı

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Yayın Bilgisi / Article Info:
Gönderi Tarihi/ Received: 28/08/2025
Kabul Tarihi/ Accepted: 27/10/2025
Online Yayın Tarihi/ Published: 20/12/2025

Atıf / Cited: Ertilav K. Resveratrol Reduces Hypoxia-Caused Increases of Apoptosis and Oxidative Neurotoxicity via TRPA1 Cation Channel Suppression in Glioblastoma Cells. *Online Türk Sağlık Bilimleri Dergisi* 2025;10(4):382-389.. doi: 10.26453/otjhs.1773319

INTRODUCTION

Cerebrovascular disease caused by ischemia/ reperfusion brain damage has a significant fatality rate globally. Cerebral hypoxia (HPX) in the brain and central nervous system is caused by ischemia/ reperfusion injury.[1] The primary way that HPX causes damage is by causing an excessive amount of free reactive oxygen species (ROS) to be produced in the mitochondria.[2] Excessive ROS generations is linked to rising cytosolic Ca^{2+} concentrations, which decrease mitochondrial function and increase ROS production.[3] A mimic of HPX is cobalt chloride ($CoCl_2$), and it has been used for induction of HPX.[4,5] The transient receptor potentials (TRP) ankyrin 1 (TRPA1) belongs to the subfamily of Ca^{2+} -permeable TRP superfamily. A well-known non-specific antagonist of TRPA1 is AP-18, while *cinnamaldehyde* (CNM) is a selective agonist of TRPA1.[6,7] Having cysteine residues in its structure, which are prime targets of ROS, makes the TRPA1 the most sensitive TRP channel to oxidative stress stimulation.[8] With the exception of SH-SY5Y cells, several studies demonstrate the involvement of TRPA1 in HPX-induced apoptosis and oxidative neurotoxicity due to the oxidative stress-dependent activation of TRPA1.[9.10]

The primary sources of resveratrol (RES), a polyphenol chemical, are peanuts, berries, and grapes.[11] Clinically beneficial, RES has demonstrated efficacy in treating a number of diseases, including neurological conditions.[12] RES reduces inflammation, oxidative damage, and mitochondrial dysfunction to serve a neuroprotective effect against HPX.[12,13] In the SH-SY5Y cells, the RES treatment reduced HPX-induced apoptosis, TRP melastatin 2 (TRPM2) activity, and ROS.[9] In the rat dorsal root ganglion, RES was found to have a TRPA1 antagonist function.[13] In a human glioblastoma cell line, the TRPA1 channel was blocked by an antioxidant (alpha lipoic acid), which decreased the apoptosis and mitochondrial oxidative stress induced by HPX.[10] RES suppressed ROS generation caused by HPX by altering the L-type Ca^{2+} channel in a mouse neuroblastoma cell line.[15] It was also shown that RES might protect PC12 neuronal cells from oxidative and apoptotic damage caused by oxygen-glucose deprivation and reoxygenation via modifying Ca^{2+} influx.[16] However, it has not been shown if RES supplementation inhibits TRPA1 to prevent apoptosis and oxidative neuronal damage after exposure to HPX ($CoCl_2$).[4,11,13]

The aim of the present study was to clarify how RES protects SH-SY5Y neuronal cells from oxidative stress, apoptosis, caspases (caspase-3, -8, and -9), and mitochondrial membrane dysfunction by modifying TRPA1 activity.

MATERIALS AND METHODS

***Ethical Approval*:** The study was performed using a commercial cell culture. Hence, this study does not need ethics committee approval.

Cells: A common cell line used in TRPA1 research, including HPX and numerous neurodegenerative disorders, is the SH-SY5Y human neuroblastoma cell line.[4,17] For this reason, the cells were employed in the current investigation as a model for neural cell culture. It was reported that the DMEM/Ham's F12 mixture with high glucose (4.5 g/l) induced oxidant and TRP stimulator actions.[18] 10% FBS and 1% penicillin/streptomycin were added to the DMEM/ Ham's F12 mixture with low glucose (1 g/l). The SH -SY5Y cell line (ATCC, VA, USA) was kept at 37 ° C with 5% CO_2 until the studies were conducted.

Study Groups: Four groups [control (CNT), RES, HPX, HPX + RES] in the 25 cm^2 sterile flask containing $2x10^6$ SH-SY5Y cells were induced. Even though 10 µM AP-18 incubation blocked the TRPA1 channel in the cells of four groups, 20 µM CNM stimulated it in the plate reader studies.[6] As HPX + AP-18 was induced, the cells of fifth group were incubated with 10 µM AP-18 for an hour following a 24-hour incubation with 200 µM $CoCl_2$ for the spectrophotometric (UV-1800, Shimadzu, Kyoto, Japan) oxidant and antioxidant analysis.

The SH-SY5Y cells of CNT groups were kept in the incubator for 24h. After receiving 50 µM RES, the cells in the RES groups were cultivated for 24h.[4] In the HPX and RES combination group, cells were treated with 200 µM $CoCl_2$ and 50 µM RES (Sigma –Aldrich Inc., St. Louis, MO, USA) for 24h.[4] For twenty-four hours, the cells in the HPX group were cultured with 200 µM $CoCl_2$.

Analyses for Apoptosis and Cell Viability: The apoptotic dye (APOPercentage, Biocolor Ltd., Belfast, UK) was pipetted into 950 µL of 1x PBS, and then added to 150 µL of the diluted cells (1 ml 1x PBS). Following a half-hour incubation period in a shaking water bath, the tubes were centrifuged (5 minutes at 1500 g), and the supernatant was discarded. 200 µL of apoptotic dye was added to the pellet following one PBS wash, and centrifugation was carried out. The extracted supernatant was transferred to wells and measured at 550 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

For every Eppendorf tube containing cells ($1x10^6$), 15 microliters of methylthiazolyl diphenyl tetrazolium (MTT) dye were added. After that, the tubes were gently shaken for ninety minutes at 37 °C. Then, all Eppendorf tubes were centrifuged for 10 minutes at 500 g. D*imethyl sulfoxide (*400 µL*)* was supplemented to each tube after centrifugation, and the supernatant was discarded. There were 250 µL

of the *dimethyl sulfoxide* suspension prepared from the pellet in each well. At 490 nm, the absorbance was measured using a UV-1800 spectrophotometer.

Analyses for Caspase-3, -8, and -9: The cleavage of the caspase substrates by caspases -3 (Ac-DEVD-AMC), -8 (Ac-VETD-AMC), and -9 (Ac-LEHD-AFC) was analyzed using a microplate reader (Infinite PRO 200, Tecan Austria GmbH, Groedig, Austria) (Bachem, Heidelberg, Germany). Under the plate reader, the excitation and emission wavelengths were kept constant at 380 nm and 460 nm, respectively. When the caspases changed, the fluorescence units were recorded.[10]

Following ApoPercentage, MTT, and substrate loadings, cells in four groups were stimulated with 20 µM CNM for 60 minutes, either with or without a TRPA1 inhibitor (10 µM AP-18). This allowed for the measurement of TRPA1-dependent apoptosis induction and caspase releases. MTT, caspases, and apoptosis data were expressed as a percentage change in cell viability from the CNT group.

Analyses for Measuring ROS and Mitochondrial Membrane Dysfunction: The JC-1 dye (2 µg/ml; Cat # T3168, Thermo Fisher Scientific) and ROS probe (DCFH-DA) (1 µg/ml; Cat # D399, Thermo Fisher Scientific) were used to incubate the cells in order to examine ROS generation and mitochondrial membrane dysfunction. Incubation of the SH-SY5Y cells was then conducted for 30 minutes in the dark at 37 °C. Cells labeled with DCFH-DA and JC-1 were then investigated for differences in fluorescence intensity using the Infinite Pro 200 plate reader.[4,10]

The cells of four groups were stimulated with 20 µM CNM for 60 minutes, either with or without a TRPA1 inhibitor (10 µM AP-18), after these DCFH-DA and JC-1 loadings, in order to quantify the TRPA1-dependent ROS and mitochondrial dysfunction. The percentage changes from the CNT for ROS and JC-1 (J-monomer/j-aggregate ratio) were shown.

Analysis of Glutathione Peroxidase (GSH-Px) Activity, Reduced Glutathione (GSH), and Lipid Peroxidation (LPx) Concentrations: Using a UV-1800, the GSH-Px activity and GSH concentration of SH-SY5Y lysates were measured spectrophotometrically at 412 nm.[19,20] A spectrophotometer set to 532 nm was used to detect LPx as malondialdehyde in the lysates.[21] Lowry's solution was utilized to determine the concentration of total protein. While GSH and LPx concentrations were measured in µM per gram of protein, GSH-Px activity was assessed in IU per gram of protein.

Statistical Analysis: To show the data, the mean standard deviation (SD) is utilized. Following group comparisons using one-way analysis of variance, the Student's T test revealed p-values below 0.05.

RESULTS

Viable cell percentage (Figure 1A) increased in the CNT, RES, and HPX + RES as compared to the HPX only, while apoptosis levels (Figure 1B) in the groups were lower ($p < 0.05$) than in the HPX only. While apoptosis levels increased in the CNT + CNM, HPX + CNM, and HPX + RES + CNM, cell viability levels were further reduced by TRPA1 stimulation (CNM). But for the CNT + CNM + AP-18, HPX + CNM + AP-18, and HPX + RES + CNM + AP-18, AP-18 and RES treatments increased cell

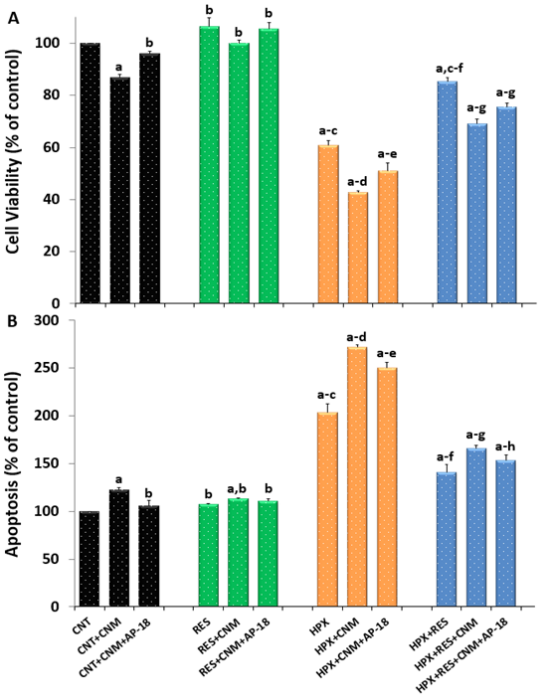

Figure 1. The twenty-four-hour incubation of RES (50 µM) modulated HPX (200 µM $CoCl_2$), causing a decrease in cell viability (A) but an increase in apoptosis (B) in the SH-SY5Y cells. (Mean ± SD and n=3). The TRPA1 channel in the cells was stimulated by 20 µM CNM, although it was inhibited by 10 µM AP-18. ($^{a}p < 0.05$ versus (*vrs.)* control (CNT) $^{b}p < 0.05$ *vrs.* CNT + CNM. $^{c}p < 0.05$ *vrs.* RES, RES + CNM, and RES + CNM + AP-18. $^{d}p < 0.05$ *vrs.* HPX. $^{e}p < 0.05$ *vrs.* HPX + CNM. $^{f}p < 0.05$ *vrs.* HPX + CNM + AP-18. $^{g}p < 0.05$ *vrs.* HPX + RES. $^{h}p < 0.05$ *vrs.* HPX + RES + CNM).

viability percentages, while their incubations decreased apoptosis percentages ($p < 0.05$).

In comparison to the CNT, RES, and HPX + RES, HPX had higher levels of caspase-3 (Figure 2A), caspase-8 (Figure 2B), and caspase-9 (Figure 2C) activities ($p < 0.05$). Through TRPA1 stimulation (CNM), the caspase activities were further increased in the CNT + CNM, HPX + CNM, and HPX + RES + CNM. The AP-18 and RES treatments, however, reduced the caspase activities in the CNT

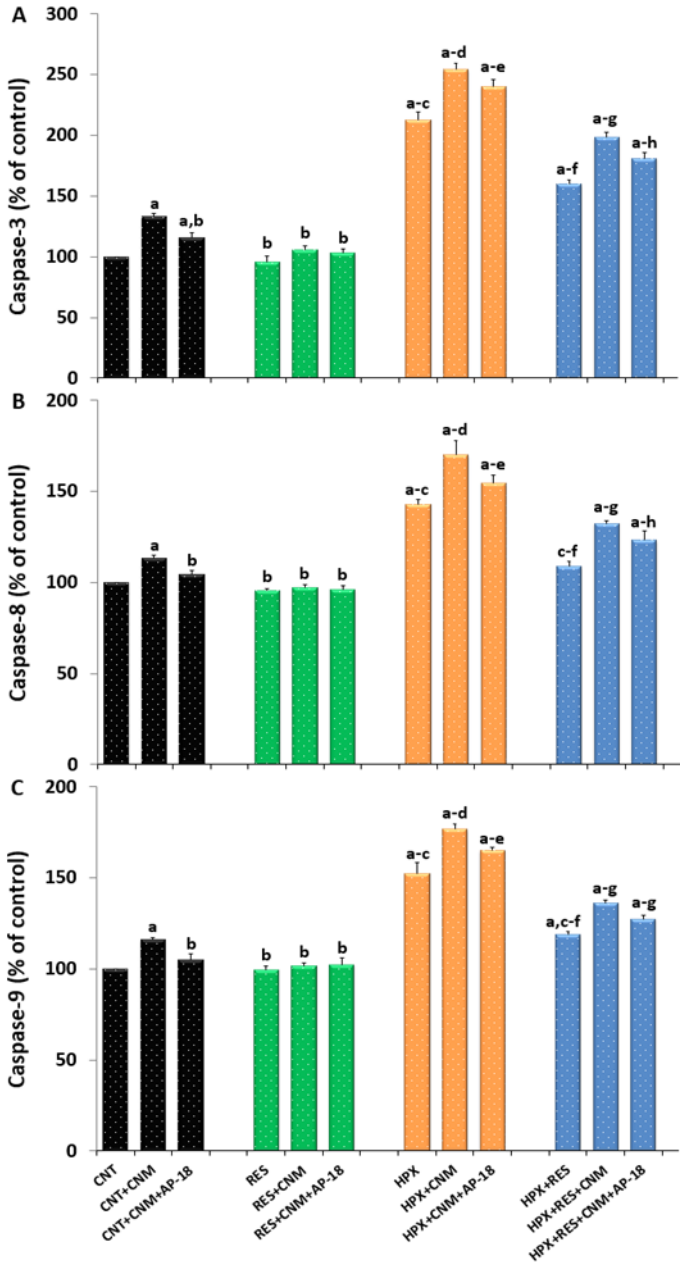

Figure 2. The twenty-four-hour incubation of RES (50 µM) decreased HPX (200 µM $CoCl_2$), causing an increase in caspase-3 (A), caspase-8 (B), and caspase-9 (C) in the SH-SY5Y cells. (Mean ± SD and n=3). The TRPA1 channel in the cells was stimulated by 20 µM CNM, although it was inhibited by 10 µM AP-18. ($^{a}p < 0.05$ versus (*vrs.)* control (CNT) $^{b}p < 0.05$ *vrs.* CNT + CNM. $^{c}p < 0.05$ *vrs.* RES, RES + CNM, and RES + CNM + AP-18. $^{d}p < 0.05$ *vrs.* HPX. $^{e}p < 0.05$ *vrs.* HPX + CNM. $^{f}p < 0.05$ *vrs.* HPX + CNM + AP-18. $^{g}p < 0.05$ *vrs.* HPX + RES. $^{h}p < 0.05$ *vrs.* HPX + RES + CNM).

+ CNM + AP-18, HPX + CNM + AP-18, and HPX + RES + CNM + AP-18 ($p < 0.05$).

The JC-1 (Figure 3A) and DCFH-DA (Figure 3B) levels were higher in the HPX than in the CNT, RES, and HPX + RES ($p < 0.05$). In the CNT + CNM, HPX + CNM, and HPX + RES + CNM, TRPA1 stimulation (CNM) additionally raised the levels of JC-1 and DCFH-DA. However, the CNT + CNM + AP-18, HPX + CNM + AP-18, and HPX + RES + CNM + AP-18 indicated lower levels of JC-1 and DCFH-DA after the AP-18 and RES treatments ($p < 0.05$).

GSH concentration (Figure 4A) and GSH-Px activity (Figure 4B) were lower ($p < 0.05$) in the CNT than in the RES. Compared to the CNT and RES, the HPX had lower GSH concentration and GSH-Px activities, while the HPX + RES and HPX + AP-18 had higher GSH concentration and GSH-Px activities ($p < 0.05$). Furthermore, the HPX + RES group had greater GSH levels and GSH-Px activities than the HPX + AP-18 ($p < 0.05$). The HPX + RES had a lower LPx concentration (Figure 4C) than the HPX only, but the HPX group had a higher LPx concentration than the CNT and RES ($p < 0.05$). In comparison to the CNT group, the LPx concentration of the RES was lower. Furthermore, the HPX + RES had a

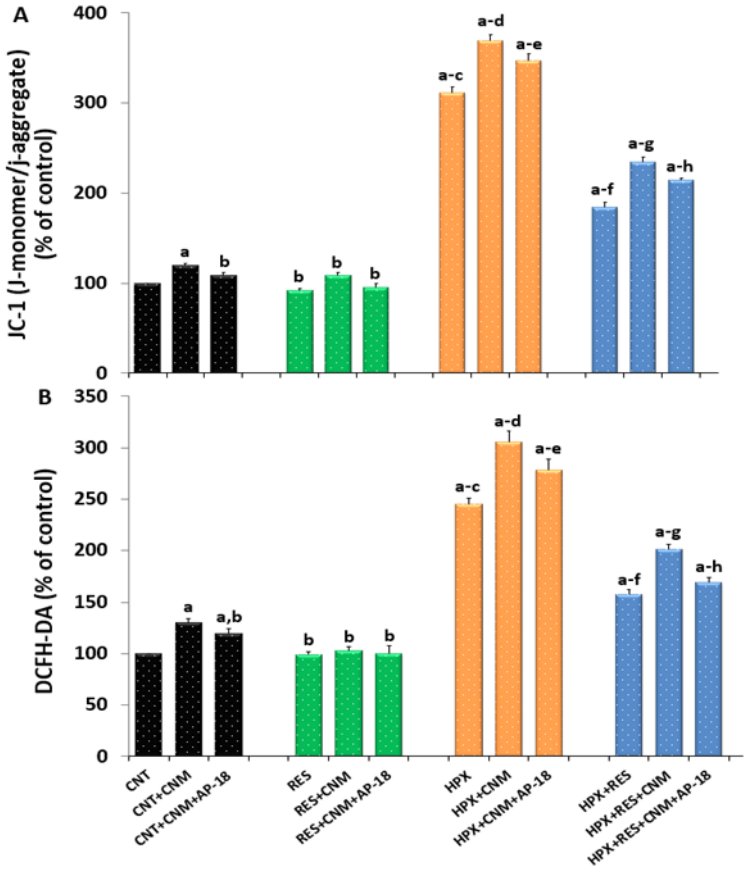

Figure 3. The twenty-four-hour incubation of RES (50 µM) decreased HPX (200 µM $CoCl_2$), causing an increase in caspase-3 (A), caspase-8 (B), and caspase-9 (C) in the SH-SY5Y cells. (Mean ± SD and n=3). The TRPA1 channel in the cells was stimulated by 20 µM CNM, although it was inhibited by 10 µM AP-18. ($^{a}p < 0.05$ versus (*vrs.)* control (CNT) $^{b}p < 0.05$ *vrs.* CNT + CNM. $^{c}p < 0.05$ *vrs.* RES, RES + CNM, and RES + CNM + AP-18. $^{d}p < 0.05$ *vrs.* HPX. $^{e}p < 0.05$ *vrs.* HPX + CNM. $^{f}p < 0.05$ *vrs.* HPX + CNM + AP-18. $^{g}p < 0.05$ *vrs.* HPX + RES. $^{h}p < 0.05$ *vrs.* HPX + RES + CNM).

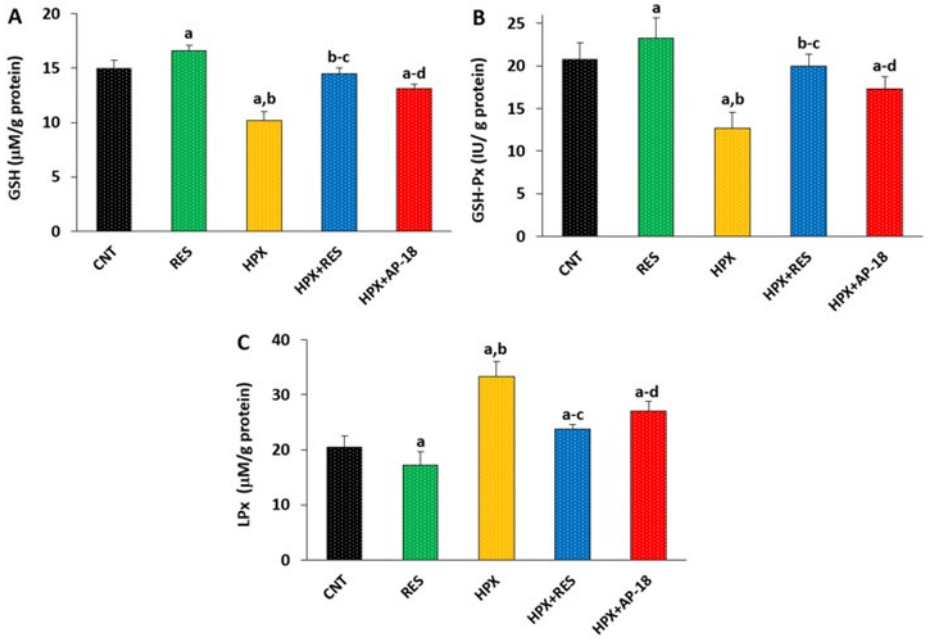

Figure 4. The incubation of RES (50 µM) modulated HPX (200 µM $CoCl_2$), causing changes in glutathione (GSH), glutathione peroxidase (GSH-Px), and lipid peroxidation (LPx) in the SH-SY5Y cells. (Mean ± SD). A. GSH. B. GSH-Px. C. LPx. ($^{a}p < 0.05$ versus (*vrs.)* CNT. $^{b}p < 0.05$ *vrs.* RES. $^{c}p < 0.05$ *vrs.* HPX. $^{d}p < 0.05$ *vrs.* HPX + RES.

lower LPx concentration than the HPX + AP-18 ($p < 0.05$).

DISCUSSION AND CONCLUSION

It was found in this study that HPX increased oxidative neurotoxicity and apoptosis by stimulating TRPA1, whereas treating with RES increased GSH and GSH-Px through the downregulation of oxidative neurotoxicity, apoptosis, and TRPA1.

In in vivo neural experiments, the formation of mitochondrial ROS is triggered by excessive Ca^{2+} entry.[2] In vitro data suggest that TRPM2 channel stimulation under hypoxic conditions causes excessive ROS production and SH-SY5Y neuronal cell death.[4] Additionally, HPX was shown to enhance the upregulation of L-type Ca^{2+} channels in PC12 cells through elevated ROS production and mitochondrial dysfunction, although these effects were mitigated by RES incubation.[13] By blocking the L-type Ca^{2+} channel in the mouse Neuro-2a neuroblastoma cells, the RES treatment reduced the ROS, apoptosis, and Ca^{2+} influx induced by HPX.[15] It has been documented that the RES therapy reduced the ROS, apoptosis, and Ca^{2+} influx caused by HPX via inhibiting glutamate receptors in SH-SY5Y cells.[22] I'm not aware of any research that looks into how TRPA1 influences the oxidative and apoptotic properties of HPX in neural cells. The apoptotic and oxidant activities of HPX in the SH-SY5Y cells were reduced by the TRPA1 antagonist (AP-18); however, in the present investigation, TRPA1 activation further enhanced these activities. However, it appears that when TRPA1 stimulation-mediated Ca^{2+} influx was reduced, oxidative damage and neuronal death were reduced; but, both events were increased when TRPA1 stimulation-mediated Ca^{2+} influx was raised.

The SH-SY5Y cells are among the neurons and cells that exhibit enhanced mitochondrial Ca^{2+} uptake in response to TRPA1 activation.[6,9,23] ROS production and cell death markers (apoptosis, caspase-3, caspase-8, and caspase-9) that result from mitochondrial membrane disruption rise as a result.[6,9,10,23] By altering Ca^{2+} influx, it was also demonstrated that RES may protect PC12 neuron cells from oxidative and apoptotic damage induced by oxygen-glucose deprivation and reoxygenation.[16] Thus, increasing mitochondrial membrane dysfunction and TRPA1 stimulations augment the oxidant (ROS and LPx) and apoptotic (caspases-3, -8, and -9) markers.[6,23] In contrast, TRPA1 suppression reduces the proportion of oxidant and apoptotic markers, like SH-SY5Y, in brain cells.[6,9,10,23] Based on available data, the enhanced mitochondrial membrane dysfunction in SH-SY5Y was caused by HPX-induced TRPA1 activation. Cell viability consequently declined, but ROS, LPx, apoptosis, and caspase-3, -8, and -9 increased. The changes were decreased by RES therapy and TRPA1 blocker (AP-18). The current results show that via blocking TRPA1, RES incubation reduced

the increases in mitochondrial dysfunction, ROS, LPx, caspases, and apoptosis that HPX caused in SH -SY5Y cells.

The components of thiol redox antioxidant system may help control the oxidative imbalance induced by HPX, as seen by the reported decreases in GSH-Px activity and GSH concentration.[4] GSH concentration and GSH-Px activity increased while the oxidative effects of HPX diminished, as seen by decreased ROS and LPx concentrations, following incubation with RES or TRPA1 suppression with AP-18. These results suggest that RES and AP-18 reduce ROS and LPx while raising GSH concentrations and GSH-Px activity. H_2O_2 is known to be converted to water by GSH-Px. GSH-Px uses GSH as a substrate in the process. The powerful scavenging of a variety of oxidants by RES is due to its antioxidant activity.[24] RES treatment has been demonstrated to restore GSH concentration and GSH-Px activity in SH-SY5Y cells while lowering LPx concentration, which is consistent with the findings.[4] The RES therapy significantly decreased the LPx generated by HPX by increasing GSH-Px activity and GSH concentration in the animals with spinal cord damage.[4,25] The current findings support these findings.

In conclusion, the results showed that TRPA1 attenuation during RES incubation protected SH-SY5Y cells from oxidative and apoptotic mediators produced by HPX, since TRPA1 inhibition reduced neuronal damage. Although RES treatment reduces the oxidative neurotoxicity and apoptosis caused by HPX, it may still cause ROS, mitochondrial dysfunction, LPx, and TRPA1-mediated caspases, which can result in HPX-induced oxidative neuronal injury and apoptosis.

Ethics Committee Approval: Human and animal data are not available.

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

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

Peer-review: Externally peer-reviewed.

Financial Support: A company (BSN Health, studies, Innov., Consult., Org., Agricul., Trade Ltd., Isparta, Türkiye) provided financial support for this study (Project No: 2024-02).

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