RESEARCH ARTICLE

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Anti-Inflammatory Effects of Resveratrol on the Radiotherapy-Induced Neuroinflammation

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Objective: Damage in the central nervous system caused by cranial radiotherapy (RT) has been linked to neuroinflammation due to microglial activation. Evidence reveals that resveratrol (RES) exerts neuroprotective effects by inhibiting neuroinflammation. There are limited studies investigating the effects of RES on microglia-related neuroinflammation developed as a result of RT. Therefore, this study was designed to investigate the effects of RES on RT-induced microglial-related neuroinflammation.

Materials and Methods: Rats were simple randomly divided into three groups. Sham (SH) group received ethanol solution on the 1st-14th days of the study. RT group was applied a single dose of total cranial 15 Gy X radiation on the 7th day of the study. RES group was administered a dose of 20 mg/kg on the 1st-14th days of the study and a single dose of total cranial 15 Gy X radiation on the 7th day of the study. The brain tissues removed at the end of the experiment were subjected to histological techniques and procedures for histological and immunohistochemical examinations. The data were evaluated statistically.

Results: RT administration caused histological changes such as neuron degeneration and edema in the brain tissue. In addition, RT administration induced a significant increase in CD68 and tumor necrosis factor-alpha (TNF-α) immunoreactivity. In the RES+RT group, it was observed that histological changes were alleviated, and CD68 and TNF-α immunoreactivities were decreased. In addition, a significant increase in the immunoreactivity of autophagy-related proteins was detected in this group.

Conclusion: Evaluating together all the data, it was revealed that RES attenuates microglia-mediated neuroinflammation and neuronal degeneration.

Key Words: *Radiotherapy, resveratrol, neuroinflammation, microglia, autophagy*

Resveratrolün Radyoterapi ile Indüklenen Nöroenflamasyon Üzerindeki Anti-Enflamatuvar Etkileri

Amaç: Merkezi sinir sisteminde kraniyal radyoterapi (RT) nedeniyle ortaya çıkan hasar, mikrogliyal aktivasyona bağlı olarak gelişen nöroenflamasyonla ilişkilendirilmiştir. Çalışmalardan elde edilen bulgular, resveratrolün nöroenflamasyonu inhibe ederek nöroprotektif etkilere sahip olduğunu ortaya koymaktadır. RT sonucu gelişen mikroglia ile ilişkili nöroinflamasyon üzerine resveratrolün (RES) etkilerini araştıran sınırlı sayıda çalışma bulunmaktadır. Bu nedenle bu çalışma, resveratrolün radyasyona bağlı mikrogliya-ilişkili nöroenflamasyon üzerindeki etkilerini araştırmak için tasarlanmıştır.

Gereç ve Yöntem: Sıçanlar rastgele üç gruba ayrıldı. Sham (SH) grubuna çalışmanın 1-14. günlerinde etanol solüsyonu verildi. RT grubuna çalışmanın 7. gününde tek doz total kraniyal 15 Gy X radyasyon uygulandı. RES grubuna çalışmanın 1-14. günlerinde 20 mg/kg doz RES ve çalışmanın 7. gününde tek doz total kraniyal 15 Gy X radyasyon uygulandı. Deney sonunda çıkarılan beyin dokuları, histolojik ve immünohistokimyasal incelemeler için histolojik teknik ve işlemlerden geçirildi. Veriler istatistiksel olarak değerlendirildi.

Bulgular: RT uygulamasına bağlı olarak beyin dokusunda nöron dejenerasyonu ve ödem gibi histolojik değişiklikler izlendi. Ayrıca RT uygulaması sonucunda CD68 ve TNF-α immünoreaktivitesinde önemli bir artış saptandı. RES+RT grubunda histolojik değişikliklerin hafiflediği, CD68 ve TNF-α immünoreaktivitelerinin azaldığı gözlendi. Ek olarak, bu grupta otofaji ile ilgili proteinlerin immünoreaktivitesinde önemli bir artış tespit edildi.

Sonuç: Tüm veriler birlikte değerlendirildiğinde RES'in mikrogliya aracılı nöroenflamasyon ve nöronal dejenerasyonu azalttığı ortaya çıktı.

Anahtar Kelimeler**:** *Radyoterapi, resveratrol, nöroenflamasyon, mikrogliya, otofaji*

Introduction

Cranial RT is a substantial adjuvant treatment of varied cancers, such as brain tumors, and particular head and neck malignancies. But, it injures surrounding healthy tissue and organs by producing both acute and long-lasting side effects. Thus, RT-related brain damage is common and seriously influences patients' quality of life (1-3). Cranial irradiation destroys the neurogenic microenvironment by activating inflammation-related genes and overexpressing pro-inflammatory factors in the central nervous system (1, 2). Pro-inflammatory factors, principally nuclear factor kappa B (NF-κB), and cytokines such as largely interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and TNF-α are upregulated within a few hours after irradiation (4).

It was reported that pro-inflammatory cytokines produced by neuronal and glial cells, especially microglia, play an important role in inflammatory reactions caused by RT application (2). Microglial cells are the resident immune cells in the central nervous system. It can become activated following exposure to radiation and activation of these cells result in increased release of pro-inflammatory cytokines (5). Growing evidence has suggested that microglial activation plays an important role in neuroinflammation, finally leading to neuroinflammation-mediated neurodegeneration (2, 3).

As the technical potential to decrease the radiation load to the organs at risk is limited, neuroprotective agents may be beneficial. Phytochemicals have been demonstrated to have neuroprotective properties against neurodegeneration as developing due to various causes such as RT, infection, trauma, ischemia, and aging (6). Recently, the use of phytotherapeutic agents has too increased. But, much research is still required to be done before its widespread use and approval. RES, a molecule belonging to the polyphenol family, is obtained from numerous natural plants (7). The main sources of RES are grapes, berries, soy, and peanuts (8). RES exhibits multiple biological features including anti-inflammatory, anti-oxidant, anti-apoptotic, and anti-aging effects (9, 10). It has been also revealed that RES, which can cross the blood-brain barrier, is a potential radioprotective agent (11, 12).

RES has been shown to be able to prevent microglia-mediated neuroinflammation in various experimental models including sepsis, ischemia, seizures, and neurodegenerative disorder models (13, 14). It can suppress the production and release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β through the inhibition of transcriptional factors such as NF-κB, and thus can protect neural tissue and cells from inflammatory damage by reducing the activation of microglial cells (10, 15).

Recently, it has been reported that RES also ameliorated microglia-mediated inflammation by promoting autophagy (16). The evidence of previous studies documented that RES stimulates autophagy via activation of various cellular signaling pathways and exerts neuroprotective effects in this way (17, 18). Autophagy, as a crucial homeostatic process for cell survival and the maintenance of homeostasis in cells, is digestion and recycling in the lysosomes of cytosolic material such as various macromolecules and organelles (19). Recent evidence has also revealed a very important role of the autophagic pathway in neuroinflammation (20, 21).

As a result of the literature review, there are limited studies investigating the effects of RES on microglia-related neuroinflammation developed as a result of RT. Based on the reported anti-inflammatory activity of RES, this study was designed to investigate the neuroprotective effects of RES on RT-induced microglia-mediated neuroinflammation using histological and immunohistochemical parameters.

Materials and Methods

Research and Publication Ethics: The study protocol was approved by the Animal Research Ethics Committee of Inonu University, Malatya, Faculty of Medicine (Protocol: 2021/22-12).

Animals and Experimental Procedure: Female Sprague Dawley rats aged 10-12 weeks, weighing 150–250g were obtained from Inonu University Laboratory Animals Research Center. Rats were provided adlibitum standard rat chow and tap water and were housed in separate cages in a well-ventilated room at 21 °C with a 12 h light:12 h dark cycle.

Twenty-eight rats were used in this experiment and the rats were simple randomly divided into three groups:

I. Sham group (n=8) was given ethanol solution (ethanol/saline; 1/23) by oral gavage on the 1^{st}-14^{th} days of the study.

II. RT group (n=10) was administered a single dose of total cranial 15 Gy X radiation on the 7^{th} day of the study (22).

III. RES+RT group (n=10) was given a dose of 20 mg/kg RES by oral gavage on the 1^{st}-14^{th} days of the study (23), and a single dose of total cranial 15 Gy X radiation was administered on the 7^{th} day of the study.

For cranial RT administration, rats were anesthetized with 75 mg/kg intraperitoneal (i.p.) ketamine and 25 mg/kg i.p. xylazine, then rats were exposed to a single dose of total cranial 15 Gy X radiation. Total cranial irradiation was performed with 6-MV photon beams delivered by a linear accelerator (Varian Trilogy DHX, Varian Medical Systems, Palo Alto, California) at an average of 23°C in-device temperature. The head of each rat was placed in a treatment field (40 × 8 cm) within the confines of the whole brain from the post-canthus line to the post-aurem line. Irradiated rats received a single dose of 15 Gy X-rays at a dose rate of 400 cGy/min and a source-to-axis distance of 98.5 cm. Sham group rats were anesthetized but were not irradiated.

On the 15^{th} day of the study, all animals were sacrificed under ketamine/xylazine (200 mg/kg/25 mg/kg) anesthesia. Thereafter, brains were removed, and then brain tissue samples were fixed in 10% formalin for histological and immunohistochemical analyses.

Histological Analysis: The brain tissues were fixed in 10% formalin. After fixation, following the dehydration process in ethanol series and then the clearing process in xylene series, the tissues were embedded in paraffin wax. Sections of 4 mm thickness were taken from paraffin blocks, then the sections were mounted on slides, and stained with hematoxylin&eosin (H&E). The histological evaluation was performed in terms of the presence of degenerate neurons in the cerebral cortex and degenerate neuron count was performed in ten randomly selected fields on each section at 40x objective (24). Leica DFC280 light microscope and Leica Q Win Image Analysis system

(Leica Micros Imaging Solutions Ltd., Cambridge, UK) were used for histological analysis.

Immunohistochemical Analysis: Immunohistochemical staining was carried out to demonstrate CD68 as a microglial activation marker, TNF-α as a neuroinflammation marker, and BECN1 and MAPLC3 as autophagy markers (CD-68, TNF-α, BECN1, MAPLC3; Santa Cruz Biotechnology, Inc., Heidelberg, Germany). The staining method was performed as described in a previous study (25).

For immunohistochemical evaluation, ten randomly selected areas in each section were examined and the following semiquantitative scale was used. According to the cell density showing immunoreactivity, the areas were graded as 1=0-25% immunoreactivity; 2=26-50% immunoreactivity; 3=51-75% immunoreactivity; 4=76-100% immunoreactivity in the total area. According to the immunoreactivity severity, the sections were graded as follows: 0=no immunoreactivity; 1=weak immunoreactivity; 2=mild immunoreactivity; 3=strong immunoreactivity. Total immunoreactivity score was obtained as (cell density showing immunoreactivity) X (immunoreactivity intensity) (25). Leica DFC280 light microscope and Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK) were used for immunohistochemical evaluation.

Statistical Analysis: Data analysis were performed using the IBM SPSS software program for Windows, version 22.0 (SPSS Inc., Chicago, IL). The normal distribution of the data was determined with the Shapiro-Wilk test. Group comparisons were conducted by Kruskal-Wallis since the distribution of the data is non-normal. When significant differences were determined, pairwise comparisons were performed using the Mann-Whitney U test with Bonferroni correction. $p<0.05$ was considered statistically significant difference. The data were summarized as the median (minimum-maximum).

Results

Histological Evaluations: It was observed no histological changes in the SH group. In this group, the cerebral cortex was regular and neurons were also intact except for a few degenerate neurons (Figure 1. A and D). On the other hand, the cerebral cortex in the RT group revealed irregular appearance and edema. In addition, it was observed extensive degenerate neurons characterized by shrunken cytoplasm, and dark pyknotic nuclei in the cerebral cortex belonging to in RT group (Figure 1. B and E). The difference between the SH group and the RT group in terms of the number of degenerated neurons was found to be statistically significant ($p<0.01$). However, RES administration attenuated radiation-induced histological changes. It was determined that the number of degenerated neurons also decreased prominently in the RES+RT group and this decrease was statistically significant when compared to the RT group ($p<0.01$) (Figure 1. C and F).

The number of degenerate neurons of each group are summarized in Table 1.

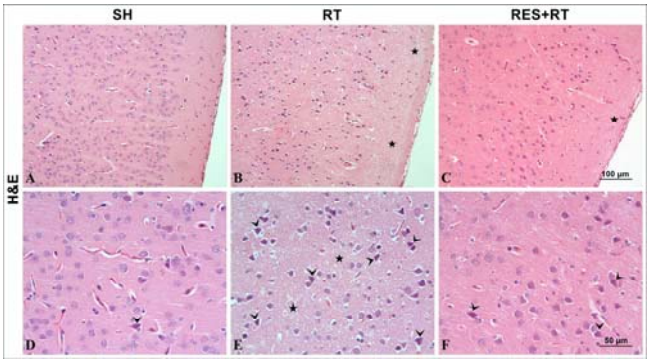

Figure 1. The appearance of the cerebral cortex in the brain tissue sections stained with H&E. Arrowheads point to degenerate neurons and asterisk point to edema

Table 1. The number of degenerate neurons (NDN) and the CD68, TNF-α, BECN1, and MAPLC3 immunoreactivity scores of each group

Groups	NDN	CD68	TNF-α	BECN1	MPLC3
SH	1.5 (0-9)	0 (0-4)	2 (0-6)	0 (0-6)	3 (1-9)
RT	5.0 (0-42)[a]	4 (0-9)[a]	5 (0-9)[a]	2 (0-6)[a]	4 (2-9)
RES+RT	1 (0-29)[b]	0 (0-8)[b]	3 (0-8)[b]	3 (0-9)[c]	6 (2-9)[c]

Data are expressed median (minimum-maximum). [a]Increase compared with the SH group ($p<0.01$). [b]Decrease compared with the RT group ($p<0.01$). [c]Increase compared with the RT group ($p<0.01$).

Immunohistochemical Evaluations

RES reduced CD68 and TNF-α immunoreactivity: CD68 and TNF-α immunoreactivity was observed as brownish in the cytoplasm of the cells (Figure 2). Sparse weakly CD68 and TNF-α positive immune reactive cells were observed in the SH group. A significant increase in CD8 and TNF-α immunoreactivity scores was determined in the RT group as compared to the SH group ($p<0.01$). In contrast, in the RES+RT group, it was recorded that both CD68 and TNF-α immunoreactivity scores were significantly reduced compared to the RT group ($p<0.01$). The immunoreactivity scores of CD68 and TNF-α of each group are summarized in Table 1.

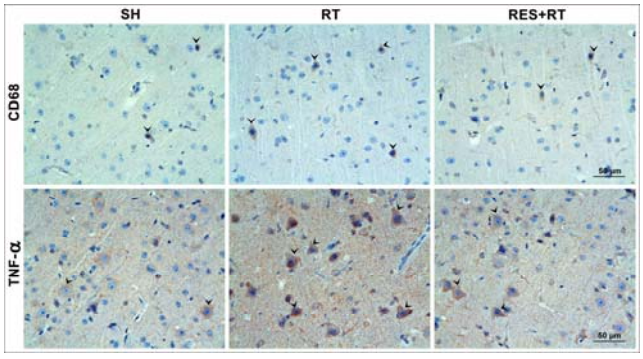

Figure 2. The CD68 and TNF-α immunoreactivity in the cerebral cortex stained with immunohistochemical staining method. Arrowheads point to positive immune reactive cells

RES increased BECN1 and MAPLC3 immunoreactivity: BECN1 and MAPLC3 positive cells were distinguished by brownish staining in their cytoplasm (Figure 3). BECN1 immunoreactivity score was significantly high in the RT group compared to the SH group ($p<0.01$), while the MAPLC3 immunoreactivity score in the RT group was similar to the SH group. On the contrary, in the RES+RT group, the immunoreactivity score obtained in both BECN1 and MAPLC3 evaluations was found to be significantly higher than in the RT group ($p<0.01$). The immunoreactivity score of BECN1 and MAPLC3 of each group are summarized in Table 1.

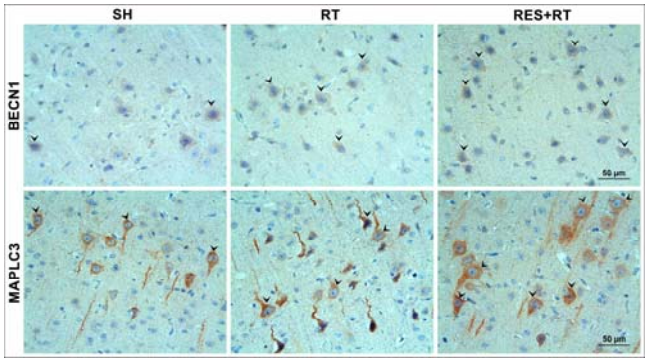

Figure 3. The BECN1 and MAPLC3 immunoreactivity in the cerebral cortex stained with immunohistochemical staining method. Arrowheads point to positive immune reactive cells

Discussion

The present study revealed that RES suppresses cranial RT-induced microglia-mediated neuroinflammation by promoting autophagy in rats.

Neuroinflammation within the central nervous system is the inflammatory reaction of microglia, and to a lesser extent of other glial cells, and neurons against infectious agents, trauma, ischemia, stroke, cranial radiation, etc. (26-28). In the present study, it was determined that RT administration significantly increased CD68-positive cell density. Consistent with this result of the present study, Ruddy et al. also reported that cranial irradiation promoted an increase in microglia proliferating (29). CD68, a microglial marker, is a membrane protein expressed by monocytes and macrophages that remarks phagocytic activity (30). It has been reported that cranial RT potently induces neuroinflammation, with markedly increased activated microglia (31).

Microglia, the resident immune cells of the central nervous system, play a crucial role in immune responses and the maintenance of neuronal tissue homeostasis. It also responds to changes in the microenvironment by upregulating various cell surface receptors and producing a wide variety of secreted factors. There is a balance between pro- and anti-inflammatory factors in the neuronal tissue under normal physiological situations. Whereas, a shift toward the pro-inflammatory state occurs by disturbing the balance after exposure to foreign conditions and substances such as radiation, infectious agents, trauma, and ischemia. Although microglial activation is necessary for protection under abnormal conditions, such activation may cause neuronal injury and death beyond a certain threshold (32, 33). Microglia abnormally activated by radiation continuously produce neurotoxic cytokines such as TNF-α, IL-6, and IL-β1 (3). In the current study, the effects of radiation on TNF-α expression were investigated and it was observed that radiation application significantly increased TNF-α expression.

TNF-α is able to be produced by other glial cells and neurons, but it is accepted that microglia are the main source of this cytokine during neuroinflammation. TNF-α, a proinflammatory cytokine, has both homeostatic and pathophysiological roles in the neuronal tissues. In pathological states, a large amount of TNF-α is expressed by microglia. This de novo expression of TNF-α is a significant indicator of the neuroinflammatory responses (28). TNF-α and other cytokines released from macrophages affect blood-brain barrier permeability, leukocyte adhesion, and microvessel diameter and cause predominantly acute RT-induced neurodegeneration including gliosis, edema, and neuronal cell death (34). Consistent with the literature, in the present study, it was also determined that the number of degenerate neurons was significantly higher in the RT group. Consistent with current results, it has been reported in many studies that radiation exposure causes neuronal damage and cell death (3, 35).

Increasing evidence has shown that RES exhibits multiple bioactivities, including anti-inflammatory, anti-oxidant, anti-apoptotic, and anti-aging effects (10, 36, 37). The current study demonstrated that RES has neuroprotection against RT-induced neurotoxicity. These neuroprotective effects of RES might be depended on the suppression of microglial activation and the subsequent decrease in the release of various neurotoxic cytokines such as TNF-α, IL-6, IL-1β, and NO. RES is able to decrease the release of pro-inflammatory factors through the inhibition of the NF-κB signaling pathway (10). Consistent with these results, the present study demonstrated that RES administration significantly decreased the intensity of CD68 and TNF-α positive cells. In addition, in the current study, it was determined that RES significantly reduced the increased number of degenerate neurons caused by RT. It has been reported that microglial activation may cause neuronal death by releasing neurotoxic pro-inflammatory molecules (14). Therefore, data of the current study indicate that the administration of RES exerted a significant anti-inflammatory effect by inhibiting microglia activation and subsequently reducing TNF-α expression.

It has been also reported that the anti-inflammatory effects of RES may be related to its stimulation of autophagy (16). Extensive research has documented that autophagy has substantial neuroprotective roles in many neurodegenerative disorders. The studies have shown that deletion/mutation of the autophagy-related genes disrupts cellular homeostasis, resulting in some neurodegenerative diseases (38-40). Autophagy, a crucial homeostatic process for cell survival, is the main regulatory catabolic mechanism by which cells digest and recycle cytosolic material, including impaired

macromolecules or damaged organelles. In this process, the degraded cytosolic material is surrounded by a double-membrane vesicle called an autophagosome and which then fuse with lysosomes. After fusion with lysosomes, the autophagosomes' content is cleared up and then the remaining products may recycle for the bioenergetics metabolites.

The formation of autophagosomes is the fundamental step of autophagy (19, 41, 42). A few autophagy-related genes and proteins are involved in this process and function in autophagosome formation (20). In the present study, the expression level of BECN1 and MAPLC3 proteins, which are autophagy-related proteins, were evaluated. BECN1, a key regulator of autophagosome formation, has an important role in autophagosome initiation and autolysosome maturation (43). MAPLC3 is a structural component of the autophagosomal membrane (44). In the present study, cell density showing BECN1 immunoreactivity was found to be significantly higher in the RT group compared to the control group. Also, in the RT group, it was determined that the cell density showing MPLC3 immunoreactivity increased, but this increase was not statistically significant compared to the control group. Besides the maintenance of cellular homeostasis, autophagy also functions as a response to cellular stress caused by exposure to a variety of chemical, and physical factors. It was documented that RT treatment also is one of the stress factors that induce autophagy in both cancer and normal cells (45). Similarly, in the current study, the increase in BECN1 and MAPLC3 expression due to RT administration indicates that RT induces autophagy. A previous study also reported that RT also induces autophagy and increases the expression of autophagy-associated proteins BECN1 and MAPLC3 (46).

Increasing evidence has revealed that RES is the potential of promoting autophagy (16). It has been reported that RES, which is suggested to modulate various cellular processes, can stimulate autophagy through different signaling pathways. RES can directly activate autophagy by regulating mTOR signaling pathways, as well as can stimulate autophagy by deacetylating histone acetylases via AMPK/SIRT1 signaling and thus exert a neuroprotective effect (47). In the present study, it was found that RES administration significantly stimulated autophagy. BECN1 and MAPLC3 positive cell density were found to be significantly higher in the RES+RT group than in both the control and RT groups. A previous study has reported that the neuroprotective effect on microglia-mediated inflammation of RES is associated with promoting autophagy (48). As mentioned above, RES administration has been found to significantly reduce the RT-induced increased CD68 and TNFα positive cell density, as well as the degenerated neuron density.

Consequently, this study has indicated RES has neuroprotective effects against RT-induced neuronal tissue damage via its anti-inflammatory activities. Evaluating together all the data, these effects of RES were related to the inhibition of microglial activation by promoting autophagy, and subsequently the reduction of pro-inflammatory factors release

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Declaration of Competing Interest: The authors report no declarations of interest.

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