

Laboratory Investigation

Neuroprotective Effect of Resveratrol on Acute Brain Ischemia Reperfusion Injury by Measuring Annexin V, p53, Bcl-2 Levels in Rats

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Background : Cerebral ischemia is as a result of insufficient cerebral blood flow for cerebral metabolic functions. Resveratrol is a natural phytoalexin that can be extracted from grape's skin and had potent role in treating the cerebral ischemia. Apoptosis, a genetically programmed cellular event which occurs after ischemia and leads to biochemical and morphological changes in cells. There are some useful markers for apoptosis like Bcl-2, bax, and p53. The last reports, researchers verify the apoptosis with early markers like Annexin V.

Methods : We preferred in this experimental study a model of global cerebral infarction which was induced by bilateral common carotid artery occlusion method. Rats were randomly divided into 4 groups : sham, ischemia-reperfusion (I/R), I/R plus 20 mg/kg resveratrol and I/R plus 40 mg/kg resveratrol. Statistical analysis was performed using Sigmasat 3.5 ve IBM SPSS Statistics 20. We considered a result significant when $p < 0.001$.

Results : After administration of resveratrol, Bcl-2 and Annexin levels were significantly increased ($p < 0.001$). Depending on the dose of resveratrol, Bcl2 levels increased, p53 levels decreased but Annexin V did not effected. P53 levels were significantly increased in ischemia group, so apoptosis is higher compared to other groups.

Conclusion : In the acute period, Annexin V levels misleading us because the apoptotic cell counts could not reach a certain level. Therefore we should support our results with bcl-2 and p53.

Key Words : Resveratrol · Brain Ischemia · Annexin V · Bcl-2 · p53.

INTRODUCTION

Cerebral ischemia is as a result of insufficient cerebral blood flow for cerebral metabolic functions. Stroke is the second leading cause of long-term disability and death worldwide^{6,7}. Oxidative stress and inflammation have an important role in cerebral infarction which mediated by ischemia and reperfusion^{18,26}. Reperfusion injury stimulates many pathological mechanisms such as leukocyte infiltration, oxidative stress, inflammation, destruction of blood-brain barrier, platelet activation, nitric oxide release, and apoptosis^{29,31,34}. Consequently, a potent anti-inflammatory and antioxidant mediators may be beneficial in the treatment of cerebral ischemia and reperfusion injury. Mitochondria produces ATP, is essential for cell function, and plays an important role in response to oxidative stress⁸. Energy failure is due to insufficient ATP for a cell to maintain its cellular functions or defenses against oxidative stresses³². Pathological events like ischemia cause necrosis but can also induce apoptosis²⁰. Severe oxidative stress causes necrosis, and milder stress causes apoptosis. Increased oxidative stress cause reduced ATP production in mitochondria, and this releases cytochrome C an AIF, which leads to apoptosis.

Recent researches prove that activation of the cysteine protease caspases is critical for apoptosis in ischemia-induced brain injury²¹. During apoptosis caspases are activated in the intracellular region and leads to degradation of cellular constituents; fi-

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nally leading to cell death. Caspase-3 is believed to be the main executioner protease of apoptotic caspase³⁶.

Resveratrol is a natural phytoalexin that can be extracted from grape's skin. Resveratrol was reported to have antioxidant, anti-inflammatory, antidiabetic, and antiaging, control of cell cycle, and apoptosis^{11,28,49}. Earlier reports suggested that antioxidants and anti-inflammatory agents had potent role in treating the cerebral ischemia-reperfusion injury^{5,49}. Antiphospholipid antibodies (aPLs) are associated with some neurologic disorders. Annexins are a family of calcium-dependent phospholipid-binding proteins. The potent anticoagulant activity of annexin V is derived from its inhibitory effect on prothrombin activation^{32,42}. Antiannexin V antibody (aAV) has an important role in thrombotic events in patients with systemic lupus erythematosus and antiphospholipid syndrome¹² aAV plays a role in cerebral thrombotic events by inhibiting the anticoagulant activity of annexin V¹⁶.

p53 is an important modulator of cell death and survival, and its inhibition could be a therapeutic approach to several neuropathologies⁹. Bcl-2, a gene located at chromosome 18q21, blocks apoptosis without affecting cellular proliferation. Increases in the expression of bcl-2 stop apoptosis and inhibits neuron death. p53 induced apoptosis activate Bax and cause neuronal apoptosis⁴¹. Bcl-2 is a distinguished anti-apoptotic protein that binds to Bax to inhibit apoptosis²⁴. p53 induced downregulation of Bcl-2 and upregulation of Bax promote cytochrome C release from the mitochondria and caspase-3 activation causes cell death^{14,35}.

In this study we have aimed to show the effects of resveratrol with the comparison results of annexin V, p53, Bcl-2 levels after ischemia in rats.

MATERIALS AND METHODS

All experimental procedures and animal care were in accordance with the guidelines of Eskisehir Osmangazi University, Faculty of Medicine. The experimental protocols used in this study were also approved by the Animal Experimental Ethic Committee of Eskisehir Osmangazi University (Date and decision number : 25.07.2013, 351).

Rat Annexin V ELISA kit (sc 4252-AK, Santa Cruz Biotechnology, TX, USA), rat p53 ELISA kit (Cloud-Clone Corp., Houston, TX, USA), rat Bcl-2 ELISA kit (Cloud-Clone Corp., Houston, TX, USA) and resveratrol (Sigma-Aldrich, St, Louis, MO, USA) were used for measuring the serum levels of these apoptotic markers.

All efforts were made to minimize animal suffering and to reduce the number of animals used. In the present study, 50 Sprague-Dawley female rats (250–300 g weight) were used. The rats were monitored at a suitable temperature ($21\pm 2^{\circ}\text{C}$) and moist environment ($60\pm 5\%$ humidity). Rats was observed in this environment for one week before starting the experiment to adapt to the environment.

The animals included in the study were divided into five groups (Table 1). Group I, Sham, no ischemia (n=10), Group II : bilateral carotid artery (BCA) occlusion for 30 min (n=10), no

resveratrol; Group III, BCA occlusion for 30 min; followed by 20 mg/kg resveratrol i.p. (n=10) followed by reperfusion for 24 h (n=10); Group IV, BCA occlusion for 30 min, followed by 40 mg/kg resveratrol i.p. (n=10); Group V, BCA occlusion for 30 min, followed by DMSO (solvent).

Experimental induction of global cerebral infarction

In this experimental study, we preferred a model of global cerebral infarction which was induced by bilateral common carotid artery occlusion method (Fig. 1)¹⁰.

Rats were anesthetized with thiopental sodium (30 mg/kg). Cervical vertebrae and the common carotid arteries were then exposed and carefully separated from the vagus nerve. These arteries were occluded for 30 min followed by reperfusion for 4 h. The rectal temperature was maintained at $37\pm 0.5^{\circ}\text{C}$ with a feedback-controlled heating pad. animals which did not lose the righting reflex or which convulsed during the ischemic episode were excluded.

Statistical analysis

Statistical analysis was performed using Sigmastat 3.5 and IBM SPSS Statistics 21. Continuous variables are expressed as mean \pm standard deviation. Relationships between Bcl-2 and p53 levels were determined using analysis of variance (ANOVA). The variables did not show normal distribution in comparison between Annexin V levels, we used Kruskal-Wallis one-way analysis of variance. As multiple comparison test annexin V levels were measured by Tukey's test. We considered a significant

Table 1. Groups of the study

Group	n
I. Sham	10
II. Ischemia	10
III. Resveratrol 40 mg/kg	10
IV. Resveratrol 20 mg/kg	10
V. Solvent (DMSO)	10

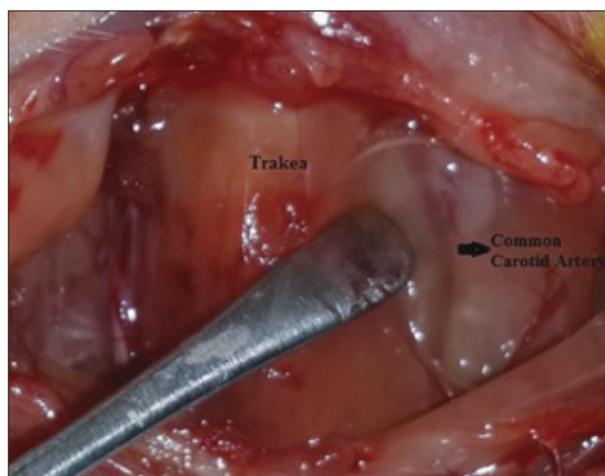


Fig. 1. Rat carotid artery.

result when $p < 0.001$.

RESULTS

After resveratrol administration, Bcl-2 levels were significantly increased when compared to the ischemia group ($p < 0.001$). Depending on the dose of resveratrol, we have observed that Bcl-2 levels increase. We have found that the solvent did not change Bcl-2 levels (Fig. 2). p53 levels were significantly increased in ischemia group, so apoptosis is higher compared to other groups. Depending on the dose of resveratrol, we observed that p53 levels decrease so the apoptosis (Fig. 3). Our data revealed that Annexin V

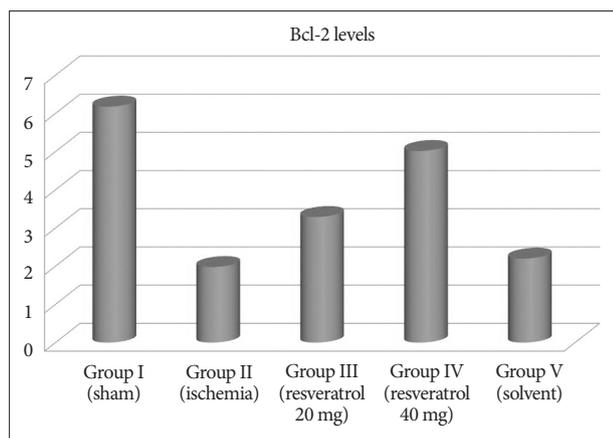


Fig. 2. Comparison of the serum Bcl 2 levels in the groups (ANOVA, $p < 0.001$).

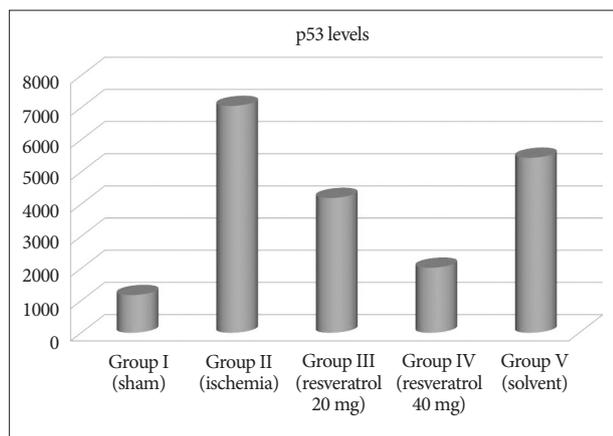


Fig. 3. Comparison of the serum p53 levels in the groups (ANOVA, $p < 0.001$).

V levels were significantly increased in ischemia group compared to other groups. However, depending on the dose of resveratrol dsaAnnexin V levels did not change (Fig. 4, Table 2).

DISCUSSION

The annual incidence of stroke around the world is 15 million, with a mortality of 5 million and permanent sequelae, disability and dependency on others of 5 million patients²³). It is estimated that the stroke rate will rise significantly in the future⁴⁵). During the stroke, several mechanisms are responsible for the neuronal injury including increased excitotoxicity, calcium overload, formation of free radicals and inhibition of protein synthesis^{33,46}). During ischemia/reperfusion, resveratrol modulate the neurotransmitters and neuromodulator protects the brain from ischemia¹⁷). Li et al.¹⁸) showed that treatment with resveratrol improve neurological function and decrease infarct volume as compared to ischemia rats. The neuroprotective mechanism of resveratrol are not clarified and further investigation must be conducted¹⁸). Apoptosis, inflammatory response, and free radical formation are responsible for the neuronal cell death. Apoptosis, a programmed cell death, activated by the cell ischemia¹). A family of proteases known as caspases plays an important role in apoptosis. There are two ways of caspase activation : cell death receptor mediated and mitochondrial. The Bcl-2 family includes both antiapoptotic proteins (Bcl-2, Bcl-xL, A1/Bfl1, Boo/Diva, Bcl-w, Mcl-1, Nrf3) and proapoptotic proteins (Bax, Bak, Bok/Mtd)¹⁷). Cytochrome C induces cell

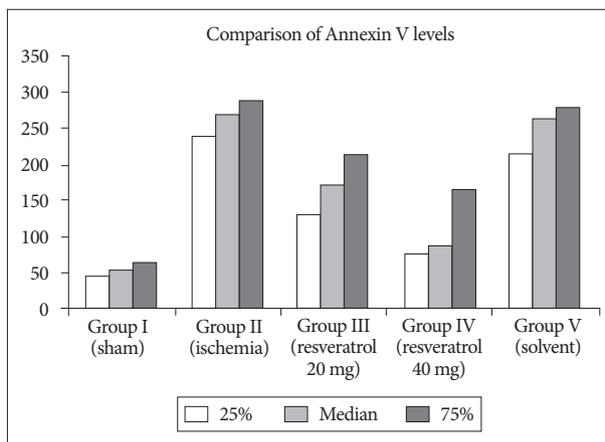


Fig. 4. Comparison of the serum Annexin V levels in the groups (Kruskal-Wallis one way, $p < 0.001$).

Table 2. Comparison of the serum Annexin V levels in the groups (Kruskal-Wallis one way, $p < 0.001$)

Groups	n	25%	Median	75%
Group I (sham)	10	45.6	54	63
Group II (ischemia)	10	239	269	289
Group III (resveratrol 40 mg/kg)	10	131	169	212
Group IV (resveratrol 20 mg/kg)	10	76	89	165
Group V [solvent (DMSO)]	10	215	262	278

death by activation of caspase 9 and 3 in the mitochondrial pathway of apoptosis⁴¹. The mitochondrial release of cytochrome C in brain ischemia is caused by proapoptotic Bax gene, but it's not the only way of apoptosis. Apoptosis can be activated in the absence of caspase activity. Apoptosis-inducing factor (AIF), which is found in the mitochondrial intermembranous space, releases as a result of absence of ATP, causing apoptosis³.

Antioxidant and anti-inflammatory agents can limit the reperfusion injury during the ischemia^{13,15,33,48}. Some research showed that resveratrol has a neuroprotective effects in rats^{29,39,47}. There is no standard treatment for the reperfusion injury. Oxidative stress and inflammation have an important role in cerebral ischemia and reperfusion injury^{15,30}. Resveratrol has a neuroprotective effect in cerebral ischemic models^{39,44}. Resveratrol can prevent a wide variety of conditions including cancer, cardiovascular, diabetes and neurological disorders^{2,25,43}. Resveratrol exerts multiple biological effects, including anti-inflammatory, antiproliferative, antiplatelet and potent antioxidant effects^{4,11}.

The present study indicates that resveratrol presents a neuroprotective effect on cerebral ischemic rats by measuring Annexin 5, p53, and bcl-2. Li et al.¹⁹ indicated that resveratrol attenuates ischemic neuronal apoptosis by up regulating bcl-2. In our study after resveratrol administration, Bcl-2 levels were significantly increased when compared to the ischemia group. Shin et al.³⁸ showed that in the ischemic brain, resveratrol modulate the proteins and protects the brain. Annexin 5 has been extensively used as an indicator of early apoptosis due to its inherent property of selectively interacting with phosphatidyl serine. The physiologic functions of Annexin 5 include suppression of blood coagulation and platelet aggregation by occlusion of exposed PS from access by coagulation factors³⁷. In our study we found that Annexin V levels were significantly increased in ischemia group compared to other groups. However, depending on the dose of resveratrol Annexin V levels did not change. Resveratrol affects the activation of a variety of intracellular signal pathways. Molchadsky et al.²⁷ showed that resveratrol induces p53 activation in vascular smooth muscle cells (VSMCs). Unlike Molchadsky et al.²⁷, Zheng et al.⁵⁰ showed that p53 decreased myocardin expression; hence inhibited differentiation from mesenchymal cells to smooth muscle cells. We observed that p53 levels decreases after the administration of resveratrol. Orsu et al.³⁰ indicated that resveratrol showed significant cerebroprotective action mediated by anti-oxidant and anti-inflammatory mechanisms. Lu et al.²² suggest that resveratrol prepared by RES loaded nanoparticles shows enhanced neuron protection against oxidative stress in cultured neurons exposed to H₂O₂ both in a short time and in longer time treatment over bax and bcl 2 genes.

In conclusion, we suggest that resveratrol is a natural drug with minimal side effects and it might be effective medical therapy for the treatment of acute ischemic stroke.

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