Research Article

Research Into The Acute Effects of Resveratrol on The Traumatic Brain Injury Model in Immatur Rats

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Summary

Aim: Traumatic brain injury (TBI) is the leading cause of death and disability in childhood. The aim of the present study is to determine the acute effects of resveratrol (Res) in the hippocampus and parietal cortex in 7-day-old rat pups subjected to contusion injury.

Methods: 28 pups were randomly allocated into four groups (per group, n=7): control group (1), TBI group (2), TBI+50 mg/ kg resveratrol group (3) and TBI+100 mg/ kg resveratrol group (4). Single doses of resveratrol were injected intraperitoneally immediately after the induction of TBI. The damage was examined by cresyl violet staining, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and active caspase-3 assay 24 h after trauma.

Results: Treatment with resveratrol significantly ameliorated the trauma induced neuron loss at the ipsi- and contralateral hippocampal brain regions and parietal cortex of rats, other than the CA2 and CA3 regions of the hippocampus in the TBI + 50 mg/ kg resveratrol group. Both doses of resveratrol treatment significantly decreased the number of TUNEL-positive and active caspas-3-positive cells in all ipsi- and contralateral hippocampus and parietal cortices. A significant difference was found between Resveratrol 50 and Resveratrol 100 mg/kg in all regions p(< 0.01).

Conclusions: In conclusion, Resveratrol 100 mg/kg especially may be proposed as a highly promising agent for preventing the unfavorable outcomes of traumatic brain damage in young children.

Key words: Resveratrol, Traumatic brain injury, Apoptosis, Hippocampus, Immature rats

Özet

Amaç: Travmatik beyin hasarı (TBH) çocukluk çağı ölüm ve sakatlıklarının önde gelen nedenidir. Bu çalışmanın amacı, resveratrol kullanımının kontuzyon hasarı oluşturulan 7 günlük şişan hipokampus ve parietal kortekstinde akut etkilerini belirlemektir.

Method: 28 şişan yavrusu rastgele dört gruba ayrıldı(her grupta n=7) kontrol grub (1), TBH grub (2), TBH+50 mg/ kg resveratrol grub (3) and TBH+100 mg/ kg resveratrol grub (4). Resveratrol, TBH uygulamasından hemen sonra intraperitoneal olarak tek sefer uygulandı. Hasar, travmadan 24 saat sonra krezilвиyle, deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) ve active caspase-3 boyanmaları ile incelendi.

Bulgular: Resveratrol ile tedavi, TBH + 50 mg/ kg resveratrol grubunda, CA2 ve CA3 bölgelerine göre hipokampusun diğer bölgelerinde ve parietal kortekste travmanın neden
olduğunu nöron kaybını ipsilateral ve kontralateral olarak anlamlı derecede azalttı. Resveratrol tedavisinin her iki dozu da ipsilateral ve kontralateral hipokampus ve parietal kortekste TUNEL- pozitif ve active caspase-3- pozitif hücre sayısı anlamlı olarak azaldı. Resveratrol 50 ve Resveratrol 100 mg/kg dozları arasında tüm bölgelerde anlamlı farklılık bulundu. p(<0.01).

**Sonuç:** Sonuç olarak özellikle Resveratrol 100 mg/kg küçük çocuklarda travmatik beyin hasarının olumsuz sonuçlarını önlemek için son derece umut verici bir madde olarak önerilebilir.

**Anahtar Kelimeler:** Resveratrol, Travmatik beyin hasarı, Apopitoz, Hipokampus, Immatur rat

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**INTRODUCTION**

Traumatic brain injury (TBI) is the leading cause of death and disability during childhood(2). Clinical experience and experimental observations in animals suggest that brain damage resulting from severe head injury can be classified into primary, occurring at impact and appearing immediately or shortly after injury, and secondary, occurring distant to the impact and which may not appear until several hours after injury(1,20). It is assumed that injured neurons have a potential for recovery and that neurodegeneration triggered by traumatic impact is a dynamic and time-related process(17). According to this viewpoint, early diagnosis and medical support are crucial for the prevention of additional brain damage following head injury(16).

Two pathogenic mechanisms, excitotoxicity and apoptotic, have been defined in secondary damage. Apoptosis may be induced by extracellular or intracellular events, such as oxidative stress or excess calcium(11). The immature brain is highly vulnerable to TBI-induced apoptotic neurodegeneration(5,14). This period of developmental vulnerability corresponds to the brain growth spurt period which coincides with the first two postnatal weeks in rats(5).

Resveratrol (3,4',5-trihydroxystilbene), a natural polyphenol, is synthesized by various plants in response to injurious substances(8). It has broad physiological and pharmacological functions including anti-oxidation, anti-inflammation and inhibition of tumor growth, antiplatelet, modulation of blood lipid metabolism, and agglomeration(7,9,10).

In addition, in multiple models of neurological injury including stroke(23), spinal cord injury(12,25) and epilepsy(24), resveratrol has demonstrated efficacy in reducing neuropathological and behavioral sequelae.

It has been reported that treatment with resveratrol immediately after traumatic brain injury reduces oxidative stress and lesion volume in adult rats(3). In addition, the potential for resveratrol to provide behavioral protection, affect contusion volumes and hippocampal neuronal numbers in adult animals has also been explored(18). In existing literature there are no studies of induced traumatic brain damage on immature rats which illustrate the effects of resveratrol on the neurological degeneration in the hippocampus and parietal cortex.

We have previously demonstrated the protective effect of resveratrol following head trauma induced hippocampal damage and spatial memory deficits in immature rats 17 days after trauma(19). We planned this study to analyse the stereological and immunohistochemical impact of varying doses of resveratrol treatment on early stages of brain damage on the head trauma model in immature rats.
MATERIAL AND METHODS

1. Animals and drugs

All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylül University, School of Medicine. Wistar Albino rats with dated pregnancies were maintained at the same center and housed in individual cages with free access to water and laboratory chow. Twenty eight litters delivered spontaneously were reared with their dams until the time of experimentation at 7 days of postnatal age. All rats were maintained on a constant 12-h-light/12-h-dark cycle at constant room temperature (21 °C) and humidity (60%).

Rats were randomly allocated into four groups (per group, n= 7): (1) control group, (2) TBI group, (3) TBI + 50 mg/ kg resveratrol group and (4) TBI + 100 mg/ kg resveratrol group.

We used a modification of a well-described percussion trauma model in immature rats, in an attempt to model infant and early childhood head trauma. In this model, the severity of the trauma-triggered neurodegeneration in the brains of 3- to 30-day-old rats had been demonstrated to be age dependent and highest in 7-day-old animals. Therefore, 7-day-old rat pups were subjected to contusion injury in our study.(5)

The contusing device consisted of a hollow tube 40 cm long, perforated at 1-cm intervals to prevent air compression. The device was kept vertical to the surface of the skull and guided a falling weight onto a circular footplate, resting upon the surface of the parietal bone. The trauma was performed to the right hemisphere of parietal cortex, the dominant hemisphere. The center of the footplate was stereotaxically positioned 3 mm anterior and 2 mm lateral to the lambda and was fixed in place under ether anesthesia. A force of 160 g cm produced by a 10-g weight was selected to produce brain contusion. All the pups were kept on a heating pad until returned to their mothers at 4 h after the trauma.

Resveratrol, (Sigma Chemicals, St, Louis, MO, USA), is prepared freshly by dissolving in 50% ethanol and diluted in physiological saline (2%). A 50mg/kg and 100 mg/kg single dose of resveratrol for resveratrol groups and equal amounts of physiological saline (2%) was used in the dilution volume of Resveratrol treatment immediately after trauma(3).

Rats were sacrificed under ether anesthesia after twenty-four hours after injury. Brain tissues were removed for light microscopic estimations. All histomorphological analyses described below were performed by an investigator with no prior knowledge of the treatment groups.

2. Histomorphological evaluation

Brain tissues were fixed in 10% formalin in phosphate buffer, processed by routine histological methods, embedded in paraffin blocks and sectioned coronally into sequential 5 m sections. Each sample was subjected to estimation of neuron number by taking three consecutive coronal sections through the hippocampus (CA1,CA2, CA3 and GD regions) and parietal cortex that corresponded approximately to Plates 8, 22 and 23, respectively, in rat atlas of Paxinos and Watson(15). All sections were stained with cresyl violet for stereological and histomorphological evaluations(19).

3. Estimation of neuron density of hippocampus and parietal cortex

Images were analyzed by using a computer assisted image analyzer system consisting of a microscope (Olympus BX-51, Japan) equipped with a high-resolution video camera (Olympus DP-71, Japan). The number of neurons in CA1, CA2, CA3, gyrus dentatus of hippocampus and parietal cortex regions were counted by help of a counting frame of 15,800 µm² viewed through a 20× lens (Olympus U) at the
monitor. The counting frame was randomly placed three times on the image analyzer system monitor and the number of neurons was counted (UTHSCA Image Tool for windows, software version 3.0) and the average was taken. Hippocampal and parietal cortex neuron density was calculated.

4. In situ cell death detection

To detect DNA fragmentation, TUNEL (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany) and active caspase-3 immunohistochemistry (AB3623, Millipore, Temecula, CA) was applied to the paraffin sections. The sections were stained with diaminobenzidine solution, counterstained with hematoxylin and analyzed by using a light microscope. For quantitative measurement of the number of cells that underwent apoptosis, 1000 cells were randomly counted in these different areas, and apoptotic cell percentages were calculated.

5. Statistical analysis

All data were analyzed Kruskal–Wallis test using SPSS 15.0 for Windows. Values are presented as mean ± SD. Differences between the two groups were examined with the Mann–Whitney U-test. p < 0.05 is accepted as statistically significant.

RESULTS

1. Effects of Resveratrol Treatment on Neuronal Density

At 24 h after trauma, the density of hippocampal neurons was significantly less in both ipsi- and contra-lateral hippocampal regions (CA1, CA2, CA3, gyrus dentatus) and parietal cortex of TBI group rats in comparison with the control group (p<0.001). Res 100 mg/kg treatment significantly preserved the neurons in both the ipsi- and contralateral hippocampal CA1, CA2, CA3 and gyrus dentatus regions; and the parietal cortex was affected by TBI. Res 50 treatment significantly preserved the neurons in both the ipsi- and contralateral hippocampal CA1; the gyrus dentatus region and parietal cortex were affected by TBI (p<0.001 or p<0.05). The CA2 and CA3 regions of the hippocampus remained unaffected with the 50 mg/kg resveratrol treatment dose. A significant difference was found between Res 50 and Res 100 in all regions (p<0.001) (Table 1, 2; Fig. 1).

2. Effects of Resveratrol treatment on Apoptosis

The effect of Resveratrol treatment on DNA fragmentation and apoptotic cell death was examined using TUNEL and active caspase-3 immunostaining. When compared with the control group, both the number of TUNEL-positive and caspase-3 positive neurons increased in both the ipsi- and contralateral hippocampus and parietal cortex in the TBI group (p<0.001) but there were significantly fewer TUNEL-positive and caspase-3 positive cells in the rats treated with Res 50 mg/kg and Res 100 mg/kg (p<0.01). When varying doses were compared, a significant difference was observed between Res 50 and Res 100 in all regions (p<0.001) (Table 3, 4 and Fig.2).
Fig 1: Effects of resveratrol on neuronal density (A1-2-3-4), TUNEL (B1-2-3-4) and active caspase-3 immunoreactivity (C1-2-3-4) in the CA1 region of hippocampus. The neuronal density is significantly less in the trauma group (A2). TUNEL and caspase-3 positive cells enhanced in the trauma group (B2 and C2). Resveratrol 100 mg treatment significantly reduced the number of apoptotic neurons (B4 and C4). Arrows indicate TUNEL-positive and active caspase-3 immun-positive cells.

Fig 2: Effects of resveratrol on neuronal density (A1-2-3-4), TUNEL (B1-2-3-4) and active caspase-3 immunoreactivity (C1-2-3-4) in the parietal cortex. The neuronal density is significantly less in the trauma group (A2). TUNEL and caspase-3 positive cells enhanced in the trauma group (B2 and C2). Resveratrol 100 mg treatment significantly reduced the number of apoptotic neurons (B4 and C4).
Table 1: The effect of resveratrol on neuronal density of the hippocampus (CA1, CA2, CA3 and GD regions) and parietal cortex of rats with traumatic brain injury in the right hemisphere

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>GD</th>
<th>Parietal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control  (n=7)</td>
<td>50.02 ± 1.94*</td>
<td>34.36 ± 1.15*</td>
<td>23.02 ± 1.63*</td>
<td>62.76 ± 1.89*</td>
<td>31.02 ± 1.27*</td>
</tr>
<tr>
<td>2. Trauma (n=7)</td>
<td>32.87 ± 0.83</td>
<td>28.97 ± 1.24</td>
<td>18.45 ± 0.66</td>
<td>50.37 ± 1.53</td>
<td>22.05 ± 0.97</td>
</tr>
<tr>
<td>3. Res 50 (n=7)</td>
<td>40.40 ± 1.15*</td>
<td>33.02 ± 0.99</td>
<td>19.96 ± 1.24</td>
<td>56.40 ± 1.63*</td>
<td>25.5 ± 0.91*</td>
</tr>
<tr>
<td>4. Res 100 (n=7)</td>
<td>47.37 ± 1.33*</td>
<td>22.2 ± 4.19*</td>
<td>21.87 ± 0.83*</td>
<td>60.12 ± 0.90*</td>
<td>29.2 ± 1.71*</td>
</tr>
</tbody>
</table>

p values:
1 vs 2 <0.001 <0.001 <0.001 <0.001 <0.001
2 vs 3 <0.001 <0.001 0.141 0.132 <0.001
2 vs 4 <0.001 <0.001 <0.001 <0.001 <0.001
3 vs 4 <0.001 <0.001 <0.001 0.031 0.001

The values are presented as Mean ± SD
* p < 0.001 compared with the trauma group

Table 2: The effect of resveratrol on neuronal density of the hippocampus (CA1, CA2, CA3 and GD regions) and parietal cortex of rats with traumatic brain injury in the left hemisphere

<table>
<thead>
<tr>
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<th>GD</th>
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</thead>
<tbody>
<tr>
<td>1. Control  (n=7)</td>
<td>50.61 ± 2.27**</td>
<td>34.45 ± 1.72**</td>
<td>23.56 ± 2.08**</td>
<td>62.78 ± 2.55**</td>
<td>32.18 ± 0.95**</td>
</tr>
<tr>
<td>2. Trauma (n=7)</td>
<td>33.62 ± 1.45</td>
<td>29.08 ± 1.85</td>
<td>18.86 ± 1.38</td>
<td>50.82 ± 1.41</td>
<td>21.48 ± 1.17</td>
</tr>
<tr>
<td>3. Res 50 (n=7)</td>
<td>40.83 ± 2.48**</td>
<td>31.16 ± 2.85</td>
<td>19.50 ± 1.48</td>
<td>55.30 ± 0.72**</td>
<td>24.16 ± 1.74*</td>
</tr>
<tr>
<td>4. Res 100 (n=7)</td>
<td>46.20 ± 1.99**</td>
<td>33.37 ± 1.22*</td>
<td>21.02 ± 0.89*</td>
<td>58.12 ± 1.18**</td>
<td>29.57 ± 1.51**</td>
</tr>
</tbody>
</table>

p values:
1 vs 2 <0.001 <0.001 <0.001 <0.001 <0.001
2 vs 3 <0.001 0.382 1.0 <0.001 0.008
2 vs 4 <0.001 <0.001 0.019 <0.001 <0.001
3 vs 4 <0.001 <0.001 0.414 0.020 <0.001

The values are presented as Mean ± SD
* p < 0.05 compared with the trauma group
** p < 0.001 compared with the trauma group

Table 3: The effect of resveratrol on TUNEL and Caspase-3 positive cell of hippocampus and parietal cortex of rats with traumatic brain injury in the right hemisphere

<table>
<thead>
<tr>
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<th>TUNEL-Positive Cell (%)</th>
<th>Caspase-3 (%)</th>
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<tr>
<td></td>
<td>Hippocampus</td>
<td>Parietal cortex</td>
</tr>
<tr>
<td>1. Control  (n=7)</td>
<td>1.78 ± 0.19**</td>
<td>4.52 ± 0.22**</td>
</tr>
<tr>
<td>2. Trauma (n=7)</td>
<td>12.15 ± 0.18</td>
<td>12.22 ± 0.89</td>
</tr>
<tr>
<td>3. Res 50 (n=7)</td>
<td>7.36 ± 0.41**</td>
<td>3.86 ± 0.24**</td>
</tr>
<tr>
<td>4. Res 100 (n=7)</td>
<td>4.40 ± 0.50**</td>
<td>5.77 ± 0.32**</td>
</tr>
</tbody>
</table>

p values:
1 vs 2 <0.001 <0.001 <0.001 <0.001 <0.001
2 vs 3 <0.001 <0.001 <0.001 <0.001 <0.001
2 vs 4 <0.001 <0.001 <0.001 <0.001 <0.001
3 vs 4 <0.001 <0.001 <0.001 <0.001 <0.001

The values are presented as Mean ± SD
* p < 0.05 compared with the trauma group
** p < 0.001 compared with the trauma group
The major findings of this study are as follows: 1) This is the first study implementing the head trauma model in the research of the stereological and immunohistochemical effects of resveratrol treatment in early brain damage in maturing rats. 2) This is the first time varying doses of Resveratrol were used and compared while implementing the head trauma model in immature rats. 3) The application of a single dose of 100 mg/kg resveratrol was significantly more effective than a dose of 50 mg/kg resveratrol.

An important consideration of hippocampal damage following head trauma is the unique pattern of neuron death.(13) Tong et al. (21) demonstrated that neuronal injury was apparent in the ipsilateral cortex, hippocampal CA2/CA3 regions, and the dentate gyrus within 24 h after controlled cortical impact in mice at postnatal day 21. However, in our previous study, we observed that neuronal loss following trauma is not limited to the ipsilateral hippocampal regions but also occurs in contralateral hippocampal areas by 24 h after trauma.(14) In the present study, we observed that following TBI, neuron numbers decreased by 24 hr after trauma in all ipsi- and contralateral hippocampal regions and the parietal cortex of 7-day old rats exposed to contusion injury. Neuronal injury in the cortex and hippocampus has been attributed to direct mechanical damage, excitotoxicity, and oxidative injury after traumatic brain injury.(21)

Apoptosis, plays a major role in the mechanism of traumatic injury in the immature brain and is very severe in the brains of 7-day-old rats(4). Apoptotic cell death is highest 24 hours after trauma and continues for 7 more days. The amount of apoptosis necessary for physiological brain development is determined by the degree of myelinization and the water content of the brain(5). The cortex and hippocampus are predominantly affected in head trauma(21). In the present study, the number of TUNEL-positive and active caspase-3-positive cells in all ipsi- and contralateral hippocampal regions and parietal cortex also increased significantly following traumatic insult, indicating that neuronal apoptosis was induced by head trauma in the immature rat brains.

### DISCUSSION

**Table 1: The effect of resveratrol on TUNEL and Caspase-3 positive cell of hippocampus and parietal cortex of rats with traumatic brain injury in the left hemisphere**

<table>
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<th>TUNEL-Positive Cell (%)</th>
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<tr>
<td></td>
<td>Hippocampus</td>
<td>Parietal cortex</td>
</tr>
<tr>
<td>1. Control (n=7)</td>
<td>1.51 ± 0.13**</td>
<td>4.61 ± 0.15**</td>
</tr>
<tr>
<td>2. Trauma (n=7)</td>
<td>11.10 ± 0.80</td>
<td>11.54 ± 1.11</td>
</tr>
<tr>
<td>3. Res 50 (n=7)</td>
<td>7.93 ± 0.54**</td>
<td>8.02 ± 1.13**</td>
</tr>
<tr>
<td>4. Res 100 (n=7)</td>
<td>4.35 ± 0.37**</td>
<td>5.40 ± 0.40**</td>
</tr>
</tbody>
</table>

**p values**

1 vs 2 < 0.001,< 0.001,< 0.001,< 0.001
2 vs 3 < 0.001,< 0.001,< 0.001,< 0.001
2 vs 4 < 0.001,< 0.001,< 0.001,< 0.001
3 vs 4 < 0.001,< 0.001,< 0.001,< 0.001

* p < 0.05 compared with the trauma group
| p < 0.001 compared with the trauma group
Recently, resveratrol (3,4′,5-trihydroxystilbene), a natural polyphenol, was found to be a potent neuroprotective agent against stroke (23), spinal cord injury (12,25), and epilepsy (24). Resveratrol was found to be neuroprotective against traumatic injury in adult animals (3,18). We have previously demonstrated that the administration of resveratrol immediately upon trauma ameliorated the histopathological and behavioral consequences of trauma in immature rats 17 days after trauma (19). In the present study, Resveratrol treatment significantly reduced hippocampal and cortical damage at 24 h after injury.

Doses in the range of hundreds of mg to several g per day have been proposed based on animal studies, but it is clear more human studies are needed to confirm these estimates (22). Changjiang et al. reported that in a rat spinal cord injury model, Resveratrol improved neuron protection and functional recovery in the rat group treated with resveratrol 200 mg/kg, i.p. three times per day for 3 days after the injury (6). Kıztıltepe et al. (12) reported that resveratrol decreases oxidative stress, increases NO release, and protects the spinal cord from I/R injury 1 mg/kg and 10 mg/kg. Singleton et al. (18) used 100mg/kg and studied the contusion volumes and hippocampal neuronal numbers in adult animals on 21 days after trauma. In the present study, we investigated whether a 50 or 100 mg/kg single dose of resveratrol given immediately following contusion injury in 7-day-old rat pups would reduce hippocampal and cortical damage at 24 h after injury. There was a statistically significant difference between the doses of 50 and 100 mg/kg of resveratrol in terms of preventing acute hippocampal and cortical damage.

Although the mechanisms by which resveratrol mediates its neuroprotection is unclear, the current study adds to the growing literature identifying resveratrol as a potential treatment in terms of preventing acute hippocampal and cortical damage.

CONCLUSION
In conclusion, a single administration of resveratrol immediately after traumatic insult prevents acute hippocampal and cortical neuron loss in the developing brains of rats. Therefore, Resveratrol 100 mg/kg especially may be proposed as a highly promising agent for preventing the unfavorable outcomes of traumatic brain damage in young children.

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