The effects of caffeic acid phenethyl ester (CAPE) on spinal cord ischemia/reperfusion injury in rabbits

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Abstract

Objective: Oxygen-derived free radicals have been implicated in the pathogenesis of spinal cord neuronal injury after both trauma and ischemia-reperfusion. Caffeic acid phenethyl ester (CAPE), an active component of propolis extract, exhibits antioxidant properties. This experimental study was designed to determine the effect of CAPE on ischemia-reperfusion of spinal cord in rabbits.

Methods: Forty-one New Zealand white rabbits were used in the study. The animals undergone aortic occlusion were divided into three groups each consisting of 11 rabbits: methylprednisolone (MP), CAPE, and control. CAPE 10 \textmu\text{mol/kg}, methyl prednisolone (MP) 30 mg/kg or similar dose saline were injected intraperitoneally before surgical intervention. Animals were subjected to 21 min of cross-clamp time. At the end of occlusion time, the clamps were removed and restoration of the blood flow was verified visually. Animals in sham group (n = 8) underwent a surgical procedure similar to the other groups but the aorta was not occluded. Neurological status was scored by assessment of hindlimb motor function deficit.

Results: The scores in CAPE group was different from control groups at 48 h (3.91 ± 0.5 vs. 2.91 ± 0.7; \( P = 0.0013 \)). Spinal cord specimens were obtained to determine the tissue levels of malondialdehyde, superoxide dismutase, catalase, and histological changes. Malondialdehyde levels in control group were increased significantly when compared to sham group (124 ± 22\textsuperscript{nmol/g wet tissue}, 41 ± 9\textsuperscript{nmol/g wet tissue}, \( P = 0.0003 \)). MDA levels in the CAPE group were lower than MP group and differences between the two groups were statistically significant (56 ± 15.265 and 107 ± 19.31\textsuperscript{nmol/g wet tissue}, \( P = 0.0001 \)). We did not observe additional tissue injury in CAPE group when compared to control group. SOD and CAT activities were not concordant in all the groups.

Conclusions: These results suggest that CAPE may be an available agent to protect the spinal cord from ischemia-reperfusion injury. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Caffeic acid phenethyl ester; Methylprednisolone; Spinal cord ischemia; Reperfusion injury

1. Introduction

Paraplegia is the most serious and not infrequent complication, with an incidence rate ranging from 4 to 33\%, after operations on the descending thoracic and thoracoabdominal aorta, despite improvements in anesthetic, surgical and perfusion techniques [1]. It is believed that the cause of spinal cord dysfunction is ischemia from hypoperfusion during cross-clamping. Injury to the gray matter of the spinal cord, with neuronal death, has generally been considered an important element in the pathology of spinal cord ischemic injury.

Caffeic acid phenethyl ester (CAPE), an active component of propolis extract, inhibits 5-lipoxygenase-catalyzed oxygenation of linoleic acid and arachidonic acid in the micromolar concentration range. At a concentration of 10 \mu\text{M}, it completely blocks production of reactive oxygen species (ROS) in human neutrophils and the xanthine/xanthine oxidase system [2]. Previous studies have demonstrated that CAPE also exhibits antioxidant property as well as anti-inflammatory, cytostatic, antiviral, antibacterial and antifungal properties [3,4]. The objective of our study was to investigate the effects of CAPE on histopathological changes, antioxidant status, lipid peroxidation, and neurologic recovery in temporary induced spinal cord ischemia in...
rabbits. We also intended to compare the effects of CAPE with methylprednisolone (MP).

2. Materials and methods

2.1. Synthesis of CAPE

Caffeic acid phenethyl ester was synthesized in the Physico-Chemistry Laboratory using the technique described by Grunberger and a prepared 25 µmol/ml of CAPE solution [5].

2.2. Animal care and surgical technique

Forty-one New Zealand white rabbits, each weighing 2–3 kg (mean 2.8 kg) were used in the study. Animal care and experiments complied with the ‘Principles of Laboratory Animal Care’ and the ‘Guide for the Care and Use of Laboratory Animals’ (NIH Publication 86-23, revised 1985) and was approved by Inonu University, School of Medicine, Animal Studies Committee on the care and use of laboratory animals.

The animals were fasted for 12 h and operated on in a room kept at 24°C. They were anesthetized with intramuscular ketamine with an initial dose of 50 mg/kg and xylazine 3 mg/kg, followed by 25 mg/kg fractionally as need during the procedure. The animals were allowed to breathe room air without mechanical ventilation. Body temperature maintained close to 38°C using a thermostatically controlled heated operation table. An intravenous catheter (24 gauge) was placed in an ear vein, and preoperatively cefazoline 10 mg/kg was administered as a single dose. Maintenance fluid of 0.9% NaCl was infused at a rate of 20 ml/h during the procedure. The animals were placed in the supine position with the pelvis partially rotated to the right. After the surgical preparation, the vertical incision was made from the left costal margin directed towards the pubis. The abdominal aorta was exposed through a retroperitoneal approach and mobilized from just inferior to the left renal vein down to the aortic bifurcation. Heparin (100 U/kg) was administered intravenously 5 min before aortic occlusion. The animals undergone aortic occlusion were divided into three groups each consisting of 11 rabbits. In MP group, 30 mg/kg methylprednisolone acetate (Depo-Medrol, Eczacibasi); in CAPE group, CAPE 10 µmol/kg (from 25 µmol/ml solution); in control group, a similar volume of sterile saline solution were injected intraperitoneally 30 min before the aortic occlusion. Group MP, CAPE and control animals underwent surgical procedures as described above and spinal cord ischemia was induced with clamping the aorta just below the renal vein with a bulldog clamp (FB328). A second similar clamp was placed above the aortic bifurcation for occluding iliac collateral circulation. Animals were subjected to 21 min of cross-clamp time. At the end of occlusion time, the clamps were removed and restoration of the blood flow was verified visually. Animals in sham group (n = 8) underwent a surgical procedure similar to the other groups but the aorta was not occluded. This group of animals was used for eliciting the effects of anesthesia and operation on results and also determining the biochemical parameters studied in the normal spinal cord tissue. The catheters were removed and the incisions closed. When the animals awakened from anesthesia, they were returned to their cages.

2.3. Evaluation of neurologic status

Neurologic status of animals was assessed blindly by two neurologists at 6, 12, 24 and 48 h. Crede’s maneuver was used for evacuation of the urinary bladder when necessary. Neurological status was scored by assessment of hindlimb motor function deficit. A score of 0 to 5 was assigned to each animal, as follows:

- Score 0 = No voluntary hindlimb function
- Score 1 = Movements of joints perceptible
- Score 2 = Active movement but able to sit with assistance
- Score 3 = Sit without assistance
- Score 4 = Weak hop
- Score 5 = Normal hop

2.4. Histopathology and biochemical analyses

After the last neurological examination at 48 h postoperation, the animals were anaesthetized with the use of ketamine (50 mg/kg) followed by transcardially perfusion of 1 l cold 0.9% NaCl. A pathological evaluation was carried out in the spinal cords and abdominal aorta. The entire spinal column and abdominal aorta with lumbar arteries was removed. For biochemical analysis, spinal cords were used in all rabbits from each group. Distal parts of the specimens were fixed in 10% buffered formalin for about 10 days before being set in paraffin blocks for sectioning. Five-micrometer sections were cut from paraffin-embedded blocks and the sections were taken to slides for hematoxylin and eosin staining (H&E). Abdominal aorta and its branches were examined for revealing possible thrombosis or embolic occlusion.

After spinal cord tissues were obtained, they were kept at −30°C until analysis (about 3 days). Tissues were weighed and homogenized in Tris–HCl buffer (pH 7.4, 50 mM) containing 0.50 ml/l Triton X-100 with a homogeniser (Tempest Virtishear, Model 278069, The Virtis Company, Inc., Gardiner, NY) and then centrifuged at 5000 × g for 30 min to remove debris. For a further extraction procedure, the supernatant was extracted in alcohol/chloroform mixture (5/3, v/v). After a second centrifugation at 5000 × g for 60 min, clear upper layer was taken and used in the enzymatic assays. All procedures were performed at +4°C. Malondialdehyde (MDA) levels were determined in the homogenate, catalase (CAT) in supernatant, and total superoxide dismu-
tase (SOD) in the extracted samples. Protein measurements were made in all stages.

Protein concentrations were determined according to Lowry's method [6]. SOD activity was measured by reduction of nitrobluetetrazolium (NBT) by xanthine-xanthine oxidase system, which is a superoxide generator. Enzyme activity leading to 50% inhibition was accepted as one unit. Results were expressed as U/mg protein [7]. CAT activity was determined according to Aebi [8]. The principle of the CAT activity was based on the determination of the rate constant ($k_{s^{-1}}$) or the hydrogen peroxide decomposition rate at 240 nm. Results were expressed as $k$/g protein.

Tissue MDA levels were determined by the method described by Wasowicz et al. [9]. Briefly, MDA was reacted with thiobarbituric acid by incubating for 1 h at 95–100°C. Following the reaction, fluorescence intensity was measured in the n-butanol phase with a fluorescence spectrophotometer (Hitachi, Model F-4010) (excitation at 525 nm, emission at 547 nm), by comparing with a standard solution of 1,1,3,3-tetramethoxypropane. Results were expressed in terms of nmol/g wet tissue.

2.5. Statistical analysis

Non-parametric analyses with Mann–Whitney $U$-test were performed on the data of the physiological and biochemical variables. $P$-values less than 0.0083 (0.05/6) were considered significant. Motor deficit scores of animals were compared using repeated measures analysis of variance (ANOVA) with the statistical significance of each comparison adjusted for the multiple comparisons using a Bonferroni correction. A value of $P$ less than 0.002 (0.05/24) was considered significant. Fisher exact probability test was used in the analysis of bladder and bowel function. Correlations among the biochemical parameters in each groups were tested for with Spearman’s test. Data are expressed as mean ± SD. All statistical analyses were carried out using SPSS statistical software (SPSS for Windows; Chicago, IL).

3. Results

3.1. Physiological variables and neurological outcome

Statistical analysis between the four groups for the various physiologic variables (mean levels of blood glucose, arterial blood gases, pH, body temperatures) did not disclose differences. Also, there was no difference in the loss of bowel and bladder sphincter control between the three groups. All rabbits survived without major complications. Twenty-one minutes of ischemia resulted in severe motor deficit in the hind limbs of all animals, while all sham animals maintained normal motor behavior (score of 5), as assessed by the motor deficit score (Fig. 1). Pretreatment with MP or CAPE did not prevent the development of paralysis. Most of animals in CAPE and MP groups exhibited score 3 or score 4 motor function at 24 h. In the follow-up period, there was mild recovery of the neurologic function of animals in both groups. The recovery was more pronounced in the CAPE group. The scores in CAPE group was different from control group at the 48th h (3.91 ± 0.5 vs. 2.91 ± 0.7; $P = 0.0013$).

3.2. Histopathology

Histopathologic analysis of the H&E-stained sections from the lumbosacral segments of the MP, CAPE and control groups revealed changes consistent with ischemic injury. The sham rabbits showed no signs of neuronal damage, with many large motor neurons in anterior horn (Fig. 2). Animals in control group with complete paralysis (score 0) had total destruction of the motor neurons with intensely eosinophilic cytoplasm, Nissl granule loss, and pronounced vacuolization of the anterior horn, as well as the presence of infiltrating neutrophils and mononuclear phagocytes. Although spinal cords from rabbits in CAPE group that scored 3 to 4 exhibited mild degrees of destruction such as the perikaryon becoming shrunken, the animals in MP group exhibited moderate changes such as marked

Fig. 1. Motor deficit score in the control, sham, methylprednisolone (MP) and CAPE groups during the 48-h follow-up period. The scores in CAPE group were different from the control group at the 48th h (3.91 ± 0.5 vs. 2.91 ± 0.7; $P = 0.0013$).

Fig. 2. Cross-section of anterior horn of rabbit spinal cord in sham group (score 5, lumbosacral segment, $\times$ 100 H&E).
triangular shape, and Nissl granule loss in some motor neurons (Fig. 3). A significant increase in neutrophils was not noted in any of the sections in both CAPE and MP groups. In addition, histologic examination of abdominal aorta and its branches were normal and reveal no thrombus formation in all animals.

3.3. Biochemical analysis

Malondialdehyde levels in control group were increased significantly when compared to sham group (124.22 ± 24.36 and 41.92 ± 10.08 nmol/g wet tissue, $P = 0.0003$) (Table 1). MDA levels in the CAPE group were lower than MP group and differences between the two groups were statistically significant (56.77 ± 15.265 and 107.74 ± 19.31 nmol/g wet tissue, $P = 0.0001$). Although SOD activity in the control group was higher than those of other three groups, CAT activity was lower and these differences were statistically significant (Table 1). According to the intracorrelation analyses, there was a negative correlation between SOD activity and MDA levels in sham group ($r = -0.690$, $P = 0.029$). On the other hand, there was a positive correlation between CAT and SOD activities in CAPE group ($r = 0.563$, $P = 0.035$).

4. Discussion

Ischemic spinal cord injury represents the main complication in surgical repair of thoracic and thoracoabdominal aneurisms and remains a persistent clinical problem. To date, numerous clinical and laboratory studies in attempt to decrease the risk of this devastating complication have been reported. The use of adjunctive shunts, partial cardiopulmonary bypass, or left atrium-femoral artery bypass to maintain spinal cord perfusion during aortic cross-clamping has many advocates [10,11]. Methylprednisolone, a glucocorticoid, is the only drug proven to be effective in improving neurologic function after traumatic spinal cord injury [12]. Although the beneficial effect of MP in reducing the incidence or the severity of ischemic spinal cord injury in humans remains unproven, the drug is administered to patients by some surgeons before cross-clamping of the aorta during operations on the descending thoracic and thoracoabdominal aorta [13].

We hypothesized that CAPE would effectively protect spinal cord by its antioxidant and antiinflammatory effects on reperfusion-induced injury. To our knowledge, this is the first study to adapt these effects of CAPE in an attempt to prevent reperfusion-induced injury. Our results demonstrate that CAPE will be able to reduce the damage to the rabbit spinal cord of reperfusion-induced injury. This result was verified by both biochemical (MDA levels), histological and neurological observations.

Infrarenal aortic occlusion in rabbits is a reliable model for systematically and rapidly observing the protective effects of investigated agents on ischemia and reperfusion injury. However, the abdominal aorta ligation in rabbits does not produce complete ischemia in the spinal cord. Therefore, we used a second clamp to distal abdominal
H2O2, and H2O2, are normally produced by the mitochondria and in generation shows a biphasic pattern ([17,18]. It was recently shown that extracellular ROS ultimate extend of ischemic injury like other researchers, try to extend the tissue damage resulting from spinal ischemia activates a cascade of events which represents an inflammatory response and the invading leukocytes contribute to the tissue damage. Inhibitions of this inflammatory cells have been shown to improve neurological function [20]. In the present study, although neutrophil leukocyte or macrophage infiltration were noted in the control group, there was no leukocyte sequestration in the both CAPE and MP groups. Furthermore, it has recently been proposed that microglia produce various cytotoxic mediators such as ROS and inflammatory cytokines [interleukin-1,6 (IL-1, IL-6), tumor necrosis factor-α (TNF-α)], playing an important role in ischemia-reperfusion injury [21]. Natarajan et al. showed that the activation of NF-kappa B by TNF is completely blocked by CAPE in a dose-and time-dependent manner [22]. Besides TNF, CAPE also inhibited NF-kappa B activation induced by other inflammatory agents including hydrogen peroxide, phorbol ester, ceramide, and okadaic acid. Thus, they demonstrated that CAPE is a potent and a specific inhibitor of NF-kappa B activation and this may provide the molecular basis for its multiple immunomodulatory and anti-inflammatory activities.

Under ischemia-reperfusion and hypoxia-reoxygenation conditions, the imbalance between restoration of oxygen supply and mitochondrial respiratory function results in the massive generation of O₂⁻ in mitochondria. Under these conditions, defensive system, such as manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase, cannot prevent the escape of ROS from mitochondria, and their effects on other intracellular sites [16]. Superoxide dismutases are protective enzymes that can efficiently and specifically scavenge the superoxide radical by catalyzing its dismutation to hydrogen peroxide and oxygen [23]. Another enzyme, CAT, acts to decompose hydrogen peroxide to water and molecular oxygen. Overexpression of CuZn-SOD reduces the infarct volume due to transient focal ischemia in transgenic mice [24]. According to our results, SOD activity in control group was higher than sham and other groups. In the light of these findings, it might be suggested that spinal cord tissue enhance its anti-

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<th>MDA (nmol/g wet tissue)</th>
<th>SOD (units/mg protein)</th>
<th>CAT (k/g protein)</th>
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<tr>
<td>I. MP (n = 11)</td>
<td>107.74 ± 19.31</td>
<td>0.638 ± 0.026</td>
<td>5.942 ± 0.693</td>
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<td>II. CAPE (n = 11)</td>
<td>56.77 ± 15.26</td>
<td>0.600 ± 0.023</td>
<td>4.404 ± 0.430</td>
</tr>
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<td>III. Control (n = 11)</td>
<td>124.22 ± 24.36</td>
<td>0.723 ± 0.030</td>
<td>2.511 ± 0.343</td>
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<tr>
<td>IV. SHAM (n = 8)</td>
<td>41.92 ± 10.08</td>
<td>0.640 ± 0.024</td>
<td>4.601 ± 0.418</td>
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<td>I-II</td>
<td>0.0001</td>
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*NS, non significant.*

The tissue damage resulting from spinal ischemia activates a cascade of events which represents an inflammatory response that occurs independently of any improvement in spinal cord reoxygenation. We propose that the inflammatory response and the invading leukocytes contribute to the ultimate extent of ischemic injury like other researchers [17,18]. It was recently shown that extracellular ROS generation shows a biphasic pattern (<1 h and >24 h) after reperfusion following ischemia, and that circulating and infiltrating leukocytes are involved in both the first and second phase of ROS production [19]. Therefore, such leucocyte-induced inflammatory responses during reperfusion may contribute to tissue damage. In the progression of neuronal death are complex. While early reperfusion can limit the extent of necrosis, reperfusion may also exert a variety of potentially deleterious effects that are collectively described as reperfusion injury. However, the precise sequence of intracellular events during reperfusion is poorly understood, and in some enzymatic reactions, such as those catalyzed by xanthine oxidase and cyclo-oxidase. During ischemia, adenosine triphosphates (ATP) are degraded to hypoxanthine and xanthine dehydrogenase is converted to xanthine oxidase (XO). When abundant amounts of oxygen are delivered to ischemic tissues, XO catalyzes the conversion of hypoxanthine to uric acid with release of the superoxide radical anions (O₂⁻). Toxic products of XO reaction including superoxide, hydroxyl radical, and hydrogen peroxide are generated in quantities that overwhelm the capacity of endogenous free radical scavengers and inflict significant injury on the previously ischemic tissues [16]. Prime targets of ROS attack are the polyunsaturated fatty acids (PUFA) in the membrane lipids causing lipid peroxidation which may lead to disorganization of cell structure and function. Further, decomposition of peroxidized lipids yields a wide variety of end products, including MDA.

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oxidant enzyme capacity by overexpression or some covalent modifications. In the CAPE group, there was no increase in SOD activity compared to both control and MP groups. This suggests that CAPE has acted in parallel with SOD enzyme and has diminished free oxygen radical formation. This action may be explained with the inhibition of polymorphonuclear leukocyte infiltration by CAPE in the ischemic tissue.

In our study, marked decrease of antioxidant enzyme, CAT, after reperfusion means that these protein structures were degraded in combating with antioxidant attack developed during reperfusion. The effect of CAPE on these enzymes is not clear. It has been proposed that during excessive production of free radicals, CAT and other antioxidant enzymes are inactivated by at least one of the oxygen reactive species [25]. These enzymes protect each other from inactivation either directly or indirectly. To our opinion, SOD seems to be protected by CAT.

In conclusion, the results suggest that CAPE reduces ischemic and reperfusion damage in transient spinal cord ischemia and provide better neurologic outcome, and MP is not a potent scavenger compared to CAPE. We also believe that it decreases ischemic injury in spinal cord by scavenging free radicals and provides better microcirculatory environment during reperfusion by preventing endothelial cell lysis by proteases from activated leukocytes. In addition, CAPE may be beneficial in humans as it has no known harmful effects on normal cells. Further studies are needed to define biochemical aspects of these events and to determine the correct dose necessary for maximal benefit.

References