The protective cardiac effects of B-myrcene after global cerebral ıschemia/reperfusion in C57BL/J6 mouse[1]

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ABSTRACT

PURPOSE: To investigate the protective effect of β-myrcene (MYR) on oxidative and histological damage in mice heart tissue caused global cerebral ischemia/reperfusion (IR) in C57BL/J6 mice.

METHODS: Animals(n=40) were randomly divided into four groups: (1)control, (2)IR, (3)MYR and (4)MYR+IR. The control group was received 0.1% carboxymethyl cellulose as a vehicle following a medial incision without carotid occlusion. In the IR group, the bilateral carotid arteries were clipped for 15min, and treated with the vehicle intraperitoneally(ip) for 10 days. MYR (200mg/kg) was received dissolved in 0.1%CMC for 10 days. In the MYR+IR group, the IR model was applied exactly as in the IR group, and then they were treated with MYR 10 days.

RESULTS: The cerebral IR caused oxidative damage (increase TBARS, decrease antioxidant parameters). Treatment of MYR was increased in GSH,GPx,CAT,SOD activity while TBARS level was decreased. In addition, degenerative changes in I/R group heart tissue were ameliorated by MYR administration.

CONCLUSİON: The administration of β-myrcene protects oxidative and histological damage in the heart tissue after global ischemia-reperfusion and may be useful safe alternative treatment for cardiac tissue after ischemic stroke.

Key words: Brain Ischemia. Reperfusion. Oxidative Stress. Mice.

Introduction

Stroke is the third most common cause of death in most industrialized countries[1]. Global cerebral ischemia occurs when the blood supply to the entire brain or a large part of the brain is disrupted, resulting in the tissue deprivation of oxygen and glucose that may give tissue damage[2]. In adult humans, global cerebral ischemic injury occurs in some conditions like cardiac arrest, coronary artery bypass surgery, cardio-respiratory failure, and others leading to the drastic reduction of the blood flow to the brain[3]. Ischemia/reperfusion (IR) injury is a complex disorder and produces a series of molecular and cellular changes such as reduction-oxidative damage, molecular inflammatory dysfunction, high-energy depletion, and ion imbalances that culminate in a severe pathological state directly related to the intensity of the injury[4]. However, a therapeutic strategy for cerebral IR injury has not been established yet[5]. Among these, cerebral IR in particular are responsible for oxidative stress due to the generation of free radicals[6], which culminates into deleterious effects during pathogenesis[7]. During reperfusion after ischemia, while restoration of oxygen and glucose supply reinstates the oxidative phosphorylation that helps to normalize energy demanding physiologic processes, a parallel cascade of deleterious biochemical processes can be triggered that may paradoxically antagonize the beneficial effect of reperfusion[8]. This phenomenon has been demonstrated in various tissues, especially in the heart, and has been termed reperfusion injury[9].

β-myrcene (7-methyl-3-methylene-1,6-octadiene) is a monoterpene that is found in many plants including hops, verbena and lemon grass[10]. It is also produced commercially in high volume for use in essential oils as intermediates in the production of terpene alcohols. β -myrcene is therefore widely used as a flavoring additive in the production of food and soft beverages, in alcoholic beverages, and in the manufacture of cosmetics, soaps, and detergents[11]. It has many pharmacological actions including analgesic, antiiflammatory and antioxidant effects[10-14]. It was thought that MYR is a strong antioxidative agent and with this property it can be prevents oxidative and histological damage in tissue. Therefore MYR were selected in this study for treatment of cardiac damage after stroke.

Thus, the current study investigated that MYR treatment ameliorates the oxidative and histological heart damage after global cerebral IR in C57BL/6 J mice.

Methods

The present study was approved by the Ethical Committee on Animal Research of Inonu University and carried out in accordance with the Guidelines for Animal Research of the National Institutes of Health (NIH).

C57BL/6J male mice (clean grade), weighting 18-22g, were supplied by the Inonu University Laboratory Animals Research Center (Malatya, Turkey), housed in sterilized polypropylene cages, and given an *ad libitum* diet of standard commercial food pellets and water. All mice were kept under a 12-h light/dark cycle at an ambient temperature of 21±2°C and a humidity level of 60±5%. A total of 40 animals were randomly divided into four groups (n = 10): (1) control (sham-operated), (2) global cerebral IR, (3) MYR, and (4) MYR+IR. This experimental design was described in our previous study[15]. MYR (200 mg/kg) was dissolved in 0.1% carboxymethyl cellulose and administered intraperitoneally (i.p.) for 10 consecutive days. The dose of MYR was selected according to Ciftci *et al.*[12] and MYR treatment was initiated concomitant with the induction of global cerebral IR. Mice in the SH group and the IR group were treated with only the 0.1% CMC solution as a vehicle. In the MYR and MYR+IR groups, mice were treated with MYR (200 mg/kg/day) for 10 days following the IR procedure, after which all animals were sacrificed under anesthesia, and tissue and blood samples were obtained for laboratory analyses.

Chemicals

Myrcene and all other chemicals used for experimental analysis were purchased from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade, or of the highest grade available.

Surgical procedure

This experimental design was described in our previous study[15]. For the induction of global cerebral ischemia, the mice were anesthetized with xylazine (5 mg/kg, *i.p.*) and ketamine (100 mg/kg, *i.p.*), and the procedure was performed according to the methods of Yonekura *et al.*[16]. Briefly, after a midline cervical incision, the bilateral common carotid arteries of animals in the IR and MYR+IR groups were isolated and occluded simultaneously for 15 min using two vascular miniclips. The same surgical procedure was applied to the SH and MYR groups, except that the carotid arteries were not clipped. Following surgery, all mice were placed in a thermal room until they recovered from anesthesia.

Biochemical analyses

The homogenization of tissue briefly was described our previous study[13,14]. The levels of thiobarbituric acid reactive

substances (TBARS), total glutathione (GSH) levels and catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were determined by spectrophotometric methods and these methods briefly given our previous study[13,14].

Histological analysis

For histopathological examination, transverse section through the bottom of the heart is collected for each animal. For light microscopic evaluation, heart samples were fixed in 10% formalin. The heart samples were processed by routine tissue techniques and were embedded in paraffin. Paraffin-embedded specimens were cut into 5 mm thick sections, mounted on slides and stained with Hematoxylen- Eosin (H-E). Sections examined under a Leica DFC280 light microscope by Leica Q Win and Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, U.K).

Statistical analysis

A computer program SPSS 11.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. For biochemical values, statistical analyses were performed using one-way ANOVA and post hoc Tukey's honestly significant difference test. All values were presented as mean ± S.E.M. Differences were considered to be significant at p<0.05.

Results

Biochemical

The TBARS, GSH, CAT, GPx levels and SOD activity for heart tissue are given in Table 1. In IR group, the results showed that SOD, GPx, CAT and GSH levels were significantly (P≤0.01) decreased whereas TBARS levels were significantly (P≤0.01) increased compared with the control and other groups. It was shown that brain IR caused a significant increase lipid peroxidations in heart tissue. Also, it was determined that there were not any significant changes between the control and MYR groups in terms of SOD, CAT, GSH, TBARS and GPx levels. In IR+MYR group, the value of TBARS, CAT, SOD and GPx were near to control and reversed the effect of brain IR on these values. However, The GSH levels were changed but it was not in a statistically significant range. Generally, the results showed that MYR significantly reduced lipid peroxidations in heart tissue.

TABLE 1 - The levels of TBARS, GSH, CAT, GPx and SOD in heart tissue of C57BL/6J mice (mean ± SEM).

	TBARS (nmol/g tissue)	GSH (nmol/ml)	CAT (k/mg protein)	GPx (U/g protein)	SOD (U/mg protein)
Control	5.23 ± 0.41^a	53.7 ± 2.81^a	0.0061 ± 0.0002^a	65.16 ± 2.14^a	17.5 ± 1.12^a
IR	8.61 ± 0.40^b	39.2 ± 3.02^b	0.0023 ± 0.0004^b	$31.,57 \pm 1.25^b$	10.2 ± 1.21^b
MYR	5.34 ± 0.25^a	58.4 ± 3.23^a	0.0058 ± 0.0005^a	59.86 ± 1.71^a	17.9 ± 1.54^a
IR+MYR	6.02 ± 0.53^a	43.1 ± 2.13^b	0.0045 ± 0.0001^c	45.54 ± 2.06^c	13.2 ± 1.32^c

Means bearing different superscripts within same column were significantly different (p<0.01).

Histopathological

We examined sections for eosinophilic stained and pyknotic nuclei cells, vacuolisation, hemorrhage, mononuclear cell infiltration, congestion and necrosis. Histopathologic damage score was calculated these findings. Statistical analysis was made with SPSS 13.0 (SPSS Inc., Chicago, Ill., USA) and MedCalc 11.0 (Belgium) statistical programs. All data are expressed as arithmetic mean ± S.E. For comparisons between groups Kruskal-Wallis and Connover tests were used. Exact *p* values were given where available, and p< 0.0001 was accepted as statistically significant. Histopathological score was given in Table 2.

TABLE 2 - Comparison of the effect of Myrcen on histolocigal changes caused by cerebral I/R on cardiac tissue. (Mean ± SE).

	Histopathologic Damage
Control	0.24 ± 0.10^a
IR	2.38 ± 0.13^b
MYR	0.90 ± 0.12^a
IR+MYR	1.57 ± 0.13^c

The mean differences the values bearing different superscript letters within the same column are statistically significant. (p≤0,0001). SE: Standart Error

In control and MYR groups showed normal cardiac histology of mouse as represented by Figure 1A and B,

respectively. In these groups heart tissue showed a normal myofibrillar structure with striations, branched appearances and continuity with adjacent myofibrils. Cardiac muscle cells were also normal; their large purple nucleus was located in the center of their pink colored cytoplasm. In addition to this, MYR treated animals had normal myocardiac morphology too. The hearts of IR treated mouse showed distinctive appearance of cardiotoxicity with various degrees of focal damages.   These damages are eosinophilic stained and pyknotic nuclei cells (Figure 2C, E), vacuolisation, hemorrhage (Figure 2A, E), mononuclear cell infiltration (Figure 2A, D), congestion (Figure 2D) and necrosis (Figure 2C). We detected these histological findings decreased in IR+MYR group (Figure 3A, 3B). MYR supplementation reduced IR- induced histopathological changes in comparison to compared to that of IR treated groups.
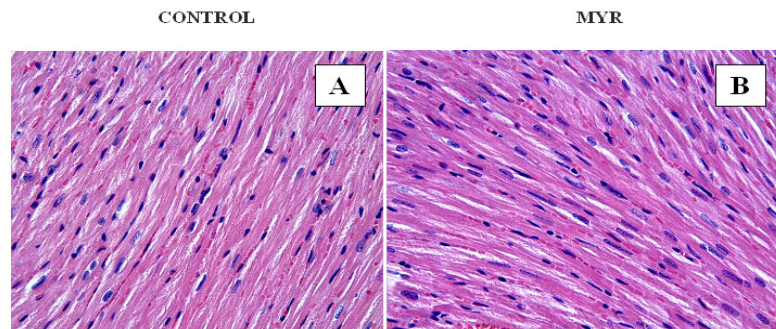
CONTROL   MYR

FİGURE 1 - Heart tissues in control (**A**) and MYR (**B**) groups show normal appearence of cardiac myofibres (H&E, x40).
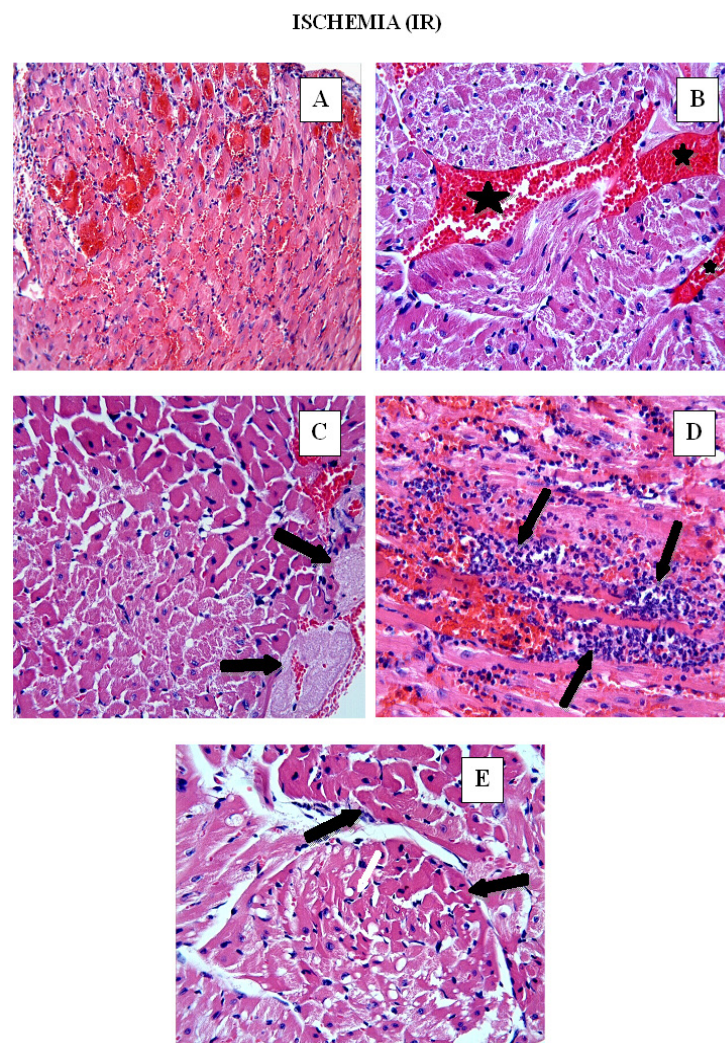
ISCHEMIA (IR)

FİGURE 2 - IR group**.** Hemorrhage (**A,C**), mononuclear cell infiltration (**A,D**), vascular congestion (asterisks) (**B**), necrosis (arrows) (**C**), eosinophilic stained and pyknotic nuclei cells (**C,E**), vacuolisation (**E**) (H&E, x20).
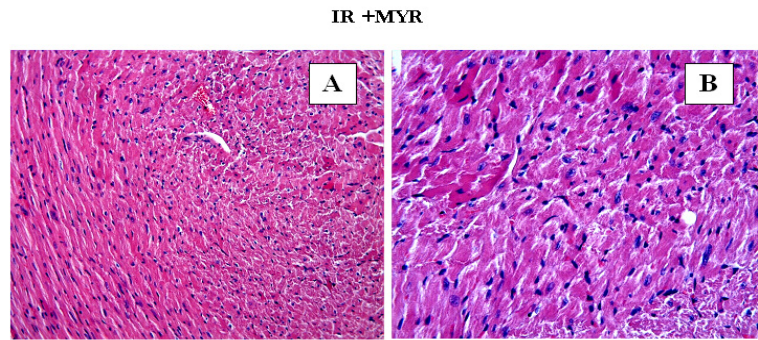
IR +MYR

FİGURE 3 - IR + MYR group. Degenerative changes were decreased in IR+MYR group compared with IR group. **A** (H&E, x20), **B** (H&E, x40).

Discussion

In the present study, the protective effect of MYR on oxidative and histological damage in heart tissue after global cerebral ischemia/reperfusion(IR) in C57BL/J6 mice. There were found that brain IR caused a significant increase lipid peroxidations in heart tissue. And treatment of MYR reduced significantly lipid peroxidation in heart tissue. Many methods have been used to induce global cerebral ischemia in experimental animal models including decapitation without recirculation, neck tourniquet, bilateral common carotid artery occlusion and four-vessel occlusion[17-20]. But, bilateral carotid artery occlusion is commonly used in C57BL/J6 mouse to design global cerebral IR model[12]. This experimental model is used there. MYR were selected in this study for treatment of cardiac damage after stroke[12]. Because It was thought that MYR is a strong antioxidative agent and it can prevent oxidative and histological damage in tissue. It was found that treatment of MYR has a protective effect against induced global cerebral IR due to decrease in oxidative stress and histopathological damage in heart. The mechanism is disclosed that reduction of production lipid peroxidation and inhibition of apoptosis.

It has been well established that oxidative stress displays an important role in the pathophysiology of IR. The excessive production of ROS occurs during IR[21,22]. However, the exact mechanisms underlying heart damage after IR injury have not been elucidated yet. Among these, cerebral IR in particular are responsible for oxidative stress due to the generation of free radicals[6]. Alteration in Ca^{2+} homeostasis in tissue due to an increase in ROS has been suggested to explain the adverse effects of cerebral IR. It is a problem which damages the myocardium after a critical period of arteries occlusion. Mitochondria serve as a source for the massive generation of ROS during IR, thus leading to serious tissue injury. Under these conditions, xanthine dehydrogenase is converted into xanthine oxidase, leading to the accumulation of purine metabolites, hypoxanthine and xanthine, which subsequently produce enormous amounts of superoxide anion radical and hydrogen peroxide[22,23]. Oxidative stress and increased Ca^{2+} cause the opening of mitochondrial permeability transition pore. This allows an entry of water and solutes from the cytoplasm resulting in mitochondrial swelling and damage. The generation of ROS also occurs in the cytoplasm through the action of NAD(P)H oxidase. The resulting oxidative stress can outweigh antioxidant defenses and lead to cell death because oxidative stress can cause a widespread damage to cellular components such as DNA, lipids, and proteins that ultimately promote cellular damage and death during cerebral ischemia and reperfusion in many organs including heart[2,7].

It was seen that global cerebral IR induced lipid peroxidation in heart tissue in term of TBARS levels in this study. But, the antioxidant defense system was suppressed by IR in heart tissue via decreased enzymatic activities of SOD, CAT, GPx and expression in levels of GSH. Recent studies[10,25] have confirmed our findings. The current study demonstrated that treatment of MYR caused a significant decrease in TBARS levels and increase in SOD, CAT, GPx activities and GSH levels in heart tissue of ischemic mice. Treatment of MYR decreases oxidative damage and ameliorates the heart damage caused by IR. There are few studies describing the pharmacological activity of MYR and these studies showed that MYR has an important analgesic, antiinflammatory and antioxidant properties[10,11,13,14,26]. These findings approve that MYR is a strong antioxidant. Hence, MYR may protect heart tissue as a possible antioxidant, prevent damage and lipid peroxidation.

In this study, there was evaluated that significant histopathological changes in IR group, compared with the other groups. In IR group was shown distinctive appearance of cardiotoxicity with various degrees of focal damages including

eosinophilic stained and pyknotic nuclei cells, vacuolisation, hemorrhage, mononuclear cell infiltration, congestion, and necrosis. There was decreased these histological findings in treatment of MYR group. MYR treatment was reduced histopathological changes compared to IR group. Many studies[12,27-29] confirmed the histological results. Also, treatment of MYR ameliorates histological changes caused by IR.

Conclusions

β-myrcene (MYR) has a modulatory effect on heart tissue after cerebral IR injury. There was relationship between oxidative status and histopathological changes. The useful effects of MYR seem to be closely linked with the suppressing of lipid peroxidation and enhancing of antioxidant enzyme activities.

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