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Antioxidant Effect of Ethanolic Extract of Propolis in Liver of L-NAME Treated Rats*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The blocking of nitric oxide synthase (NOS) activity may cause vasoconstriction with formation of reactive oxygen species. Propolis is a natural product collected from plants by honeybees. Propolis has biological and pharmacological properties.
Objectives. This study was designed to investigate the effects of propolis on catalase (CAT) activity, nitric oxide (NO) and malondialdehyde (MDA) levels in the liver tissues of NOS inhibited rats by Nω-Nitro-L-arginine methyl ester (L-NAME).
Material and Methods. Rats were given a NOS inhibitor (L-NAME, 40 mg/kg, intraperitoneally) for 15 days to provoke hypertension and propolis (200 mg/kg, by gavage) the last 5 of the 15 days.
Results. Nitric oxide levels in the liver tissue of the rats given L-NAME significantly decreased ($p < 0.01$). That parameter did not significantly alter in the liver of rats treated with propolis compared to the control group. CAT activity and MDA levels in the liver of the rats administrated L-NAME significantly increased compared to the control group ($p < 0.01$). These parameters significantly decreased in the liver of the rats given L-NAME + propolis compared to the L-NAME group ($p < 0.01$).
Conclusions. The present data shows that L-NAME in the liver may enhance oxidative stress *via* inhibited nitric oxide synthase. Our results also suggest that this effect is suppressed by the antioxidant properties of propolis in the liver tissue of NOS inhibited rats (**Adv Clin Exp Med 2015, 24, 2, 227–232**).

Key words: L-NAME, liver, oxidative stress, propolis, rat.

Nitric oxide (NO) is a molecule with pleiotropic effects in different tissues [1]. NO is generated from L-arginine, which is converted into L-citrulline [2], a family with 4 essential types: endothelial, neuronal, inducible and mitochondrial [3]. They can be obtained in virtually all the tissues and they can even co-exist in the same tissue. NO is a well-known vasodilating factor, but it acts as a neurotransmitter when produced by neurons. It is involved in defense roles when it is produced by immune and glial cells [1, 4]. The application of Nω-Nitro-L-arginine methyl ester (L-NAME), which is a nonselective nitric oxide synthase (NOS) inhibitor, induces NO deficiency and hypertension and increases oxidative stress [5]. Dietary L-NAME increases microvascular pressure in the liver *in vivo* and *in vitro* [6]. Oxidative stress and the changing of the cellular redox state are related to many types of acute and chronic liver injuries [7]. Microvascular changes lead to alterations in organ perfusion and lead to hypertension related to liver damage. Essential hypertension is generally associated with

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liver damage [5]. It depends on the degree of the microcirculation exposed to the risen blood pressure [5]. Hypertension is a major risk factor that predisposes the liver to disorders. The liver actively detoxifies and handles endogenous and exogenous chemicals, making it vulnerable to injury [5]. The presence of different oxidizing species within the body can also have an important function in the development and progression of many disease processes. In most recent works, oxidative stress has been analyzed using a lipid peroxidation assay. Malondialdehyde (MDA), the last product of lipid peroxidation, was used as an indicator of local tissue damage and liver injury [2, 8]. Lipid peroxides could change biological membrane properties, resulting in severe cell damage [9, 10]. To reduce such damage, cells have defence mechanisms including both enzymatic and nonenzymatic processes. For example, enzymatic, antioxidative enzymes such as catalase (CAT) and nonenzymatic, which are antioxidant molecules such as flavonoids. Exogenous antioxidative compounds must be delivered when reactive oxygen species overrun the endogenous protective systems. Bioflavonoids are effective molecules as antioxidants. These compounds have the various antioxidant features to preclude oxidative stress [11]. These molecules block the oxidation of lipids and lead some roles, such as hypotensive properties [12]. In most studies, the biological or pharmacological properties were associated with phenolic compounds, chiefly to flavonoids and aromatic acids and ester. Propolis, which contains rich flavonoids, is a resinous matter that honeybees collect from various plant exudates and use to fill gaps and seal parts of the hive [13]. Therefore, we performed the present study to evaluate the antioxidant and antihypertensive potential of ethanol extract of propolis on the well-established L-NAME-induced rat model by monitoring the extent of lipid peroxidation and NO, and the status of CAT as an enzymatic antioxidant in the rat liver.

Material and Methods

Experimental Section

Twenty eight male Wistar rats weighing 200–250 g were placed in a quiet, temperature (21 ± 2°C) and humidity (60 ± 5%) controlled room in which a 12–12 h light–dark cycle was maintained. All experiments were performed between 9:00 and 17:00. All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Ethical Committee on Animal Research at Firat University, Elazig.

Preparation of Propolis Extract Solution

Propolis is generally extracted with ethanol or water, and these extracts have been used in folk medicine. The composition of propolis depends on the solvent used for its extraction. In addition, biological activities could be related to its chemical composition [12]. This bee product was collected from the village of Kocaavsar in Balikesir, Turkey. The propolis extract was prepared to 30 g of propolis, completing the volume to 100 mL with 70% ethanol. The extracts were then protected from light, filtered twice, dried and stored in sealed bottles at 4°C until used [14].

Experimental Design

The experimental groups were designed as four groups comprised of seven rats each: (Group 1) sham-oparated (control), (Group 2) propolis group, and (Group 3) L-NAME group and (Group 4) L NAME + propolis group. The control group received normal saline intraperitoneally (i.p.) for 15 days. The propolis group received propolis (200 mg/kg) by gavage [15]. The L-NAME group received the non-specific NOS inhibitor L-NAME (40 mg/kg, i.p.) for 15 days [15]. The L-NAME + propolis group received both L-NAME (40 mg/kg, i.p.) for 15 days and propolis (200 mg/kg, gavage) for the last 5 days. The L-NAME (Fluka Chemie, Switzerland) was dissolved in normal saline (0.09% NaCl w/v). The propolis extract was dissolved in distilled water.

Preparation of Tissues for Biochemical Analyses

After these treatments, the rats were anesthetized with 75 mg/kg sodium pentobarbital, their chests were opened, the *vena cava* was cut and then 30 mL of 0.9% NaCl was injected into the heart to rinse blood from the body in the anesthetized rats. The liver tissue of the rats was kept at –80°C until used. These liver tissues were segregated into 2 sections for assay of enzymatic activity, lipid peroxidation and NO levels. The liver tissues were homogenized in 100 mL of 2 mM phosphate buffer, pH 7.4 using a PCV Kinematica Status Homogenizer. The homogenized samples were sonicated for 30 s, the sonications interrupted with a 30 s pause on ice and then centrifuged at 12.000 g for 15 min at 4°C. The supernatants, if not used for enzyme assay, were stored in a deep freeze at –80°C. These samples were used for total protein and CAT activity analyses. The other parts of the tissue homogenates were used for lipid peroxidation analyses.

After being washed 3 times with ice-cold 0.9% NaCl, the liver tissue parts were homogenized with 1.15% KCl. These tissue homogenates were used for assay of MDA and NO levels.

Measurement of Total Protein

Total protein levels of the tissues were tested according to the colorimetric method of Lowry et al. using BSA as the standard [16].

Assay of CAT Activity

CAT activity in the rat liver tissues was analyzed using the method by Aebi [17]. It was expressed as kU/g protein, where k is the first-order rate stable.

Measurement of NO Levels

NO levels in the liver tissues were determined by a modification of the Griess reaction [18]. Since NO is an unstable molecule; it converts to its end products (nitrate and nitrite) in a short time. Therefore, the measurements of nitrate and nitrite were used in this study, where total nitrite concentrations equal NO. The levels of nitrite and nitrate were analyzed using the Griess reaction. For measuring total nitrite, the samples were incubated with cadmium for 2 h, which converts all nitrate to nitrite. Serum nitrate concentrations were calculated by subtracting the direct nitrite value from total nitrite value.

Measurement of MDA Levels

MDA levels in the liver tissues were measured spectrophotometrically with a thiobarbituric acid (TBA) solution [19]. The data was expressed as nm/g tissue.

Statistical Analysis

The data were analyzed with SPSS 16.0 for Windows by using one-way analysis of variance (ANOVA). Differences between ranks were determined using Duncan's multiple range analysis in which the significance level was described as $p < 0.01$.

Results

Results of CAT Activity

The effects of L-NAME and propolis on CAT activity and NO and MDA levels in rat liver are shown in Table 1. In the liver of rats exposed to L-NAME had important effects on CAT activity. As an important part of the antioxidant system, the CAT activity increased in the liver tissues of L-NAME-induced rats ($p < 0.01$) (Table 1). The CAT activity decreased ($p < 0.01$) in the propolis group compared to the L-NAME and L-NAME + propolis groups (Table 1). There was a statistically significant decrease in the CAT activity of the L-NAME + propolis group compared to the L-NAME group ($p < 0.01$) (Table 1).

Results of NO Level

The effects of the propolis and L-NAME on NO levels are demonsrated in Table 1. NO levels were found to be significantly decreased in the liver tissues of the rats applied to L-NAME ($p < 0.01$) compared to the control group (Table 1). The NO level increased ($p < 0.01$) in the liver tissues of rats treated with propolis compared to the L-NAME and L-NAME + propolis groups and was close to the values of the control group (Table 1). There was a statistically significant increase in the NO level of the L-NAME + propolis group compared to the L-NAME group ($p < 0.01$) (Table 1).

Table 1. Alteration with propolis treatment of CAT activity, NO and MDA levels in the liver tissues of L-NAME induced rats. All data points are the average of n = 7 with ± STD DEVs. Data with different superscript letters (a, b, c) means statistically significant among of all

Parameters / Groups	CAT (kU/g protein)	NO (μmol/g tissue)	MDA (nmol/g tissue)
Control	5.56 ± 0.44^{c}	6.98 ± 0.72^{b}	8.27 ± 0.99^{c}
L-NAME	8.28 ± 0.90^{a}	4.12 ± 0.52^{c}	12.86 ± 1.74^{a}
Propolis	5.50 ± 0.45^{c}	7.35 ± 0.49^{a}	7.32 ± 0.63^{d}
L-NAME + propolis	6.20 ± 0.39^{b}	$4.49 \pm 0.78^{b, c}$	10.02 ± 0.39^{b}

groups ($p < 0.01$).

Results of MDA Level

The effects on MDA levels of the L-NAME, propolis and L-NAME + propolis applications are shown in Table 1. There was a significant increase ($p < 0.01$) in the MDA level of rats exposed to L-NAME compared to the control, propolis, and L-NAME + propolis groups (Table 1). Propolis treatment to the NOS-inhibited rats created significant decreases ($p < 0.01$) in MDA level compared to the L-NAME applied group (Table 1).

Discussion

Endothelial dysfunction induced by reactive oxygen species (ROS) (especially superoxide) causes decreased NO bioavailability and deteriorated vasodilation. Increased production of ROS has been shown in experimental and human hypertension [20]. Hypertension is the main risk factor that predisposes the tissues to damage, which is responsible for morbidity and mortality in patients. Changes to the microvascular system alter organ perfusion and lead to hypertension-related damage [7]. Previous studies have shown that application of the NOS inhibitor L-NAME have resulted in increased ROS mediated tissue injury [21–23]. As a result, the harmful effects of ROS could be equalized by NO. Some researchers have emphasized that bioflavonoids may play a preventive role in tissues by increasing the presence of NO. NO deficiency model hypertensive rats are helpful resources for the work of the protective effects of natural products under hypertension and oxidative stress [5]. The antihypertensive effects of various foods and natural products have been reported [24]. It has been suggested that bioflavonoids may also exert a direct effect on radical scavenging enzymes occurring in hypertension related organ damage [15, 25]. Pharmacological agents used to lower blood pressure can help prevent/reduce hypertension related organ injury. Polyphenols have been demonstrated to protect from this damage by the blood-pressure-lowering activity too [26]. The helpful effects of many free radical scavengers and antioxidants have been shown on hypertension and oxidative stress [27]. In some studies, flavonoids were found to significally improve a liver injury. Different flavonoids have also shown some hepatoprotective activity [2]. The study has indicated various biological activities of flavonoids, including vasodilator effects in isolated aorta stimulated with noradrenaline, and flavonoids can show antihypertensive effects in hypertension [24]. Inadequate NO production leads to vasoconstriction and blood pressure increases, which eventually mediates the complex hemodynamic disorders associated with chronic diseases [28]. Partial NO blockage over an extended period can produce a stable hypertension with vasoconstriction and organ damage. L-NAME causes an increase in water intake with or without elevation in systemic arterial pressure. Similarly, L-NAME causes an increase in water excretion, associated with the pressure of antidiuretic hormone release from the pituitary due to the significant neural activity of NO too [5]. Hypertensive humans show increased levels of superoxide and hydrogen peroxide in plasma [20]. Moreover, enhanced endothelial superoxide anion production has been identified in vessels of spontaneously hypertensive rats, a valuable animal model of essential hypertension in humans, and these effects are related to impairment of endothelium dependent relaxation. Various foods and natural products have different activities such as an inhibitory activity against angiotensin converting enzymes, vasodilatory action or inhibitory effects on the release of noradrenaline from sympathetic nerves [24]. It is well known that propolis and its metabolites are free radical scavengers and antioxidants [29]. Some data has indicated that an ethanol extract of propolis and its main compounds would be useful for improving hypertension as a functional food [24]. Flavonoids are ubiquitous in plant foods and drinks and therefore an important supply is consumed in our daily diet. These flavonoids are differently associated with the sensory and nutritional quality of our plant foods. The *in vitro* antioxidant activities have been recognised for decades, but it is still not clear whether there are other helpful effects *in vivo*. Many other biological activities for flavonoids have been described but we are not sure how to use flavonoids to actually contribute to human health. However, some recent studies suggest that they may be significant as anticancer agents [30].

In this study, it has been observed that propolis, which is a natural bee product, has the ability to reduce the hypertensive effect and oxidative stress in liver of rats. Finally, if there is not enough L-arginine to compete with L-NAME, NOS, which carries out NO synthesis, inhibits. Thus, the occurrence of NO may lessen. Hypertension occurs by the reduction of NO because of the inhibition of acute or chronic NOS, so the reduction in NO synthesis acts as an important role in hypertension physiopathology. There should be enough arginine to compete with L-NAME for increasing the production of NO and activating NOS. It may be thought that this requisite can be obtained with a compound rich with arginine like propolis. This case is important for providing arginine to compete with L-NAME and for the carrying out of

enzyme activation. The activated enzyme increases the formation of NO which shows a vasodilator effect [34]. The study for new antioxidants as potential drugs is an active field of pharmacology of natural products and ethnopharmacology. This work provides evidence of the protective effects of propolis in hypertensive target organs under a NO deficient environment using L-NAME. Oxidative stress increased by the administration of L-NAME. As a result, CAT activity increased for inhibition of oxidative stress too. After propolis treatment, CAT activity parallelly decreased with decreasing oxidative stress.

In this study, CAT activity in the liver tissue increased in the L-NAME-induced group. Stress appeared by L-NAME triggered CAT activity. But propolis altered the decreasing CAT activity in rat liver. Propolis caused a slight decrease in CAT activity in the liver of L-NAME-treated rats. Our data shows that a propolis remedy suppresses oxidative stress as monitored by the height activity of the basis antiperoxidative enzyme, CAT, and reduced lipid peroxidation products in rat liver. The results demonstrate L-NAME-induced oxidative stress and enhanced CAT activity. Oxidative stress and MDA levels increased with the administration of L-NAME. Oxidative stress was decreased with a treatment of propolis exract. Propolis proved to be beneficial in decreasing the levels of MDA in the liver. Therefore, propolis extract provided protection against free radicals and lipid peroxidation measured as MDA in rat liver tissue. Propolis showed antioxidant effects against L-NAME--induced oxidative stress in rats. The results of this study are consistent with the literature [15]. L-NAME–treated rats also showed increased tissue lipid peroxidation (MDA levels) and decreased liver antioxidant capacity. These results indicate vascular and systemic oxidative stress [32]. Various propolis extracts are generally considered to be antioxidants. They could demonstrate various results when tested in animal models and human subjects. In recent years, there has been a great deal of studies carried out on the biological activities of propolis [15, 29, 33–35]. If antioxidant defense systems intake sufficient antioxidants including micronutrients, it may prevent lipid peroxidation. Oxidative factors may markedly increase oxidative cell injury. Propolis has antioxidant properties and scavenges free radicals, thus it can prevent tissue damage [29]. In conclusion, in the present study, application of L-NAME to the Wistar rats resulted in well-developed oxidative stress. Also, we have reported effects against oxidative stress of the antioxidant potential of propolis extract. This study will shed light on the new research of pharmacology of natural products, allowing various usages of propolis to keep the inner balance of the body constant and give direction to the developments of new natural extracts and preparations.

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