Propolis attenuates oxidative injury in brain and lung of nitric oxide synthase inhibited rats

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**Abstract**

**Background:** The blocking of nitric oxide synthase (NOS) activity may reason vasoconstriction with formation of reactive oxygen species. Propolis has biological and pharmacological properties, such as antioxidant. The aim of this study was to examine the antioxidant effects of propolis which natural product on biochemical parameters in brain and lung tissues of acute nitric oxide synthase inhibited rats by Nω-nitro-L-arginine methyl ester (L-NAME).

**Methods:** Rats have been received L-NAME (40 mg/kg, intraperitoneally), NOS inhibitor for 15 days to produce hypertension and propolis (200mg/kg, by gavage) the lastest 5 of 15 days.

**Results:** There were the increase \((P<0.001)\) in the malondialdehyde levels in the L-NAME treatment groups when compared to control rats, but the decrease \((P<0.001)\) in the catalase activities in both brain and lung tissues. There were statistically changes \((P<0.001)\) in these parameters of L-NAME+propolis treated rats as compared with L-NAME-treated group.

**Conclusion:** The application of L-NAME to the Wistar rats resulted in well developed oxidative stress. Also, propolis may influence endothelial NO production. Identification of such compounds and characterisation of their cellular actions may increase our knowledge of the regulation of endothelial NO production and could provide valuable clues for the prevention or treatment of hypertensive diseases and oxidative stress.


**Introduction**

Nitric oxide (NO) is recognized as the endothelium-derived relaxing factor responsible for vascular dilation. NO is synthesized by NO synthase (NOS), which consists of three isoforms, type I NOS, type II NOS, and type III NOS. Large quantities of type I NOS and NO are known to be present in the brain and neural tissue. However, the role of NO in the sympathetic nervous system is controversial (1). NO is a molecule with pleiotropic effects in different tissues. NO is a well-known vasorelaxant agent, but it works as a neurotransmitter when produced by neurons and is also involved in defense functions when it is produced by immune and glial cells. The ubiquitous localization of NO demonstrates its implication in a wide range of physiological process. The relevance of NO in brain is determined by the neuronal, glial and vascular physiological effects, opening the possibility of pharmacological treatments directed to NO metabolic pathways (2). Furthermore, since NO has been shown to be an inhibitor of smooth muscle contraction and of
mast cell degranulation, any strain-related differences in bronchoconstriction following antigen challenge could also be a reflection of differences in endogenous NO generation in lung tissue. To assess this possibility in our model, relation of oxidative stress and synthesis of endogenous NO was inhibited by NO synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) (3). Alterations in nitric oxide synthesis or bioavailability causes vasoconstriction and might be involved in the pathogenesis of hypertension. NOS inhibitors such as L-NAME are usually used in hypertensive models (4). Reactive oxygen species (ROS) production increases in atherosclerotic risk factors such as hypertension. This is important because of essentially every aspect of atherosclerotic lesion formation is influenced by oxidative events. For example, ROS induce lipid peroxidation when beginned lesion (5).

In hypertensive animals, superoxide dismutase make converts superoxide anions radical (O$_2^-$) to H$_2$O$_2$ when ROS are increased. Catalase (CAT) removes H$_2$O$_2$ by breaking down directly to O$_2$. Thus, H$_2$O$_2$ is reduced, suggesting that an imbalance between oxidant and antioxidant mechanisms is a contributing factor (6). These conditions are associated with oxidative stress. The beneficial effects of many free radical scavengers and antioxidants on hypertension and ischemia-reperfusion injury have been demonstrated.

In recent years, most of interest have been focused on the therapeutic properties of exogenous antioxidants in biological systems, and on the mechanisms of their biological activities. Natural products are a promising source for the discovery of new pharmaceuticals (7). Flavonoids are potent antioxidants, free radical scavengers and metal chelators: they inhibit lipid peroxidation and exhibit various physiological activities, including antihypertensive and antiarthritic activities. Methods for identification of flavonoids are of interest because of the widespread occurrence of these compounds in different natural products (8). Propolis is a resinous natural product collected from cracks in the bark of trees and leaf buds which are enriched with the salivary enzymes of honeybees. In the last decade, various studies dealing with propolis about chemical composition and biological activities have been published (8,9).

In the present study, we evaluated malondialdehyde (MDA) levels and CAT activities in brain and lung tissues of L-NAME-induced rats. In addition, the effects of potent antioxidant propolis treatment on these parameters were investigated. MDA, a stable metabolite of the free radical-mediated lipid peroxidation cascade, is used widely as marker of oxidative stress. CAT is an important endogenous antioxidant, the levels of which are influenced by oxidative stress.

**Materials and Methods**

**Experimental section**

The ethical rules raised by “Guide for the Care and Use of Laboratory Animals” (Guide for the Care and Use of Laboratory Animals 1996) (10) were obeyed during this study which was closely scrutinized by the Ethical Committee on Animal Research at Firat University, Elazig, Turkey.

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 h.

**Preparation of propolis extractive 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 the present work, propolis was collected from a farm at village Kocaavasar in Balikesir, Turkey. Propolis was dissolved in 30 % ethanol, protected from light and moderately shaken for 1 day at room temperature. Afterward, the extracts were filtered twice, dried and stored insealed bottles at 4 °C until used (11).

**Experimental design**

Rats were divided into four groups of seven rats each: (1) control group, (2) propolis group, (3) L-NAME group and (4) L-NAME+ propolis group. L-NAME (Fluka Chemie, Switzerland) was dissolved in normal saline (0.09% NaCl w/v). The ethanolic extract of propolis was dissolved in distyle water. The rats in control group were injected normal saline intraperitoneal (i.p.) for 15 days. Propolis group received propolis 200 mg/kg with gavage (12). L-NAME group received non-specific NOS inhibitor L-NAME (40 mg/kg, i.p.) for 15 days (12). 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 (12).

**Preparation of tissues for biochemical analyses**

After these treatments, rats were anaesthetised with 75 mg/kg sodium pentobarbital, chests were opened, vena cava was cut and then into the heart 30 mL of 0.9 % NaCl was injected to rinse blood from the body in anaesthetised rats. Tissues of rats were removed and frozen in liquid nitrogen. Tissues were stored at −80 °C until used. The tissues were separated into two parts for determination of CAT activity, lipid peroxidation. Tissues were weighed and then homogenized in 100 mL of 2 mM phosphate buffer, pH 7.4 using PCV Kinematica Status Homogenizer. Homogenized samples were then sonicated for 1.5 min (30 s sonication interrupted with 30 s pause...
on ice). Samples were then centrifuged at 12,000g for 15 min at 4 °C and supernatants, if not used for enzyme assays immediately, were kept in the deep freeze at −80 °C. Supernatants were used for determination of total protein and measurement of CAT activity. The second part of tissues homogenate was used for lipid peroxidation analysis. Tissues were washed three times with ice-cold 0.9% NaCl solution and homogenized in 1.15% KCl. The homogenates were assayed for MDA, the product of lipid peroxidation.

**Protein assay**

Supernatants of tissues were used for assay of total protein. Total protein was quantified by the colorimetric method of Lowry et al. Using BSA as the Standard (13).

**Analysis of CAT activity**

The CAT activity in the rat tissues was analysed by measuring the decomposition of hydrogen peroxide at 240 nm, according to the method of Aebi (14). It was expressed as kU/g protein, where k is the first-order rate constant.

**Measurement of MDA level**

MDA levels were measured spectrophotometrically using thiobarbituric acid (TBA) solution (15). The reaction mixture containing tissue homogenate, phosphoric acid, TBA and sulfuric acid was heated 60 minutes in a boiling water bath. After cooling, n-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation and absorbance was determined at 532 nm. For quantification an external standart curve was prepared using 1, 1, 3, 3 tetraethoxypropane. Data were expressed as nm/g tissue.

**Statistical analysis**

The data were analysed with SPSS 9.0 for Windows by using oneway analysis of variance (ANOVA). Differences between means were determined using Duncan’s multiple range test in which the significance level was defined as P<0.01.

**Results**

It has been showed effects of L-NAME and propolis on CAT activity and MDA levels on rat brain and lung tissues in table 1 and 2. Treating L-NAME produced important effects on CAT activity in tissues of rats. An important part of antioxidant system, the CAT activity decreased in the brain and lung tissues of L-NAME treated rats compare with control group (P<0.001) (Table 1). There was statistically significant increase in CAT activity of L-NAME+ propolis group compared with L-NAME treated group (P<0.001) in the brain and lung tissues of rats (Table 1). Effects on MDA levels of L-NAME is an important indicator of oxidant status. The data of L-NAME, propolis and L-NAME+ propolis groups in the brain and lung tissues are indicated in table 2. There was statistically significant increase (P<0.001) in the MDA levels by NOS inhibition caused by L-NAME treated rats compare with control group but in propolis group has been occurred significant reduction (P<0.001) compared with control group (Table 2) in the brain and lung tissues of rats. Propolis treatment to the NOS inhibited rats created significant reduction (P<0.001) in MDA levels compared to L-NAME treated group in the brain and lung tissues (Table 2).

**Discussion**

The increasing vasoconstriction with inhibited NOS leads to hypertension. ROS, biologically believed to be closely related superoxide and hydrogen peroxide radicals occur due to changes in vascular structure to with occurrence of hypertension. The production of ROS may contribute to the oxidative stress in other organs such as brain and lung (5, 16). In the present work, antioxidant properties of propolis were determined by measuring CAT activities and lipid peroxidation levels in the tissues of rats induced by NOS inhibitor L-NAME. The antioxidant systems that both enzymatic and non-enzymatic are effective in removing and prevention of the formation of ROS. In this study, it has been observed statistically significant decreasing the CAT activities according to other experimental groups in result of stress,

**Table 1.** Changes in CAT activities in the brain and lung with administration of propolis in L-NAME-induced rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CAT Activities in Tissues (kU/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Control</td>
<td>35.46 ± 3.04a</td>
</tr>
<tr>
<td>Propolis</td>
<td>40.26 ± 2.68b</td>
</tr>
<tr>
<td>L-NAME</td>
<td>29.76 ± 1.89c</td>
</tr>
<tr>
<td>L-NAME+propolis</td>
<td>39.03 ± 8.78e</td>
</tr>
</tbody>
</table>

All data points are the average of n=7 with ± STDEVs. *statistically significant (P<0.001)

**Table 2.** Changes in MDA levels in the brain and lung with administration of propolis in L-NAME-induced rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA Levels in Tissues (nmol/ g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Control</td>
<td>10.05±0.58e</td>
</tr>
<tr>
<td>Propolis</td>
<td>6.25±0.44e</td>
</tr>
<tr>
<td>L-NAME</td>
<td>11.53±0.84e</td>
</tr>
<tr>
<td>L-NAME+propolis</td>
<td>8.25±0.57e</td>
</tr>
</tbody>
</table>

All data points are the average of n=7 with ± STDEVs. *statistically significant (P<0.001).
which created with L-NAME. Our data have been showed parallelism with results of other researchers (17, 18). The possible mechanism for decrease in CAT activity may be due to inhibition of the enzyme by $O_2^-$ by generating ferroxy catalese, which does not decompose $H_2O_2$ rapidly thereby resulting in further damage to cells. The increase of $O_2^-$ increases arterial pressure by inactivating NO and producing peroxynitrite, a stronger and relatively long lived oxidant which is cytotoxic and can initiate lipid peroxidation without the requirement of transition metals. The reduced capacity of CAT to neutralize ROS results in increased generation of hydroxyl radical, which initiates the peroxidation of polyunsaturated fatty acids (18).

ROS are known to be affected cellular biomolecules in cells and it has been reported in many studies (5, 16). Lipids, which assumed an important role in the strucual and functional are occured peroxidation of lipids as result of the attack of ROS. As a result of this chain, malondialdehydes that called lipid peroxidation products may release. In this study, L-NAME treated group’s MDA values were determined statistically significantly higher than other experiment groups in brain and lung tissues. Hypertension, which occurred by L-NAME and as result of this evolved oxidative stress made increase to produce MDA. Oxidative process, which created by the effect of endogenous and exogenous stress factors induces MDA. The data that we have observed show conformity with other researchers’s data (4, 19).

Hypertension and other external influences stimulate $O_2^-$ from the vascular NADPH oxidase. These results in oxidation of the endothelial nitric oxide synthase (eNOS) cofactor tetrahydrobipterin (BH$_4$). Activity of eNOS, which oxidized cofactor stops and accordingly, vasodilation disappear by blocking synthesis of NO. Also, other oxidants derived from peroxynitrite contribute to this event. The eNOS, which oxidized cofactor or oxidized BH$_4$ trigger production of $O_2^-$. In this way, the presence or absence of BH$_4$ determines synthase of NO or whether a $O_2^-$ synthase of eNOS. Treating of BH$_4$ both decreases vascular superoxide production and increases vascular NO production by regulate the vasodilation induced from endothelium. The BH$_4$ is known controlling hypertension by regulating blood pressure in humans. ROS can cause damage in other organs including blood vessels too (5). In many vascular beds, ROS play a key role in endothelial dysfunction. Elevated $O_2^-$ may decrease both synthesis and bioactivity of endothelium derived NO. In addition, deple tion in BH$_4$ results in increased $O_2^-$ synthesis, hypertension and remodeling within pulmonary vasculature. Therefore, the study investigates endothelial role, and more specially the function of NO and ROS, in pulmonary arteries from mice exposed or not to chronic hypoxia (24). Previous observations demonstrated that administration of NOS inhibitor, L-NAME results in increased BP and ROS mediated tissue damage (5, 19, 20). Oxidative stress and hypertention are considered one of the main factors responsible for these neurological alterations. In pulmonary circulation as in other vascular beds, endothelial cells play a critical role in maintaining homeostasis, via the release of vasculoprotective factors such as NO (20).

A potential function of ROS in the development of pulmonary hypertension is supported by experimental data showing that several antioxidants prevent some cardiopulmonary alterations triggered by chronic hypoxia (20). It is well known that propolis is free radical scavenger and antioxidant (8). Propolis is an important antioxidant for prevent hypertension, which created by treating L-NAME (21). This case has been showed in previous study that we have done (21). Propolis has many amino acids and phenolic compounds in the content of itself. The arginine is one of amino acids, which including propolis. Phenolic compounds found in structure of propolis, due to the antioxidant properties acted an important role in the prevention of many diseases which threaten life of alive. In addition, the blood pressure lowering effect by increasing the permeability of capillaries of phenolic compounds have been revealed with studies (12, 21, 22). It has been showed that it has the blood pressure lowering effect in previous study that we have done (12). Flavonoids, which concentrated in propolis has powerful antioxidant properties, also effective in removing free radicals and protective effect against lipid peroxidation in cell membranes. In a study, Isla et al. investigated the antioxidant activity of extract of Argentina propolis. That study showed the effective effect of propolis against oxidative modification of serum lipids. Researchers determined the amount of oxidation of lipoprotein by experiment of TBA reactive substances and observed propolis is a natural antioxidant. Researchers have been found that there is a positive correlation between proportional reduction of MDA production with the amount of flavonoids, flavonoids reduces the formation of free radicals, flavonoids have a protective effect on serum lipids against oxidation (23). Hypertensive patients show increased levels of plasma superoxide and hydrogen peroxide. Recently the antihypertensive effects of 25% ethanol extract of Brazilian green propolis were reported in spontaneously hypertensive rats. However, little information is available about the effects of absolute ethanol extract of Brazilian green propolis and its main constituents on hypertension in spontaneously hypertensive rats. Generally, propolis is known to yield different constituents depending on the liquid used for extraction and the concentration. Propolis extraction methods may influence its activity, since different solvents solubilize and extract different compounds. The most common extracts used in biological assays are...
ethanol, methanol and water. Its chemical composition is very complex; more than 300 components have already been identified, and its composition is dependent on the local flora. Moreover, propolis composition is highly variable, creating a problem the medical use and standardization (24). Biological and therapeutic actions of propolis are generally attributed to its constituents of plant origin, mainly phenolics (25). Flavonoids are well-known to possess antioxidant activity, mainly via their free radical scavenging activity and metal chelating properties (26). As produced by the bees, propolis is a strongly adhesive, resinous substance used to seal holes in their hives, smooth out the internal walls and protect the entrance against intruders. Although a common source of the resin is Populus balsamifera L. (and other Populusspecies), the precise composition of raw propolis varies with the source. In general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris. Raw propolis is processed using water washing and solubilizing in 95% ethanol to remove the wax and organic debris, creating propolis tincture, propolis balsam, or ethanol extract of propolis (25). Besides, propolis-containing products have been intensely marketed by the pharmaceutical industry and health-food stores (27). The ethnopharmacological approach, combined with chemical and biological methods, may provide useful pharmacological leads.

Due to the presence of some of these effective compounds such as flavonoids (flavones and flavanones), phenolic acids and their esters in propolis and propolis extract, if the positive physiological properties and the non-toxicity of the propolis sample are proven it could be used as a mild antioxidant and preservative. In this study, the extract of propolis has not been standardized based on a major ingredient. But, it is known that one of the major components of ethanolic extract of propolis is caffeic acid that derivatives polyphenolic components and flavonoids in particular are caused their strong antioxidant properties (28, 29). They use medically most properties of many flavonoids because of ability to scavenge free radicals (30). Caffeic acid phenethyl ester shows antioxidant activity in ethanolic extract of propolis (31). Differences in chemical composition are associated with variations in biological and pharmacological activity of propolis (32). The biological properties of flavonoids are considered in an evaluation of the medicinal and nutritional values of these compounds. In a work has identified new phenolic compounds of which had strong activity against lipid peroxidation together with O2• scavenging activity in the rats (33).

Phenolic acids and flavonoids known antioxidant characters based on the the hydrogen donor due to the chemical properties and the radical scavenging capabilities. In the work, we have indicated that significantly decreasing in MDA levels were observed in brain and lung tissues of rats in the propolis group according to control, L-NAME and L-NAME+propolis groups. These results, increasing MDA levels with oxidative stress show to be reduced with the application of propolis and therefore propolis can play a role as an antioxidant agent.

In conclusion, in the present study, application of L-NAME to the Wistar rats resulted in well developed oxidative stress. Also, we reported that propolis extract has effects against oxidative stress and hypertension by the antioxidant potential of itself. The results of this work shed light on the future planned research. That will contribute to the scientific literature about hypertension and its both cause and result developed oxidative stress.

Acknowledgement

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