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Original article

Brown propolis attenuates cerebral ischemia-induced oxidative damage via affecting antioxidant enzyme system in mice

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ABSTRACT

Oxidative stress plays a critical role in ischemic brain injury. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are the enzymes underlying the endogenous antioxidant mechanisms affected by stroke and are considered as oxidative stress biomarkers. Brown propolis (BP) is a bioactive natural product with a set of biological activities that in turn may differ depending on the area from which the substance is originated. The aim of this study was to investigate the effect of water-extracted brown propolis (WEBPs), from two regions of Iran, against cerebral ischemia-induced oxidative injury in a mouse model of stroke. Experimentally, the chemical characterization and total polyphenol content were determined using GC/MS and Folin–Ciocalteu assay respectively. Seventy-two adult male mice were randomly divided into the surgical sham group, control group (treated with vehicle), and four groups of WEBPs-treated animals. The WEBPs were administered at the doses of 100 and 200 mg/kg IP, during four different time points. Oxidative stress biomarkers (SOD and GPx activity, SOD/GPx ratio), lipid peroxidation (LPO) index (malondialdehyde content) and infarct volume were measured 48 h post stroke. Behavioral tests were evaluated 24 and 48 h after stroke. WEBPs treatment resulted in significant restoration of antioxidant enzymes activity and a subsequent decrease in LPO as well as the infarct volume compared to the control group. Sensory-motor impairment and neurological deficits were improved significantly as well. These results indicate that Iranian BP confers neuroprotection on the stroke-induced neuronal damage via an antioxidant mechanism which seems to be mediated by the endogenous antioxidant system.

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1. Introduction

Brain ischemia commences a twisted cascade of metabolic events results in cell death, some of which involve the production of nitrogen and oxygen free radicals such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), called oxidative stress [1]. Oxidative stress is one of the most prominent phenomena that plays a critical role in the pathogenesis of cerebral ischemia and a lot of subsequent post-stroke injuries [1,2]. Free radicals cause damage to lipids, proteins, and DNA through the involvement of other pathological processes (e.g. neuroinflammation) and reaction with cellular macromolecules which in turn results in necrosis or apoptosis [1,3,4]. Lipid peroxidation (LPO) is the oxidative degradation of polyunsaturated fatty acids which destroyed the cellular membrane phospholipids and ultimately cell dysfunction in numerous mammalian tissues, including the brain [5].

In order to reduce the oxidative stress-induced tissue damage, endogenous antioxidant systems have developed protective mechanisms including enzymatic [e.g. superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT)] and non-enzymatic (e.g. ascorbic acid, glutathione, and flavonoids) antioxidant processes [5,6]. Several enzymatic antioxidant

Abbreviations: SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde; BP, brown propolis; WEBPs, water-extracted brown propolis; LPO, lipid peroxidation; TTC, 2, 3, 5-triphenyltetrazolium chloride; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; TBA, thiobarbituric acid; KhWEBP, water-extracted brown propolis from Khorasan Razavi province of Iran; KeWEBP, water-extracted brown propolis from Kerman province of Iran.

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molecules (SOD and GPx) and also malondialdehyde (MDA) level are commonly being used as potential oxidative stress biomarkers and LPO index respectively [5]. The assessment of oxidative stress biomarkers and LPO index in cerebral ischemia is very important for a better understanding of its pathophysiology and the neuroprotective mechanisms of plant extracts [5]. MDA is an end-product formed during LPO and is considered as an estimate of the oxidative stress-induced cell dysfunction [5,7]. SOD is the most studied antioxidant enzyme in brain ischemia. It converts superoxide anions (O_2^-) into H_2O_2 in the existence of copper and iron (first step of the antioxidant pathway) whereas GPx converts H_2O_2 to H_2O (second step along the antioxidant pathway) [1,8].

Propolis is a bioactive bee product of plant origin which is rich in numerous compounds especially polyphenols [9,10]. It possesses a diversity of botanical source, which is consistent with the native flora. Thus the combinations and properties of propolis may also be different [10,11]. The difference in the chemical composition of propolis collected from various regions makes a set of biological activity such as anti-inflammatory [12] antioxidant [13,14] and neuroprotective [9].

It is a folk medicine employed by human since the ancient period for its pharmaceutical properties [11,14]. There are some studies about the therapeutic effects of propolis on biochemical and physiological changes in organisms [6]. Propolis has attracted much attention in recent years as a protective compound due to its antioxidant property [6,13]. It possesses neuroprotective effect in the retina in vitro and in vivo through inhibiting of oxidative stress responses [6]. Propolis has been categorized into three major classes depends on its colure of which are green, red and brown [15]. Green and red propolis, from tropic regions, has been broadly studied due to their chemical characteristics and biological activities [9,16]. Several in vitro and in vivo studies have proved that water and ethanol extracts of propolis from Brazil [9] China [17] and Turkey [6] have antioxidant properties and protect the neuron against oxidative damage at least partly by inhibiting the production of free radicals and lipid peroxidation (LPO) [6,9,17]. In addition, propolis contains a wide variety of natural phenolic compounds, mainly flavonoids. These compounds, recognized as multifunctional molecules, have potent antioxidants and anti-inflammatory effects which might be advantageous in multi-causal diseases treatment such as stroke [14,18]. Variation in the phenolic content of propolis is also mainly attributable to the difference in the preferred regional plants collected by honeybees [18,19]. The protective effect of brown propolis (BP) of Iran following stroke has not been previously reported. The present study was conducted to assess the chemical characterization and phenolic content of BP, from two regions of Iran, as well as to examine the effect of water-extracted BP (WEBPs) on stroke outcome, oxidative stress biomarkers (SOD and GPx activity) and LPO index (MDA content) in a mouse model of permanent middle cerebral artery occlusion (MCAO).

2. Material and methods

2.1. Chemicals and reagents

2, 3, 5-triphenyltetrazolium chloride (TTC) was obtained from Merck KGOA (Darmstadt, Germany). Oxidative stress biomarkers assay kits (SOD, GPx, and MDA) were purchased from ZellBio GmbH Ulm (Deutschland, Germany). Ketamine hydrochloride, xylazine, diethyl ether and formaldehyde were obtained from Sigma-Aldrich (Poole, Dorset, UK).

2.2. Water-extracted brown propolis (WEBPs) preparation and total phenolic determination

Brown propolis was collected from beehives at the village Lalehzar in Kerman province and from another region (Hezar-Masjed) in Khorasan Razavi, Iran, in late winter 2015 and then was sent to Soren Tech Toos, Inc. Ltd. (Mashhad, Iran). Extraction of samples and evaluation of their total phenolic content, using the Folin–Ciocalteu colorimetric assay plus minor modifications, were carried out by a company-specific method [15,20,21]. Briefly, two brown propolis samples were collected from beehives located in Khorasan Razavi and Kerman provinces, Iran, in late winter 2015. For better extraction of propolis compounds in form of water extract, the following protocol was performed. In the first step, samples were extracted by ethanol. Propolis was added into 3 vols of ethanol and mixed for 18 h at 1 °C. The mixture was centrifuged at 7000 rpm for 15 min at 20 °C. The supernatant was collected and the pellet was re-extracted with 100 ml ethanol. After mixing the obtained supernatants, it was used for water extraction step. 100 ml ethanol extracts of propolis were poured into the 500-ml flask and kept on a magnetic stirrer. 400 ml of 20 mM phosphate buffer was added to the ethanol extract of propolis and mixed for 20 min at 20 °C. The mixture was centrifuged at 7000 rpm for 15 min and the supernatant was collected. Water soluble compounds remained in the aqueous phase which was a solution with light yellow color and the lower part was dark brown and very sticky. The water extracts of propolis were freeze dried in order to remove ethanol and to enhance the sample concentration. Total phenolic content was declared as mg per gram (mg/g).

2.3. Gas chromatography-mass spectrometry (GC–MS) for chemical characterization

The GC–MS analysis was executed by an Agilent Gas Chromatograph 6890 added to an Agilent 5973N mass spectrometer instrument equipped with a 30 m long, 0.25 mm id, 0.25 mm film thickness BPX5 capillary column. The temperature was put from 50 to 300 C at a rate of 3 C/min. Helium was used as a transporter gas, flow rate 1 ml/min. The injection was done in split/spliless manner at 220 C, ionization voltage 70 eV. The chemical characterization was determined by chemstation software and Kovats's retention index as follows:

$$I = 100 \times \left[n + \frac{log(t'_{r(unknown)}) - log(t'_{r(n)})}{log(t'_{r(N)}) - log(t'_{r(n)})} \right]$$

Where: I = Kovats retention index, n = the number of carbon atoms in the smaller n-alkane N = the number of carbon atoms in the larger n-alkane and t'_r = the adjusted retention time.

2.4. Animals and treatments

Animals were handled in accordance with the criteria outlined in the Guide for Care and Use of Laboratory Animals (US NIH publication, revised 1996; http://books.nap.edu/readingroom/books/labrats/). Experimental procedures were approved by the Research Ethics Committee of the Rafsanjan University of Medical Sciences, Iran (approval code: IR.RUMC.REC.1394.165). A total of 72 adult male Balb/C mice weighing 30–35 g was maintained on a 12-h light-dark cycle with food and water available ad libitum. After acclimatization, animals were randomly divided into 6 groups of 12 mice each (6 for infarct volume and behavioral tests and 6 for oxidative stress biomarkers and MDA content) as follows: group 1: sham (craniotomy without MCAO), group 2: control (animals subjected to ischemia and treated with vehicle), and four WEBPs –treated groups. The WEBPs were achieved from two different

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