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Assessment of DNA damage and lipid peroxidation in diabetic mice: Effects of propolis and epigallocatechin gallate (EGCG)

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ABSTRACT

There is growing recognition that polyphenolic compounds present in many plants and natural products may have beneficial effects on human health. Propolis – a substance produced by honeybees – and catechins in tea, in particular (−)-epigallocatechin gallate (EGCG), are strong antioxidants that appear to have anti-obesity and anti-diabetic effects. The present study was designed to elucidate the anti-diabetic effect of the water-soluble derivative of propolis (WSDP), which contains phenolic acids as the main compounds, and EGCG in alloxan-induced (75 mg/kg, *iv*) diabetes in mice. Intraperitoneal administration of EGCG or propolis at doses of 50 mg/kg body weight (bw) to diabetic mice for a period of 7 days resulted in a significant increase in body weight and in haematological/immunological blood parameters, as well as in 100% survival of the mice. A significant decrease in lipid peroxidation in liver, kidney and brain tissue was also observed in diabetic mice treated with these two agents. Additionally, EGCG and propolis clearly reduced DNA damage in peripheral lymphocytes of diabetic mice. Our studies demonstrate the anti-oxidative and anti-inflammatory potential of WSDP and EGCG, which could exert beneficial effects against diabetes and the associated consequences of free-radical formation in kidney, liver, spleen and brain tissue. The results suggest that dietary supplementation with WSDP or EGCG could potentially contribute to nutritional strategies for the prevention and treatment of *diabetes mellitus*.

1. Introduction

Diabetes is a chronic metabolic disorder that continues to present a major worldwide health problem. In view of its epidemic proportions, diabetes stands out as one of the most urgent medical problems of the 21st century [1]. According to the World Health Organization (WHO), in the year 2000 the prevalence of *diabetes mellitus* was 171,000.000, and it is estimated that the prevalence of the disease will reach 439,000.000 by 2030 [2]. It is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action associated with chronic hyperglycaemia and disturbances of carbohydrate, lipid, and protein metabolism.

The development of insuline-dependent *diabetes mellitus* (IDDM) is a complex multi-factorial process involving environmental, dietary, viral, genetic, and autoimmune factors. Diabetes is associated with the generation of reactive oxygen species (ROS) causing oxidative damage, in particular to heart, kidney, eyes, nerves, liver, small and large blood vessels and the gastrointestinal system [1,3,4]. ROS have been implicated as important causes of β-cell damage related to IDDM.

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) is widely used to provoke experimental diabetes *via* selective induction of dysfunction of pancreatic β-cells. The mechanism of alloxan-induced diabetes is generally thought to involve ROS, which initiate damage that ultimately leads to β-cell death. Alloxan can induce several processes: oxidation of –SH groups, inhibition of glucokinase, generation of free radicals and disturbances in calcium homeostasis [5]. Such data support the use of alloxan-induced diabetes as a model for the status of oxidative stress experienced by diabetic patients.

Diabetes and hyperglycaemia can be sources of DNA damage *via* the oxidation of DNA bases and sugar-phosphate binding sites [6].

Abbreviations: AO, acridine orange; EGCG, epigallocatechin gallate; EtBr, ethidium bromide; HPLC, high-performance liquid chromatography; IDDM, insuline-dependent *diabetes mellitus*; LMP, low melting-point; LPO, lipid peroxidation; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MDA, malondialdehyde; MN, micronucleus; NMP, normal melting-point; ROS, reactive oxygen species; TBA, thiobarbituric acid; TC, total cholesterol; TG, triglyceride; WSDP, water-soluble derivative of propolis.

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The occurrence of these alterations can result in mutagenic effects and/or DNA-replication arrest, and could be associated with risks for developing cancer in *diabetes mellitus* patients [1,7]. As a result of the plethora of scientific evidence proposing the involvement of oxidative stress in the pathogenesis of diabetes and its complications, interest has grown in the use of natural antioxidants as a new strategy for alleviating the oxidative damage associated with diabetes.

Propolis is a complex resinous material collected by honeybees from buds and exudates of certain plant sources neighbouring their hives. Propolis consists of sap, bark and bee excreta, and accumulates in bee hives. The chemical consistency of propolis is highly dependent on the flora of the region from where it is collected [8–10]. Propolis contains at least 200 compounds that have been identified in different samples, with more than 100 being present in any given sample. These include fatty and phenolic acids and esters, substituted phenolic esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones), terpenes, β-steroids, aromatic aldehydes and alcohols, and derivatives of sesquiterpenes, naphthalene and stilbenes [11–18]. The main types of flavonoid are rutin, quercetin, galangin [19] and caffeic acid phenethyl ester [14–16,18,20]. Propolis possesses a broad spectrum of biological activities and has a historical utilization in folk medicine. Thus, it is extensively used in health food, pharmaceutical preparations [21–24] and beverages with the aim of maintaining or improving human health [11–13,17]. It was reported that propolis enhances immune system activities [23–26], oxygen-radical scavenging [27–29], antimicrobial, anti-inflammatory [30] and anti-tumour activities [26,31–34].

Tea catechins, especially (−)-epigallocatechin gallate (EGCG), lower the incidence of cancers [35], collagen-induced arthritis [36], oxidative stress-induced neurodegenerative diseases [37], and cytokine-induced inflammation *in vivo* [35]. Also, EGCG can reduce body weight and body fat [35]. In addition, the role of EGCG in the regulation of diabetes and blood glucose levels [35] is beginning to emerge.

Despite the importance of EGCG and propolis, relatively little is known about the mechanism of their action in regulating body weight and diabetes, as well as their capacity to protect blood, liver, and kidney cells from alloxan-induced DNA damage. In this study, we investigated DNA damage evoked by alloxan in normal mouse peripheral blood lymphocytes, liver and kidney cells by means of the alkaline comet assay. The alkaline comet assay introduced by Singh et al. [38] is a rapid and sensitive procedure for quantifying DNA lesions in mammalian cells. Not only single-strand breaks but also alkali-labile sites, DNA cross-links, and incomplete excision-repair sites can be detected [39]. To search for the mechanisms underlying this effect we checked the efficacy of the free-radical scavengers WSDP and EGCG in modulating the DNA-damaging potential of alloxan. In addition, micronucleus (MN) formation in reticulocytes as markers of cyto- and genotoxicity, and an oxidative stress marker such as lipid peroxidation (LPO) were evaluated to explore the mechanism of the protective effects of WSDP and EGCG.

2. Materials and methods

2.1. Animals and reagents

Male CBA inbred mice (2–3 months old, weighing 20–25 g) were obtained from Department of Animal Physiology, Faculty of Science, University of Zagreb. The animals were kept in individual cages during the experiment under a 12 h/12 h light/dark cycle. They were fed a standard laboratory diet (4 RF 21, Mucedola, Italy) and tap water *ad libitum*. Maintenance and care of all experimental animals were according to the guidelines in force in the Republic of Croatia (Law on the Welfare of Animals, N.N. #19, 1999) and carried out in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH) 86-123. The strips to test blood glucose (Betachek Visual blood-glucose test strips, Australia); the kits of total cholesterol (TC) and triglyceride (TG) Cholesterol Reagent and Triglycerides Reagent (Thermo Electron, Australia), alloxan and EGCG were from Sigma, USA.

2.2. Water-soluble derivative of propolis (WSDP)

A water-soluble derivative of propolis (WSDP) was prepared by the method described previously [25]. Briefly, propolis from beehives kept at the outskirts of Zagreb (Croatia) was extracted with 96% ethanol. The extract was filtered and evaporated to dryness in a vacuum evaporator. The resultant resinous product was added to a stirred solution of 8% L-lysine (Sigma) and freeze-dried to yield the WSDP, a yellow-brown powder. The WSDP was stored under sterile conditions at −20 °C to minimize bacterial contamination. Before use the WSDP was dissolved in distilled water.

2.3. HPLC analysis of WSDP

WSDP was analyzed by high-performance liquid chromatography (HPLC) according to the method described by Pietta et al. [40] with minor modifications, as described below. A Shimadzu AV system with UV–visible detector (model SPD-10A) and a VP-class software v3.0 was used for the analysis. Before injection into the column, WSDP was dissolved in mobile phase A (4.64 mg/mL) and filtered through nylon 0.45-μm filter (Nalgene®, USA). Twenty microlitres were injected onto the C_{18}-column 100 × 0.2-mm with 5-μm particle size (Bischoff Chromatography, Germany). HPLC separations were performed by elution of the column with a mixtures of (A) 0.2% (v/v) formic acid (J.L. Baker, USA) in ultra-pure water (Millipore, UK) and (B) 100% acetonitrile (LiChrosolv®, Merck, Germany) with the following gradient: 10–40% (B) in 40 min, 40–55% (B) in 10 min, and then reducing (B) to 10% in 10 min. The flow rate was set to 1.5 mL/min at an oven temperature of 30 °C. The chromatographic data were acquired simultaneously at 290 nm. For the quantitative analysis of constituents in WSDP, caffeic acid, chlorogenic acid, *p*-coumarinic acid, ferulic acid, isoferulic acid and the flavonoid-aglycones: quercetine-dihydrate, chrysine, galangin, naringenin were purchased from Sigma. Working solutions were prepared in methanol (Merck, Germany) to obtain five calibration concentrations ranging between 2 and 200 μg/mL with $r^2 > 0.994$. All solvents used were of HPLC-grade.

2.4. Experimental design

Forty-four mice were used in the experiment, as follows: Group (i): control animals (normal, non-diabetic mice, $n = 5$) received 0.5 mL distilled water intraperitonealy (*ip*) per day by injection for 7 days; Group (ii): alloxan controls; injected *iv* with alloxan in a single dose of 75 mg/kg body weight; served as untreated diabetic group ($n = 13$); Group (iii): received water extract of propolis (WSDP) *ip* in a daily dose of 50 mg/kg bw for 7 days, starting 2 days after alloxan injection; served as propolis-treated diabetic group ($n = 13$); Group (iv): received EGCG *ip* in a daily dose of 50 mg/kg bw for 7 days, starting 2 days after alloxan injection; served as EGCG-treated diabetic group ($n = 13$). Five mice of each group were killed on the 9th day after alloxan injection by cervical dislocation. After disinfection of the external abdomen, each animal was inoculated with 3 mL of saline solution and after gentle agitation of the abdominal region the solution containing peritoneal cells was removed for cellular evaluation. The following parameters were analyzed: the total number of cells present in the peritoneal cavity, functional activity of macrophages, haematological parameters, LPO, micronucleus frequency and DNA strand breakage. The remaining animals, 8 animals of each diabetic group (untreated diabetic group, WSDP- and EGCG-treated diabetic group) were used for the survival analysis.

2.5. Induction of experimental diabetes and determination of glucose level in serum

Diabetes was induced in Swiss albino mice by a single intravenous injection of alloxan monohydrate (75 mg/kg bw, *iv*) in total volume of 0.5 mL of saline solution, freshly prepared. The blood-glucose level was measured before alloxan injection and 48 h after treatment, to monitor the immediate diabetogenesis. After 48 h, the animals with blood-glucose levels above 11 mmol/L were selected for the study (diabetic mice) and then treated with WSDP or EGCG. Blood-glucose levels were determined by test strips of blood glucose (Betachek Visual blood glucose test strips, Australia). The diabetic animals showed the following signs of the condition: polydipsia (abnormal thirst), polyuria (increased urine volume), weight loss (due to mean mass loss), asthemia (weakness due to the inability to use glucose as a source of energy), dehydration (due to the animal body's attempt to eliminate the excess blood glucose, as the normal process of storing glucose in the body cells is impaired).

2.6. Effect of WSDP or EGCG on body weight in alloxan-induced diabetic mice

During the study period of 50 days the mice were weighed every 4 days on an electronic balance, and their body weights were recorded. From this data, the mean change in body weight was calculated.

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