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Original article

Protective effects of propolis on methotrexate-induced testis injury in rat

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ABSTRACT

Propolis is an adhesive substance which is collected and used by honeybees. Propolis is a potent antioxidant and a free radical scavenger. This study was designed to determine whether propolis could protect against dysfunction and oxidative stress induced by methotrexate-induced injury in rat testis.

A total of 40 male Wistar albino rats were divided into four groups: group 1 was the untreated control. On the eighth day of the experiment, groups 2 and 3 received single intraperitoneal injections of methotrexate (MTX) at 20 mg/kg. Groups 3 and 4 received 100 mg/kg/day propolis (by oral gavage) for 15 days by the first day of the experimental protocol. Then the rats were decapitated under anesthesia, and their testes were removed. The histopathological and biochemical analysis along with apoptosis assessment of testis tissues were compared. Immunohistochemical analysis of Heat shock protein-70 (HSP-70) and Proliferating Cell Nuclear Antigen (PCNA) were performed. The phenolic characterization of propolis was performed by Liquid chromatography–mass spectrometry (LC–MS/MS).

Methotrexate caused tended to increase in malondialdehyde level and in the number of apoptotic cells; it also caused a decrease in MSTD and JTBS, PCNA and HSP-70 expression and xanthine oxidase levels in group 2. Propolis prevented the rise in malondialdehyde, xanthine oxidase levels and HSP-70 expression and improved testicular morphology and JTBS.

It was found that, methorexate gives rise to serious damage in the testes and propolis is a potent antioxidant agent in preventing testicular injury.

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1. Introduction

Cancer is one of the major causes of death in the world. Chemotherapy is one of the most widely used methods for the treatment of cancer. However, chemotherapy often causes significant unwanted toxicity [1]. Chemotherapy drugs are more toxic than any other pharmaceutical agents, and many of these drugs are cytotoxic agents acting on all dividing cells both normal and cancer [2]. Methotrexate (MTX) is a folic acid antagonist having antiproliferative, anti-inflammatory and immunomodulatory effects used in the treatment of many diseases including cancer, rheumatoid arthritis and psoriasis [3–6]. Like other chemotherapeutic agents, MTX has toxic effects on dividing cells. However, the mechanisms of MTX-induced toxicity have not been completely determined [7]. Various hypotheses have been proposed due to oxidative stress. In the pathogenesis of MTX-induced testicular damage, it has been reported that oxidative stress plays an important role [8]. MTX has testicular toxicity and this side effect which may cause subsequent infertility. The administration of MTX has been reported to cause a decrease in sperm number, disorganization in the seminiferous tubules of the testis, and sperm DNA damage [6,9].

Propolis is an adhesive substance which is collected and used by honeybees [10]. Propolis has been utilized in traditional medicine for decades. Propolis comprises more than 300 pharmacologically active compounds including polyphenols, terpenoids, steroids, and amino acids [11]. It has antioxidant, anticancer, anti-inflammatory, immunoregulatory, antibacterial and bacteriostatic activities [12]. It also has s strong cytoprotective effect against different exogenous toxic stimuli [10].

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Many types of cell stress, including extreme temperature, hypoxia, inflammation, ischemia, oxidative stress and toxic compounds, cause the synthesis of heat shock proteins (HSPs) [13]. HSPs are classified into subgroups based on molecular weight. The HSP-70 stress protein group is related most directly with the cytotoxic effect of chemicals [14]. HSP-70 and its isoforms involve different stages of spermatogenesis.

The purpose of this study was to investigate the role of oxidative stress in MTX-associated testicular damage and also to show the probable protective effects of propolis against MTX-induced testicular damage at the histopathological, immunohistochemical and biochemical levels.

2. Material and methods

Sexually mature, 8 weeks old, male Wistar rats which were obtained from the Hakan Çetinsaya Experimental and Clinic Research Center, Erciyes University, Kayseri, Turkey, were used for this study. They were housed in plastic cages placed in a well-ventilated rat house and allowed ad libitum access to rat chow and water and were subjected to a natural photoperiod of 12-h light: dark cycle. This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Erciyes University (Permit Number: 13/83). The rats were randomly assigned to four groups of eight rats per group. Group 1 was the experimental control group and received 0.1 ml DMSO by oral gavage. The rats in group 2 received MTX. Group 3 rats were treated with both MTX and propolis. Group IV rats were treated with propolis. Propolis was dissolved in DMSO. On the first day of the experimental protocol, groups 3 and 4 received 100 mg/kg/day propolis (oral gavage) for 15 days. On the eighth day of the experiment, groups 2 and 3 received single intraperitoneal injections of MTX at 20 mg/kg (Methorexate DBL 500 mg/20 ml, Hospira UK Limites, Warwickshire, UK). The total duration of the experiment was 15 days.

At the end of the experimental period, the animals' blood samples were collected from the inferior vena cava and testis tissues were removed quickly after i.p. injection of 75 mg/kg ketamine and 10 mg/kg xylazine anesthesia. Testis tissue was used for biochemical analysis or for histology. Serum was separated by centrifugation (during 10 min at 3000 rpm) from the blood samples.

2.1. Ethanolic extraction of propolis

Propolis was firstly cooled and powdered by a warning blender (Warning Products, Torrington, CT, USA). The propolis was extracted using ethanol (96%) at room temperature for one week. To extract 30 g propolis was dissolved with 100 ml ethanol. After extraction the sample was filtered by paper filter and extract filtrated was evaporated to remove the residual solvent using a rotary evaporator at 40 °C. The dry extract was used to analysis phenolic composition.

2.1.1. Preparation of dry propolis extract for LC–MS/MS

The dry propolis extract were diluted to 1000 mg/l in methanol and the mixture was filtrated with 0.2 μm microfiber filter prior to LC–MS/MS analysis.

2.1.2. LC–MS/MS instrumentation conditions

LC–MS/MS analyzes of the phenolic compounds of propolis were performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The liquid chromatography was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven and SIL-30AC autosampler. The chromatographic separation was performed on a C18 reversed-phase Inertsil ODS-4(150 mm × 4.6 mm, 3 μm) analytical column. The temperature of column was fixed at 40 °C. The elution gradient consisted of mobile phase A (water, 5 mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium formate and 0.1% formic acid). The gradient program with the following proportions of solvent B was applied *t* (min), B%: (0, 40), (20, 90), (23.99, 90), (24, 40), (29, 40). The flow rate of solvent was maintained at 0.5 ml/min and injection volume was 4 μl. MS detection was performed using Shimadzu LCMS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. LC–MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan). The multiple reaction monitoring (MRM) mode was used to quantify the analyzes: the assay of investigated compounds was performed following two or three transitions per compound, the first one for quantitative purposes and the second and/or the third one for confirmation. The optimum ESI conditions were interface temperature: 350 °C, DL temperature: 250 °C, heat block temperature: 400 °C, nebulizing gas flow (nitrogen): 3 l/min and drying gas flow (nitrogen): 15 l/min [15].

Table 1
Phenolic compounds of propolis extract.

Phenolic compound	mg/g Dry extract
Hesperidin	1.89 ± 0.09
Quinic acid	0.39 ± 0.02
Malic acid	0.45 ± 0.02
trans-Aconitic acid	0.08 ± 0.00
Gallic acid	0.72 ± 0.04
Chlorogenic acid	0.18 ± 0.01
Protocatechuic acid	1.46 ± 0.07
Tannic acid	0.23 ± 0.01
trans-Caffeic acid	37.97 ± 1.97
Vanillin	50.18 ± 2.46
p-Coumaric acid	2.99 ± 0.15
Rutin	0.01 ± 0.00
Hyperoside	0.01 ± 0.00
Myricetin	0.09 ± 0.01
Fisetin	0.06 ± 0.00
4-Hydroxybenzoic acid	0.05 ± 0.00
Salicylic acid	0.03 ± 0.00
Quercetin	3.18 ± 0.23
Kaempferol	2.09 ± 0.11
Naringenin	10.61 ± 0.58
Hesperetin	0.20 ± 0.01
Luteolin	0.56 ± 0.04
Apigenin	18.28 ± 0.97
Rhamnetin	8.71 ± 0.53
Chrysin	168.91 ± 8.95

2.2. Histopathological evaluation

The testicular tissue was examined and evaluated in random order under blinded conditions with standard light microscopy by a histologist. Mean seminiferous tubule diameter (MSTD) was measured in micrometers (Analysis LS Research Program). More than 20 seminiferous tubular sections per testis were each given a Johnsen's score (JTBS) from 1 to 10 as described previously [16]. In this classification system, all tubular sections in each section of the testicular biopsy are evaluated systematically and each is given a score from 1 to 10. Complete spermatogenesis with many spermatozoa present is evaluated as score 10.

2.3. Immunohistochemistry

HSP-70 and Proliferating Cell Nuclear Antigen (PCNA) expression was detected immunohistochemically using a polyclonal

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