Biomedicine & Pharmacotherapy 95 (2017) 252–263

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Indian propolis ameliorates the mitomycin C-induced testicular toxicity by reducing DNA damage and elevating the antioxidant activity

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ARTICLE INFO

Keywords:
Testicular toxicity
Chemoprotection
Indian propolis
Mytomycin C
Spermatozoa
DNA damage

ABSTRACT

Development of excellent curative therapy for most of the malignancies has resulted in a growing population of cancer survivors who are at increased risk for a variety of health problems including infertility. Therefore, fertility preservation has become an important issue during cancer treatment in recent years. Combination therapy with natural agents such as vitamins, antioxidants, dietary supplements, and plant products are considered as an attractive option to mitigate normal tissue toxicity imparted by chemotherapy. The aim of the present study was to explore the beneficial effect of hydroethanolic extract of Indian propolis (HEIP) on mitigating mitomycin C (MMC)-induced testicular damage and its mechanism of action. Healthy adult male mice were injected intraperitoneally with saline, MMC, HEIP and HEIP followed by MMC after 1 h. The animals were dissected at 35 days after various treatments to analyze testicular function. MMC administration resulted in significant reduction in testicular function in a dose-dependent manner at 35 days after treatment which significantly improved by HEIP pre-treatment. At 24 h after treatment, MMC induced significant increase in oxidative stress, γ-H2AX foci and expression of RAD51 and KU80 in testicular cells. Prior treatment with HEIP decreased the oxidative stress, reduced DNA damage and restored the testicular testosterone and inhibin B level. In conclusion, co-administration of Indian propolis extract may play a promising beneficial role in fertility preservation of males undergoing chemotherapy.

1. Introduction

For more than seven decades, chemotherapy still stands as the first line of therapy for all types of cancers [1]. Chemotherapeutic agents activate the cell death pathways in the cancer cells, and in rapidly proliferating somatic cells. Apart from causing cell death, they induce debilitating mutations and long-term DNA/cellular damages in the germ cells and somatic cells [2].

Mitomycin C (MMC) is a potent DNA alkylating agent, used as first line of drug in treatment of pancreatic, gastric, breast, prostate, and bladder cancers [3]. MMC induces cell death by forming DNA adducts, DNA cross links, RNA adducts and its cross links [3–5]. Once the adducts and cross links are formed, in the subsequent replications it creates mutations and thus resulting in DNA damage. MMC in its original form is not cytotoxic; it needs to be activated by enzymatic reduction in the biological system. It forms bis-electrophile byproduct which leads to alkylation of DNA, RNA and proteins [6,7]. A previous study has shown that a reactive byproduct of MMC formed after enzymatic reduction can inhibit rRNA by reacting with the selenothiol moiety of thioredoxin reductase [7]. These highly reactive bis-electrophiles are responsible for the damages incurred to the cells. Earlier studies have shown that bone marrow, cardiac system, neurological, nephrological and gonadal systems are acutely and chronically affected by the chemotherapy [8]. Several studies have shown that naturally or/and chemically derived adjuvants can effectively protect the reproductive systems from the chemical insults caused by chemotherapy. These chemo-protective adjuvants not only provide protection against the damages caused by chemo agents, but also help in good quality life during and after the

Abbreviations: HEIP, hydroethanolic extract of Indian propolis; MMC, mitomycin C; PBS, phosphate buffer saline; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; EBSS, Earles balanced salt solution; CMA3, chromomycin A3; SCD, sperm chromatin dispersion test; NMPA, normal melting point agarose; LMPA, low melting point agarose; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; EDTA, ethylenediaminetetraacetic acid; DTNB, 5, 5′-dithiobis (2-nitro-benzoic acid); TCA, trichloroacetic; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substance; BSA, bovine serum albumin; PI, propidium iodide

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http://dx.doi.org/10.1016/j.biopha.2017.08.065
Received 14 May 2017; Received in revised form 11 August 2017; Accepted 11 August 2017

chemotherapy [9–11].

Propolis is a complex resinous mixture containing more than 300 compounds collected by honey bees from various flora to construct the hive. The composition in the propolis varies between the geographical locations due to the plant diversity. The compositions of propolis which are having biological activity include flavonoids, phenolics, and aromatic compounds, they also constitute bee wax, terpenes and volatile oils [12]. Human beings have used propolis since ancient times for treating variety of diseases [13] which is supported by *in vivo* and *in vitro* studies suggesting them to have a plethora of biological activities [13,14]. In our previous study, we have reported that propolis mitigates MMC-induced bone marrow toxicity [15]. The present study was taken up to further explore whether hydroethanolic extract of Indian propolis (HEIP) can help in recovery of testicular function following MMC administration and augment testicular toxicity in mice.

2. Materials and methods

2.1. Animal model

Swiss albino male mice aged 8 weeks were obtained from inbred colonies maintained in central animal facility, Manipal University. The animals were maintained under standard conditions of temperature (23 ± 2° C), humidity (55 ± 5%), light (12:12 h of light) and dark, food and water *ad libitum*. A prior approval was obtained from the Institutional Animal Ethical Committee, Kasturba Medical College, Manipal University, Manipal (IAEC/KMC/80/2013) to carry out the experiment.

2.2. Preparation of hydroethanolic extract of Indian propolis (HEIP)

The collection of Indian propolis and method of extraction has been described in our earlier report [15]. Briefly, after complete separation of honey from locally collected bee hive, the resinous material was cut into small pieces and enclosed in a Whatman filter paper. Cold extraction of propolis was prepared by immersing bee hive in 50% ethanol for 48 h at room temperature. The extract thus collected was filtered using cotton bed, concentrated by evaporating the alcohol, freeze-dried by lyophilization and stored at 4 °C. For the experiment, propolis solution was prepared every time by dissolving the dried extract in phosphate buffered saline (PBS, pH 7.4) just before each injection.

2.3. Mitomycin C (MMC)

The Mitomycin (MMC, Molecular formula, $C_{15}H_{18}N_4O_5$) drug was obtained from Biochem Pharmaceuticals industries limited (Mumbai, India) and then reconstituted with sterile PBS to obtain a concentration of 1 mg/mL. Fresh stock solution was prepared each time and protected from exposure to light.

2.4. Experimental outline

Male Swiss albino mice (8 weeks old, body weight 30 ± 2 g) were divided into 4 groups of 12 animals each and treated as follows:

- Vehicle Control: Mice were intraperitoneally (i.p) injected with PBS.
- MMC alone: Injected with MMC at a dose of 2, 4 and 8 mg/kg body weight, single dose (i.p).
- HEIP: Injected with 400 mg/kg of HEIP, single dose (i.p). This dose was selected based on our previous report [15].
- HEIP + MMC: Injected with Single dose of HEIP (400 mg/kg) followed by single dose of MMC (2, 4 and 8 mg/kg) after a gap of 1 h.
- HEIP: Injected with 400 mg/kg of HEIP, daily, 5 days a week for 4 weeks.
- HEIP + MMC: Injected with Single dose of HEIP (400 mg/kg) followed by single dose of MMC (8 mg/kg) after a gap of 1 h. HEIP injection was continued further, 5 days a week, for 4 weeks.

The mice were sacrificed at various time intervals after MMC injection (7, 21 and 35 days) to collect the testes and epididymis. The testis weight was recorded and the right-side testis was stored in Bouin's fixative for histological analysis. The left side testis was stored in −80 °C in phosphate buffered saline (PBS) for the analysis of testicular hormones (testosterone and inhibin), lipid peroxidation, reduced glutathione (GSH) level and the activity of super oxide dismutase (SOD) and catalase (CAT). The cauda epididymis was gently squeezed in a Petri dish containing EBSS medium. Sperm density, motility pattern and head morphology was assessed by methods described elsewhere [16,17].

2.5. Sperm chromatin maturity assessment by chromomycin A3 (CMA3) staining

Sperm suspension was placed on a coverslip, treated with 100% ethanol and air dried. Spermatozoa were later fixed in methanol/glacial acetic acid (3:1) for 20 min at 4 °C and then air dried at room temperature for 20 min. Each coverslip was treated with 10 µL of CMA3 solution (0.4 mg/mL) in McIlvaine's buffer (0.2 M Na_2HPO_4 and 0.1 M Citric acid, pH-7.0) for 20 min in dark and air dried [18]. The coverslips were inverted onto the mounting media (DAKO) placed over a clean glass microscopic slides. Minimum of 500 spermatozoa were scored under 400× magnification using fluorescence microscope. The spermatozoa with bright yellow fluorescence were considered as immature whereas those with dull yellow staining were considered as mature.

2.6. Sperm DNA damage assessment by sperm chromatin dispersion test

Sperm DNA damage was assessed by sperm chromatin dispersion (SCD) test [16]. Sperm count was adjusted to 5–7 millions/mL by using PBS and mixed with 1% Low Melting Point Agarose (Sisco Research Laboratories Ltd., Mumbai, India, Cat No. 9012-36-6). The sperm suspension was layered on slide pre-coated with 0.75% NMPA Normal Melting Point Agarose (Sisco Research Laboratories Ltd., Mumbai, India, Cat No. 9012-36-6). Slides were then covered with coverslip to allow the gel to solidify. After removing the cover slip the slide was subjected to denaturation using 0.08N HCl solution for 7 min. Then it was subjected to lysis by placing in lysis solution 1 (0.4 M Tris, 20 mM DTT, 1% SDS, 50 mM EDTA, pH 7.5) for 20 min followed by lysing solution 2 (0.4 M Tris, 2 M NaCl) for 15 min in dark and finally in neutralization solution (0.4 M Tris) for 5 min. The slides were dehydrated using gradient ethanol solutions (70%, 90% and 100%) and air dried. The slides were then stained with ethidium bromide (2 µg/mL) for fluorescence microscopic analysis and a minimum of 500 spermatozoa were scored under 400× magnification. The sperm with large halo, medium halo, no halo and fragmentation were scored separately. Spermatozoa with no halo, small hallo and fragmentation were considered as DNA damaged spermatozoa and expressed in percentage.

2.7. Testis histology

Testicular tissues were fixed in Bouin's fixative for 48 h and stored in 70% ethanol till further use. For histopathology, the tissues were dehydrated using alcohol in the order of 70%, 95% and absolute alcohol. The tissues were later embedded in paraffin and 5 µm thin sections were taken using a rotatory microtome. The sections were fixed on poly L-lysine coated slides and air dried. The sections were deparaffinized using xylene, stained with hematoxylin and eosin followed by mounting in DPX. The slides were observed under a light microscope to assess the histological changes in the testes. Number of tubules, tubular diameter, number of tubules with complete spermatogenesis (having all population of spermatogenesis including mature spermatozoa in lumen