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The effect of natural antioxidants in cyclophosphamide-induced hepatotoxicity: Role of Nrf2/HO-1 pathway

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ABSTRACT

Hepatotoxicity induced by cyclophosphamide (Cyclo) is a major concern in clinical practice. This study was designed to investigate the possible cytoprotective effect of natural antioxidants as oleuropein and quercetin against Cyclo induced hepatotoxicity *via* the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway. Male Wistar rats were randomly divided into six groups and treated for 10 days as follow: Group I (Normal control) received saline, group II (Oleu control): received orally oleuropein 30 mg/kg/day, group III (Quer control): administered orally quercetin 50 mg/kg/day, group IV (Cyclo): received saline and injected with single intraperitoneal (i.p) dose of Cyclo 200 mg/kg at day 5, group V (Oleu ttt): treated with oleuropein plus Cyclo i.p. injection at day 5, and group VI (Quer ttt): treated with quercetin plus Cyclo i.p. injection at day 5. Injection of Cyclo showed marked increase in serum transaminases and alkaline phosphatase, hepatic malondialdehyde (MDA) and tumor necrosis factor-alpha (TNF-α) levels along with significant reduction in hepatic reduced glutathione (GSH), superoxide dismutase (SOD), and catalase levels in addition to down-regulation of hepatic Nrf2 and HO-1 expressions and reduction in hepatic nuclear Nrf2 binding activity when compared with normal group. Histopathological examination of Cyclo treated rats revealed hepatic damage. Both oleuropein and quercetin exhibited an improvement in the biochemical and histopathological findings. In conclusion, the natural antioxidants oleuropein and quercetin counteract the Cyclo induced hepatotoxicity through activation of Nrf2/HO-1 signaling pathway with subsequent suppression of oxidative stress and inflammation.

1. Introduction

Cyclophosphamide (Cyclo) is a cytotoxic oxazaphosphorine alkylating agent. It is widely used in clinical practice as a chemotherapeutic drug in the treatment of different types of neoplasms, and as an immunosuppressive drug in autoimmune diseases and organ transplantation [1]. Despite the effectiveness of Cyclo, its use is often limited by associated harmful fatal effects, including hepatotoxicity [2]. The exact mechanism of the hepatotoxicity induced by Cyclo remains unclear.

The cytotoxicity of Cyclo resulted mainly from its metabolic conversion in the liver to toxic metabolite acrolein. This conversion is associated with increased production of reactive oxygen species (ROS) which subsequently lead to oxidative stress, lipid peroxidation and cell damage [3]. Furthermore, it was reported that the imbalance between oxidant and antioxidant status lead to the production of pro-inflammatory mediators which contribute to liver damage after Cyclo injection [4]. Thus, many studies documented that oxidative stresses and inflammation work in concert and play an important role [5,6].

Cellular defense against oxidative stress is mediated and regulated by a stress sensor transcription factor called nuclear factor erythroid 2-related factor 2 (Nrf2) [7]. In normal conditions, Nrf2 is sequestered in the cytoplasm in association with its protein inhibitor Kelth-like ECH-associated protein-1(Keap-1). Under stress conditions, inducers like inflammatory, hypoxic, oxidative and xenobiotic stimuli [8] activate the Nrf2 in which dissociation from Keap-1 occurred and the dissociated Nrf2 translocated into the nucleus where it binds to the antioxidant response elements (ARE) which induces the transcription of specific genes coded for antioxidant and phase II detoxifying enzymes including superoxide dismutase, catalase, glutathione S-transferases and heme oxygenase-1 (HO-1) [8–10].

Heme oxygenase is the rate-limiting enzyme in heme breakdown process. It has two isoforms; the inducible HO-1 and the constitutive HO-2. The HO enzyme metabolizes the heme into biliverdin (transformed into bilirubin by biliverdin reductase), free iron and carbon monoxide (CO) [11]. Bilirubin and biliverdin are potent antioxidants while, CO has anti-inflammatory and antiapoptotic activities [12]. The HO-1 which mediated by Nrf2 signals plays an important role in defense mechanisms against oxidative damages and is activated by many

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stimuli involving hepatic oxidative stress [4,6,13,14]. Moreover, several studies documented that the activation of Nrf2/HO-1 signaling pathway could protect against Cyclo induced oxidative tissue damage [4,6,14].

A great attention has been focused on the use of dietary antioxidants in conjunction with chemotherapeutic drugs to alleviate their deleterious side effects and oxidative organ damage produced upon administration [15]. The polyphenols derived from natural plants could exert beneficial pharmacological effects like antioxidant, anti-inflammatory, antitumor, neuro-, and hepatoprotective properties [16,17]. Oleuropein is a phytochemical compound present in olive leaf, seed, and pulp [16] while, quercetin is a dietary flavonoid founds in our foods, as berries, onion, and apples [18]. Both oleuropein and quercetin are polyphenols and their chemical structure includes hydroxyl groups and double bonds that lead them to possess antioxidant activity and scavenge the free radicles in addition to their antiinflammatory effects [16,18,19].

The hepatoprotection of oleuropein and quercetin was reported in many animal models including; thioacetamide induced liver cirrhosis [20,21], cisplatin induced liver damage [15,22,23], and carbon tetrachloride (CCl_4) induced liver injury [24,25]. While their effect against Cyclo induced hepatic injury still to be identified. Thus, the aim of this study was to investigate the effect of natural antioxidants as oleuropein and quercetin against Cyclo induced hepatoxicity in rats and explore for the first time their hepatoprotective mechanism *via* possible involvement of Nrf2/HO-1 pathway.

2. Materials and methods

2.1. Animals

Forty eight male Wistar rats weighing 200 ± 20 g were bred and housed in controlled room temperature (25 ± 2 °C) with a 12 h light/dark cycle. They were fed with rat chow and water *ad libitum*. The protocol of this study was approved by the ethical committee in Mansoura University.

2.2. Experimental design

Animals were randomly divided into six groups of rats ($n = 8$ in each group):

Group I (Normal control): rats received saline for 10 days.

Group II (Oleu control): rats received oleuropein (Oleu, GNC, Pittsburgh, PA, USA, Code 196131) 30 mg/kg/day by oral gavage for 10 days.

Group III (Quer control): rats received quercetin (Quer, GNC, Pittsburgh, PA, USA, Code 092711) 50 mg/kg/day by oral gavage for 10 days.

Group IV (Cyclo): rats received saline for 10 days and injected with a single intraperitoneal (ip) dose of cyclophosphamide (Cyclo, Baxter Oncology GmbH, Halle, Germany) 200 mg/kg at day 5.

Group V (Oleu ttt): rats treated with oleuropein 30 mg/kg/day by oral gavage for 10 days plus Cyclo 200 mg/kg i.p. injection at day 5.

Group VI (Quer ttt): rats treated orally with quercetin 50 mg/kg/day for 10 days plus Cyclo 200 mg/kg i.p. injection at day 5.

The doses of oleuropein and quercetin were determined from recent studies that showed pharmacological beneficial effects [25–28] while, the used Cyclo dose was reported to induce hepatotoxicity [29].

2.3. Sample collection

At the end of the study, all animals were sacrificed and blood samples were collected and centrifuged at 4 °C for 15 min. Sera were kept at −20 °C as aliquots for further biochemical assay of liver enzymes. Liver samples were collected rapidly, a part of it was frozen immediately in liquid nitrogen and transferred to a −80 °C for homogenate preparation to be used in subsequent biochemical assays while the other part of the liver was stored in 10% neutral buffered formalin for histopathological examination.

2.4. Assessment of liver function tests

Liver enzymes including alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were estimated in serum by using commercially available kits provided by Bio-Diagnostic Company, Giza, Egypt.

2.5. Preparation of liver homogenate

Liver samples was homogenized in a 10-fold volume of ice-cold sodium, potassium phosphate buffer (0.01 M, pH 7.4) containing 1.15% KCl. The homogenates were centrifuged at 600 g at 4 °C for 10 min. The supernatant, referred to as homogenate, was stored at −80 °C until use.

2.6. Determination of hepatic oxidative stress markers

Reduced glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and catalase levels were estimated in hepatic tissue homogenate by colorimetric method using commercially available kits provided by Bio-Diagnostic Company, Giza, Egypt.

The GSH was determined as described by Beutler (1963) [30] and MDA was estimated as described by Ohkawa et al., (1978) [31]. In addition, SOD was measured as described by Nishikimi et al., (1972) [32] and the catalase was assessed as described by Aebi (1984) [33].

2.7. Assessment of tumor necrosis factor alpha (TNF-α)

The TNF-α level was determined by ELISA technique in hepatic tissue homogenate using a commercially available ELISA kit (Uscn Life Science Inc., Houston, USA) in accordance with the manufacturer's instructions.

2.8. Real time quantitative-polymerase chain reaction (qPCR) analysis for hepatic Nrf2 and HO-1

Total RNA was extracted from hepatic tissue homogenate using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to a standard protocol. The purity of the RNA (A260/A280) and its concentration were assessed spectrophotometrically. The isolated total RNA was converted into complementary DNA (cDNA) using SuperScript Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). Real-time PCR was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR® Green PCR Master Mix (Applied Biosystems) in a final volume of 10 µl with the following thermal cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The sequences of PCR primer pairs used for Nrf2 was: forward primer: 5′-ACGGTGGAGTTCAATGAC-3′; reverse primer: 5′-GAAGAATGTGTTGGCTGTG-3′, HO-1 was: forward primer: 5′-AGAGCCTGCAGCTTCTCAGA-3′, reverse primer: 5′-ACAAA GTCTGGCCATAGGAC-3′ and for beta-actin was: forward primer: 5′-TGTTTGAGACCTTCAACACC-3′, reverse primer: 5′-TAGGAGCCAGG GCAGTAATC-3′. Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the *β*-actin genes. The relative quantification was then calculated by the expression $2^{-\Delta\Delta Ct}$ [34].

2.9. The Nrf2-binding activity assay

The Nrf2 and ARE binding activity were determined in the nuclear extract of hepatic tissue for all studied groups by using the nuclear extract kit and Trans AM Nrf2 ELISA kit (Active Motif, Carlsbad, USA) according to the manufacturer's guidelines. The ELISA assay based on the presence of an immobilized oligonucleotide containing the ARE