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Original article

Protective effects of crocin and zinc sulfate on hepatic ischemia-reperfusion injury in rats: a comparative experimental model study

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ABSTRACT

Objectives: The aim of this study was to investigate the comparative protective effects of separate and combined pretreatment with Cr and ZnSO4 on serum levels of miR-122, miR-34a, liver function tests, protein expression of Nrf2 and p53, and histopathological changes following IR-induced hepatic injury.

Materials and methods: Fifty-six male Wistar rats randomly assigned into seven groups (n = 8). Sham (S), IR, crocin pretreatment (Cr), and crocin pretreatment + IR (Cr + IR), ZnSO4 pretreatment (ZnSO4), ZnSO4 pretreatment + IR (ZnSO4 + IR) and their combination (Cr + ZnSO4 + IR) groups. In sham, ZnSO4 and Cr groups, animals received normal saline (N/S, 2 ml/day), Cr (200 mg/kg) and ZnSO4 (5 mg/kg) for 7 consecutive days (intraperitoneally; i.p), then only laparotomy was performed. In IR, Cr + IR, ZnSO4 + IR and Cr + ZnSO4 + IR groups, rats received N/S, Cr and ZnSO4 with same dose and time, then underwent a partial (70%) ischemia for 45 min that followed by reperfusion for 60 min. Blood sample was taken for biochemical and microRNAs assay, tissue specimens were obtained for antioxidants, protein expression, histopathological and immunohistochemical evaluations.

Results: The results showed that Cr and ZnSO4 increased antioxidants activity and expression of Nrf2, decreased serum levels of liver enzymes, miR-122, miR-34a, p53 expression and also ameliorated histopathological abnormality. However, their combination caused more improvement on IR-induced liver injury.

Conclusion: This study demonstrated that Cr, ZnSO4 and their combination through increasing antioxidant activity and Nrf2 expression, decreasing the serum levels of liver enzymes, miR-122, 34a, p53 expression, and amelioration of histopathological changes, protected liver against IR-induced injury.

1. Introduction

Liver ischemia-reperfusion (IR) injury is a common problem in multiple clinical situations such as liver transplantation, trauma, hypovolemic shock and liver resection [1]. A series of physiological and biochemical alterations occur following hepatic IR injury. The tissue deprivation of oxygen and nutrients in the ischemic phase, impair mithocondrial activity which result in liver cell injury. Tissue reperfusion in the second phase of IR has more deleterious impacts on tissue functions, and structures secondary to activating the other ROS-dependent processes such as inflammation, and apoptosis in liver cells [2].

MicroRNAs (miRNAs, miRs) are endogenous non coding RNAs responsible for the posttranscriptional regulation of mRNA translation and stability [3]. MiR-122 accounts for near 70% of total miRNAs pool in the liver tissue [4]. It has been shown to exert various effects in liver such as development, liver physiology [5] and lipid metabolism [6]. It is well known that miR-122 is a non-invasive and early releasing circulating biomarker for determination of hepatic disorders [7]. A previous study demonstrated that there is a close correlation between the serum level of miR-122 and enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) following warm IR-induced liver injury in rats [8].

Another microRNA which shows the liver injury is miR-34a. The elevated serum level of miR-34a represents liver injury [6]. In agreement, it have been shown that miR-34a increase during stress-induced injuries [9,10]. Moreover, suppression of miR-34a has been shown to protect liver against IR-, and non-alcoholic fatty liver disease (NAFLD)-induced injury [11]. Moreover, it has been shown that the expression of microRNAs, the recent regulators of gene expression, is modified by

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oxidative stress [12].

Oxidative stress is representative an oxidant/antioxidant imbalance situation [13], that leads to decrease the antioxidant enzymes activity such as catalase (CAT), superoxide dismutase (SOD), and gluthatione peroxide (GP_X), through generation of ROS [14]. These enzymes with scavenging of the intracellular ROS and conversion of H_2O_2 to H_2O and O_2, protect cells against IR-induced injury [15]. Moreover, heme oxygenases-1 is a stress protein and antioxidant element may play an important role in various liver diseases such as IR injury, liver failure, alcoholic or viral hepatitis, chronic inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma due to its antioxidative, antiapoptosis, and potent cytoprotective properties [16].

The p53 (MWt: 53 kDa and 6 replicates antibody) is one of the most important tumor suppressor that operated through inhibition of cell cycle and apoptosis [17]. This protein is organized into several functional domains. The N-terminal region contains two transactivation sub-domains followed by a proline-rich region that plays a selective role in transactivation and apoptosis. The central region comprising a protease-resistant core domain has sequence-specific DNA-binding activity. The core domain is joined to the tetramerization domain by a 25 amino acid linker region that contains a nuclear localization signal. Eventually, at the extreme C-terminus is a highly basic region which can associate in a non-sequence dependent manner with multiple forms of both DNA and RNA [18]. One study showed that the suppression of p53 leads to a decrease in NAFLD-induced hepatic injury [19]. It activated by multiple stimuli such as hypoxia, DNA injury, administration of cytotoxic drugs, ROS and ultra violet (UV) radiation [20].

Nuclear factor-erythroid 2-related factor-2 (Nrf2) as a key transcription parameter preserves organs against oxidative-induced injury [21]. It is retained in the cytoplasm by keap1, released and transferred to the nucleus for inducing transcription of antioxidant response element (ARE) which induce gene expression of its downstream targets. Evidences have been shown that Nrf2 has a protective role against several liver problems such as cholestatic liver injury [22], viral hepatitis [23], NAFLD [24], and drug-induced liver injury [25].

Crocin is a water soluble carotenoid and the most important active constituent of saffron. Crocin has many beneficial protective effects against renal [26], gastric [27], retinal [28], and brain [29] IR-induced injuries.

Zinc (Zn) is the most abundant divalent cation in human body that involve in metabolism, detoxification, antioxidant system and gene regulation [30]. It has been demonstrated that Zn deficiency aggravate ROS-induced insult in the rats liver [31], and Zn dyshomeostasis is involved in the pathophysiology of IR-induced injury [32]. In agreement, Masuda et al. at 2011 showed that zinc supplementation potentially protected liver against IR injury through decreasing oxidative stress agents, and liver functional enzymes (AST and ALT) [33]. The above mentioned studies have shown that both zinc and crocin have significant hepatoprotective effect but according to our best knowledge there is no comparative study about their effect on IR-induced hepatic injury. Therefore, this study was designed to 1. Compare the hepatoprotective effects of Cr and ZnSO4 against IR-injury; and 2. Investigate whether co-administration has more protective effect on early stage of IR-induced liver injury.

2. Materials and Methods

2.1. Animals

Male Wistar rats (200–250 g) were purchased from the animal house of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Animals were fed on a conventional diets and tap water *ad libitum*. They were maintained under standard conditions of humidity, temperature (20–24 °C), and 12-h light–dark cycle. Animals were deprived of food, but not water, overnight before experiments. All experiments were performed in accordance with ethics committee of Ahvaz Jundishapur University of Medical Sciences (APRC-9421).

2.2. Animal grouping

In the current study fifty six male Wistar rats were randomly assigned into seven groups, each consisting of 8 rats. They were:

Sham group: Rats received normal saline (N/S; 2 ml/day) [26] for 7 consecutive days, intraperitoneally (ip) [34], then only laparotomy was done.

IR group: Rats received N/S (2 mg/day, 7 consecutive days, ip) then IR induction was carried out as mentioned earlier.

Cr pretreatment group: Rats received Cr (200 mg/kg, 7 consecutive days, ip) [35], then only laparotomy was done.

Cr + IR group: Rats received Cr (200 mg/kg, 7 consecutive days, ip), then subjected to IR induction.

ZnSO4 pretreatment group: ZnSO4 (5 mg/kg, 7 consecutive days, ip), [36,37], then only laparotomy was done.

ZnSO4 + IR group: Rats received ZnSO4 (5 mg/kg, 7 consecutive days, ip), then subjected to IR induction.

ZnSO4 + Cr + IR group: Rats received Cr + ZnSO4 (200 + 5 mg/kg, 7 consecutive days, ip), then subjected to IR induction.

2.3. Surgical procedure

In this experimental, partial (70%) ischemia induced for 45 min. followed by reperfusion for 60 min. as described previously [38]. Multiple evidences illustrated that this model of ischemia/reperfusion is one of the models which evaluate early-stage changes following hepatic ischemia/reperfusion injury [39–41]. For induction of IR, rats anesthetized using a mixture of ketamine and xylazine (Alfasan Co. Woerden-Holland, 80 + 10 mg/kg, ip, respectively) [42], then IR induction was done. At the end of experiment, rats were killed by cardiac puncture and two samples of liver tissue were taken, rinsed with N/S and stored at −80◦C for measurement of protein expression of p53 and antioxidants assay. In addition, pieces of liver fixed in the formalin 10% solution for histopathological and immunohistochemical evaluations.

2.4. MicroRNAs extraction and cDNA synthesis

Total microRNAs extracted from the frozen serum samples using miRNeasy/Plasma kit (QIAGEN, GmbH, Germany) according to the manufacturer's protocol. The concentration and purity of RNA was determined by spectrophotometry at wavelengths of 260/280 nm (Nanodrop thermo scientific S.N:D015). The cDNA was synthesized from one microgram of the total RNA using miScript II RT Kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions.

2.5. Quantitative real-time PCR

The levels of microRNAs were measured by quantitative real-time polymerase chain reaction (qRT-PCR) using a Light Cycler® 96 Real time PCR System (Roche Diagnostics, Indianapolis, IN, USA). All PCR amplifications were performed in duplicate reactions and in final volume of 20 µl containing 2 µl cDNA, 10 µl 2 × QuantiTect SYBR Green PCR Master Mix, 2 µl 10 × miScript Primer Assay [miR-122 (MS00000315), or miR-34a (MS00000224); QIAGEN], 2 µl 10 × miScript Universal Primer [(MS0003374); (QIAGEN)], and 4 µl RNAase free water using the following protocol: Initial activation step at 95 °C for 15 min to activate HotStar Taq DNA Polymerase followed by 45 cycles at 94 °C for 15s, 55 °C for 30s, and 70 °C for 30s. In addition, the no-template negative control (H_2O) was routinely run in every PCR. The levels of microRNAs expression were normalized with RNU6 and the fold change was calculated using the $2^{-\Delta\Delta Ct}$ formula.