Biomedicine & Pharmacotherapy 89 (2017) 98–107

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Original article

Combination of Sitagliptin and Silymarin ameliorates liver fibrosis induced by carbon tetrachloride in rats

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ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form 19 January 2017
Accepted 7 February 2017

Keywords:
Liver fibrosis
Carbon tetrachloride
Sitagliptin
Silymarin

ABSTRACT

Liver fibrosis is a common pathological condition that occurs in most conditions associated with chronic liver injury. Silymarin is a herbal product widely used for its hepatoprotective effect. Sitagliptin, a dipeptidyl peptidase-4 inhibitor (DPP4-I), is clinically used as an oral antidiabetic agent. This study was designed to investigate the effects of Sitagliptin, Silymarin, and their combination on established liver fibrosis in carbon tetrachloride (CCl_4) rat model. Male albino rats received intraperitoneal injections of CCl_4 three times a week for 7 weeks, as well as daily oral treatments of Sitagliptin (100 mg/kg) or Silymarin (100 mg/kg) or their combination during the 7 weeks of intoxication. Hepatic fibrotic changes were evaluated by measuring hepatic enzymes (ALT, AST, ALP, and GGT) and markers of fibrosis (transforming growth factor β1 (TGF-β1), tissue 4-hydroxyproline level, histopathological score), oxidative stress (MDA, GSH, and NOx levels), inflammation (interleukin-6) as well as markers of HSCs activation (α-smooth muscle actin (α-SMA) expression). The injected rats with CCl_4 for 7 weeks resulted in a marked elevation of hepatic fibrotic changes and reduction of GSH level, while the combination therapy showed a significant decrease in the former one and a significant increase in the later. In conclusion, this study shows that the combination therapy is more beneficial than monotherapy in ameliorating liver fibrosis in rats. Our findings suggest that Sitagliptin alone or in combination with Silymarin may introduce a new strategy for treating liver fibrosis in humans.

1. Introduction

Despite the medical development in the field of liver diseases, progressive liver fibrosis is still the common end stage for most chronic liver injuries which leads to irreversible cirrhosis with the risk of liver failure and hepatocellular carcinoma [1]. After a chronic hepatic injury for any reason, the damaged hepatocytes, metabolites of toxic agents, and infiltrating inflammatory cells are strong activators of Kupffer cells which release a number of soluble agents including cytokines, such as platelet-derived growth factor (PDGF), interleukin 6 (IL-6), and transforming growth factor-β1 (TGF-β1) as well as reactive oxygen species (ROS) such as malondialdehyde (MDA) and nitric oxide (NOx) [2]. These factors act on the hepatic stellate cells (HSCs) lead to their activation and start expressing new proteins, such as α-smooth muscle actin (α-SMA), and new receptors, such as PDGF and TGF-β1 receptors [3]. Therefore, activated HSCs secrete large amounts of extracellular matrix (ECM) proteins which accumulate and initiate fibrogenesis [4].

Carbon tetrachloride (CCl_4) is a common hepatotoxin that is widely used to induce toxic liver injuries in experimental animal models [5,6]. Liver fibrosis induced by CCl_4 is associated with the exacerbation of lipid peroxidation and the depletion of antioxidant status [7].

Silymarin (Si) is a standardized extract obtained from the seeds of milk thistle plant *(Silybum marianum)* containing at least three flavonolignans (including silybin, silychristin, and silydianin) [8]. It is used clinically for the treatment of liver diseases as "hepatoprotective" agent in Europe and Asia [9]. Silymarin has the ability to free radicals scavenging and stabilizing effect on the cytoplasmic membranes. Also, it has anti-inflammatory, immunomodulatory, and lately antifibrotic properties in the liver [10]. In experimental animals, Silymarin has a protective effect on the liver, which is particularly vulnerable to poisoning by several hepatotoxic substances such as carbon tetrachloride, thioacetamide, and D-galactosamine [11].

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http://dx.doi.org/10.1016/j.biopha.2017.02.010
0753-3322/

Sitagliptin, a Dipeptidyl peptidase 4 inhibitor (DPP4-I), has been developed as a new possible treatment for type-2 diabetes mellitus (DM) [12,13]. Dipeptidyl peptidase 4 (DPP4), also known as CD26, is a type II transmembrane protein [14]. It causes degradation of the two incretin hormones in serum, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [15]. Both hormones inhibit glucagon secretion and stimulate insulin secretion from the β-cells [16,17]. Sitagliptin can exert its antidiabetic action due to the enhancement of the function of incretin. Therefore, continuous inhibition caused by Sitagliptin to DPP4 could lead to a higher bioavailability of these hormones and consequently lower the serum glucose level [12,13].

Besides its role in the regulation of incretin, DPP4 has various actions in different organs [18,19]. DPP4 is highly expressed in liver, which plays a role in the fibronectin-mediated interaction of hepatocytes with ECM [18,20]. It has been suggested that DPP4 is involved in the development of several liver diseases, such as chronic hepatitis C (CHC) and hepatocellular carcinoma (HCC) [21,22]. Other reports showed that DPP4 is expressed on the surface of reactive fibroblasts including activated HSCs [23,24]. Moreover, it was reported that the serum DPP4 activity was augmented in tetrachloromethane-induced cirrhotic rats [25]. Furthermore, DPP4 mRNA was significantly increased in non-alcoholic fatty liver disease (NAFLD) compared to that in control livers [26]. However, the effect of Sitagliptin against the progression of liver fibrosis has yet to be clarified.

Therefore, the present study was designed to assess the potential effects of Sitagliptin, Silymarin, and their combination against liver fibrosis induced by CCl_4 in rats and to foresee an unusual clinical application of Sitagliptin alone and in combination with Silymarin. We also attempt to investigate the related mechanisms possibly involved therein.

2. Materials and methods

2.1. Animals

Male albino rats (150–200 g) were obtained from the animal house at the College of Veterinary Medicine of Cairo University (Cairo, Egypt). All rats were placed under specific pathogen-free conditions with a 12-h light-dark cycle, constant temperature ($25 \pm 2\,^{\circ}C$) and 50% relative humidity, and provided ad libitum access to standard rodent chow (El-Nasr, AbuZabal) and filtered water. All rats were acclimatized for one week prior to use in experiments. All rats received human care in compliance with the National Institutes of Health and the Research Ethics Committee criteria for care of laboratory animals at Tanta University.

2.2. Drugs and chemicals

CCl_4 was purchased from Adwic Chemicals Co. (Cairo, Egypt). Silymarin (milk thistle powder containing 80% silymarin) was purchased from Bulk Sigma (St. Louis, MO) and prepared by dissolving in physiological saline before use. Sitagliptin (Januvia) was purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA) and prepared by dissolving in distilled water before use. All other chemicals and solvents were of analytical grade.

2.3. Experimental design

The rats were divided into five groups, 8 rats in each group as follows:

(A) Control (CTRL): rats received three times a week olive oil (1 ml/kg, i.p.) in addition to saline (1 ml/kg/day, orally) for 7 weeks.
(B) CCl_4: rats received 1.5 ml/kg of CCl_4 diluted in olive oil (1:7) by intraperitoneal injection (i.p.) three times a week for 7 weeks [27].
(C) CCl_4 + Sitagliptin (SITA): rats received CCl_4 as mentioned before in addition to Sitagliptin (100 mg/kg/day, orally) [28] for 7 weeks.
(D) CCl_4 + Silymarin (SILY): rats received CCl_4 as described before in addition to Silymarin (100 mg/kg/day, orally) [29] for 7 weeks.
(E) CCl_4 + Sitagliptin (SITA) + Silymarin (SILY): rats received CCl_4 as previously mentioned in addition to Sitagliptin (100 mg/kg/day, orally) and Silymarin (100 mg/kg/day, orally) for 7 weeks.

2.4. Biochemical analysis

Twenty-four hours after the last injection of CCl_4, the rats were anaesthetized by thiopental and the blood was collected via cardiac puncture. The isolated blood was then centrifuged at 3000 rpm for 10 min and the serum was pipetted off carefully and stored at $-20\,^{\circ}C$ until used to assess liver enzymes and transforming growth factor-β1. The rats were euthanized and their livers were carefully removed and rinsed thoroughly with saline; portions of the livers were fixed in 10% neutral buffered formalin for histopathological and immunohistochemical examinations, while the rest of tissues were stored at $-20\,^{\circ}C$ for determination of malondialdehyde, glutathione, nitric oxide, 4-hydroxyproline, and interleukin-6 levels.

2.4.1. Biochemical markers of liver

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGT) levels were determined using commercial kits from Spectrum Diagnostics (Cairo, Egypt).

2.4.2. Hepatic oxidative stress parameters

2.4.2.1. Measurement of hepatic lipid peroxide level. Hepatic malondialdehyde concentration was estimated in the liver homogenate as previously described [30]. In brief, liver tissues (0.25 g/rat) were homogenized in 10 volumes of ice-cold 1.15% (w/v) potassium chloride solution using polytron homogenizer (PT 3100) (kinematica instruments, Luzerne, Switzerland). 3 ml of 0.5% (w/v) Trichloroacetic acid (TCA) and 1 ml of 0.6% (w/v) Thiobarbituric acid (TBA) were added to 0.5 ml of the homogenate; the solution was mixed and then heated for 45 min in a boiling water bath. After cooling, 4 ml of *n*-butanol was added and vigorously shaken. After the phase separation, the *n*-butanol layer was isolated. The absorbance of the pink colored product was measured at 535 nm, using double-beam spectrophotometer (Shimadzu UV-PC 1601, Kyoto, Japan). MDA concentration was expressed as μmol/g tissue using a standard curve.

2.4.2.2. Measurement of hepatic nitric content. Total nitrate/nitrite contents were estimated as previously described [31]. In brief, (0.25 g/rat) of liver tissues were homogenized in 10 volumes of ice-cold saline (0.9% NaCl) using polytron homogenizer (PT 3100) (kinematica instruments, Luzerne, Switzerland). 1 ml of anhydrous ethanol was added to 0.5 ml of the homogenate to precipitate the proteins. After centrifugation for 10 min at 3000 rpm, 0.5 ml of the clear supernatant was rapidly added to 0.5 ml vanadium chloride (VCl_3) and then followed by addition of 0.5 ml of freshly prepared Griess reagent. The samples were incubated at 37 °C for 30 min. The absorbance was measured using double-beam spectrophotometer