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In vivo systemic chlorogenic acid therapy under diabetic conditions: Wound healing effects and cytotoxicity/genotoxicity profile

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ABSTRACT

Oxidative stress occurs following the impairment of pro-oxidant/antioxidant balance in chronic wounds and leads to harmful delays in healing progress. A fine balance between oxidative stress and endogenous antioxidant defense system may be beneficial for wound healing under redox control. This study tested the hypothesis that oxidative stress in wound area can be controlled with systemic antioxidant therapy and therefore wound healing can be accelerated. We used chlorogenic acid (CGA), a dietary antioxidant, in experimental diabetic wounds that are characterized by delayed healing. Additionally, we aimed to understand possible side effects of CGA on pivotal organs and bone marrow during therapy. Wounds were created on backs of streptozotocin-induced diabetic rats. CGA (50 mg/kg/day) was injected intraperitoneally. Animals were sacrificed on different days. Biochemical and histopathological examinations were performed. Side effects of chronic antioxidant treatment were tested. CGA accelerated wound healing, enhanced hydroxyproline content, decreased malondialdehyde/nitric oxide levels, elevated reduced-glutathione, and did not affect superoxide dismutase/catalase levels in wound bed. While CGA induced side effects such as cyto/genotoxicity, 15 days of treatment attenuated blood glucose levels. CGA decreased lipid peroxidation levels of main organs. This study provides a better understanding for antioxidant intake on diabetic wound repair and possible pro-oxidative effects.

1. Introduction

Normal wound healing is a complex process that involves a series of coordinated events, including bleeding, coagulation, the initiation of an acute inflammatory response to the initial injury, and regeneration. It also encompasses migration and proliferation of connective tissue and parenchyma cells, the synthesis of extracellular matrix proteins, remodeling of new parenchyma and connective tissue, and collagen deposition (Guo and Dipietro, 2010; Velnar et al., 2009). Delayed wound healing is common in patients with diabetes mellitus (Deodhar and Rana, 1997; Greenhalgh, 2003). Diabetes-induced impairment of wound healing is characterized by the impairment of the inflammatory response, angiogenesis, fibroplasia and defects in collagen deposition, and differentiation of the extracellular matrix (Dadpay et al., 2012). Because of protein glycosylation, the thickness of capillary basal membranes is increased, thus altering capillary permeability. The migration of inflammatory cells to and from the wounded area is delayed (King, 2001), resulting in chronic inflammation. The inflammatory response following injury is important for rapid wound healing (Ahmed, 2005). Studies have shown that defects in the inflammatory phase of healing directly result in the subsequent failure of fibroblast growth and collagen synthesis (Komesu et al., 2004). Free radicals and their scavenging systems are also known to play important roles in the normal and delayed healing types of wounds, particularly during the inflammatory phase. During the development of diabetes, persistent hyperglycemia increases

Abbreviations: CAT, catalase; CGA, chlorogenic acid; GSH, reduced glutathione; MDA, malondialdehyde; MNPCE, micronucleated polychromatic erythrocytes; NCE, normochromatic erythrocytes; NO, nitric oxide; PBS, phosphate buffered saline; PCE, polychromatic erythrocytes; PMNL, polymorphonuclear leukocytes; ROS, reactive oxygen species; SOD, superoxide dismutase.

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the production of free radicals via the oxidation of glucose, as well as non-enzymatic protein glycation (Rasik and Shukla, 2000). The diabetic wound also demonstrates a number of abnormalities reminiscent of endothelial dysfunction. Diabetic wounds also have evident reduction in cutaneous blood flow, and abnormal angiogenesis. This supports the theory of impaired endothelial function and consequently delayed wound repair (Kalani et al., 1999; Martin et al., 2003).

Antioxidants are postulated to help control the oxidative stress of wounds and thus accelerate wound repair (Fitzmaurice et al., 2011). Chlorogenic acid (CGA) is a bioactive natural phenolic compound found in diet (Clifford, 1999). CGA is formed by the esterification of *trans*-cinnamic acids (caffeic, coumaric, and ferulic acid) with (−)-quinic acid, comprising a group of isomers (mainly 3-O-caffeoylquinic acid (3-CQA), 4-O-caffeoylquinic acid (4-CQA), and 5-O-caffeoylquinic acid (5-CQA). Antioxidant, free radical scavenging, anti-inflammatory, radioprotective, antiulcerogenic, and analgesic properties of CGA have been shown (Bagdas et al., 2013, 2014a, 2014b, 2014c, 2014d; Cinkilic et al., 2013; Dos Santos et al., 2006; Oboh et al., 2013; Shimoyama et al., 2013; Yun et al., 2012). In our previous studies, we reported beneficial effects of systemic CGA on skin flap survival in diabetic rats. Both systemic and intra-arterial administrations of CGA induced better healing on skin flap surgeries (Bagdas et al. 2014a, 2014c). In addition, CGA has *in vivo* wound healing effect by topical administration (Chen et al., 2013). We also reported the accelerating wound healing properties of CGA in nondiabetic rats (Bagdas et al., 2014d). Furthermore, the antidiabetic activities of CGA in diabetes have been shown recently. It has been reported to modulate blood glucose levels and exhibit protective effects against tissue changes (Bagdas et al., 2014b; Hunyadi et al., 2012; Karthikesan et al., 2010a, 2010b). Taking into account these facts, we hypothesized systematically given CGA may improve skin wound healing by its hypoglycemic, anti-inflammatory and antioxidant properties. Despite the above-mentioned information, to date there are no published studies that investigate the possible side effects of chronic CGA therapy.

Firstly, the present study seeks to determine the effects of systematically given CGA on wound healing, oxidative stress biomarkers of skin and blood glucose. Secondly, we aimed to understand possible side effects of CGA on pivotal organs and bone marrow during long term CGA therapy.

2. Materials and methods

2.1. Animals and study plan

The study was performed on 8–12 weeks of age, male Sprague Dawley rats weighing 250–300 g. Rats (supplied by Experimental Animals Breeding and Research Center, Uludag University) were housed in conventional cages with 1 rat per cage in a temperature and humidity controlled room (21 ± 2°C, 50 ± 5%) on a 12-h light/dark cycle. The rats were provided free access to water and food. The study was approved by the Local Ethics Committee for Animal Experiments, Uludag University (Approval number: 2012-07/04).

Forty diabetic rats were divided randomly into two groups of equal size and experimental wounds were created on their backs. We tested material supplied as 3-CQA ((1S,3R,4R,5R)-3-[[3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid), but which is referred to as 5-CQA under the IUPAC nomenclature. This is the most widespread chlorogenic acid isomer and the dominant isomer in coffee beans. CGA (Acros Organics, Geel, Belgium; CAS no 327-97-9) was freshly dissolved in a 10 mM PBS (pH 7.4 at 37 °C in a water bath) and protected from light until time of use. CGA (50 mg/kg/day) or phosphate buffered saline (PBS; as vehicle control) was administered intraperitoneally (i.p.) for 15 days. Since CGA is not well absorbed from the digestive tract when administered orally to rats, it was given i.p. because this compound easily enters blood vessels after being administered parenterally (Azuma et al., 2000). Drugs were given i.p. at a total volume of 1 ml/kg body weight. Treatments began immediately after wound creation and repeated daily at the same time point. Open wound size was determined on days 4, 8, 12, and 16 as described below. Every measuring day, five rats from each group were sacrificed with an anesthetic overdose and tissues were collected for various analyses as mentioned below. No rats died during the study.

In the present study, we chose 50 mg/kg i.p. dose of CGA for an effective therapeutic dose according to our earlier studies (Bagdas et al., 2013, 2014c, 2014d). We also reported the wound healing effects of CGA in different doses in nondiabetic healthy rats (Bagdas et al., 2014d). In the current study, we used diabetic animals to understand the possible bioactivities of CGA under diabetic conditions.

2.2. Induction of diabetes and estimation of blood glucose level

First, all the rats were weighed, and the blood glucose levels were assessed in a drop of blood obtained from the tail vein using a glucose meter (One Touch Select; LifeScan, Milpitas, CA, USA). Experimental diabetes mellitus was induced by a single 45 mg/kg i.p. injection of streptozotocin (Sigma Chemical Co., St. Luis, MO, USA) to overnight fasted rats. Following the induction of diabetes, rats received a solution of 6% sucrose in their drinking water for 24 hours. One week later, blood glucose assessments were repeated, and the rats with fasting blood glucose levels more than 300 mg/dl were considered to be diabetic (Junod et al., 1969). The diabetic rats were used for wound surgery, and the diabetic status was controlled by assessments of the blood glucose levels every wound size measuring day. During this period, the clinical signs of diabetes mellitus such as polyuria, polyphagia and weight loss were observed in the diabetic rats.

2.3. Wound creation

Excisional wounds were created as described previously (Bagdas et al., 2014d). Surgical area was first shaved with electric razor and then manually depilated (Veet® hair removal cream) under light anesthesia one day before the operation. On day 0, overnight fasted rats in all groups were anesthetized by inhalation chamber of sevoflurane (4.0–5.0%, vol). After induction, anesthesia was maintained with facial mask by inhalation of sevoflurane (2.5–3.5%, vol). Carprofen (Rimadyl®, Pfizer Inc., Zaventem, Belgium) was injected to all animals (4 mg/kg s.c.) once just before the operation for analgesia. Each rat was positioned in sternal recumbency and surgically draped. Surgical area was prepared with polyvidone-iodine (Betadine®, Kansuk, Istanbul, Turkey) for antisepsis. One full thickness experimental wound (1.5 cm × 1.5 cm) was created on each side, nearly 1 cm from the dorsal midline just caudal to the caudal border of the scapula on each rat. The skin, including panniculus carnosus, was excised with a no. 11 scalpel blade and scissors to create wounds perpendicular to the spine. Hemorrhage was controlled by sterile surgical sponge compresses. During the study, the wound areas were not covered by bandages.

2.4. Evaluations of wound healing

Each wound was evaluated daily for the presence of exudate and wound healing until the end of the experiment. The day that the first granulation tissue was observed, and the days that the wound was covered and completely filled with granulation tissue and epithelialized were recorded. In addition, each rat was weighed every planimetry day.

Planimetry was performed on days 0, 4, 8, 12, and 16 on anesthetized animals (the anesthesia protocol used to create the wounds was repeated) by tracing the perimeter of the square wounds onto a sterile piece of clear acetate film with a special marking pen. The examiner traced the wound margin at the border between the normal skin and the wound. The outlined area was defined as 'total wound area'. Thereafter, the examiner traced the margin at the leading edge of the advancing epithelium. The area within the margin of the advancing epithelium was defined as 'unhealed wound area'. Wound tracings were scanned and transferred to a computer, and the area (cm^2) and perimeter were calculated for each wound tracing using the SIGMA SCAN software (Sigma Scan, SPSS Inc., Chicago, IL, USA). The percentage of total wound healing was calculated for wounds on the right side by using a previously described two-step formula (Swaim et al., 1993).

Step 1

$$\text{Open wound day}_n \text{ as percentage of original} = \frac{\text{Open wound area day}_n}{\text{Original wound area day}_0} \times 100$$

Step 2

$$\%\ \text{total wound healing day}_n = 100 - \text{Open wound day}_n \text{ as percentage of original.}$$

The unhealed wound area and the percentage of total wound healing were recorded at each day of measurement and used for statistical analyses.

2.5. Determination of oxidative stress markers and hydroxyproline content

Biochemical analyses were performed as described in our previous report (Bagdas et al., 2014a). Wound tissue samples were rapidly collected after rats were sacrificed. Samples were washed well in cold 0.9% NaCl and immediately were frozen at –20 °C until use. On the assessment day, tissues were cut into pieces on ice and homogenized by a Heidolph DIAX 900 homogenizer in a 2 ml volume of ice-cold 10% trichloroacetic acid. Then, the samples were vortexed for 10 s and centrifuged (4000 rpm, 15 min, 4 °C). The supernatants were used for analysis. Furthermore, tissue protein levels were determined in 50 µl of homogenate, according to the method of Lowry et al. (1951).