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ORIGINAL STUDIES

Single oral dose of chlorogenic acid attenuates the experimental carrageenan-induced oxidative stress

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Abstract

Background. Carrageenan-induced inflammation implies the release of inflammatory cytokines and also, oxidative stress, with high amounts of reactive oxygen species (ROS) production.

Aims. The present study aimed to investigate the effects of a unique dose of chlorogenic acid, orally administered at one hour after a paw inflammation was produced.

Methods. The paw edema and oxidative stress parameters in rats that were injected with 0.2 ml carrageenan solution 1% for paw inflammation production were investigated, and after 1 hour they received a single dose of either chlorogenic acid (100 mg/kg or 150 mg/kg) or indomethacin (1 mg/kg), by oral gavage. The paw edema was investigated using the plethysmometer test (at 2, 6 and 24 hours after carrageenan injection) and oxidative stress by spectrophotometry. Malondialdehyde (MDA), glutathione (GSH) and oxidized glutathione (GSSG) levels were investigated from blood samples, paw inflamed skin and kidneys.

Results. The present study showed that chlorogenic acid (both doses) and indomethacin produced significant decreases of inflamed paw volume, compared to the control group (rats without treatment). Lipid peroxidation was reduced significantly and the antioxidant protection investigated through the GSH/GSSG ratio was increased significantly in all rats that received medication, in all the tested samples: inflamed skin, serum, kidneys.

Conclusions. Chlorogenic acid may reduce efficiently oxidative stress and edema if it is administered during the first phase of carrageenan-induced inflammation.

Keywords: chlorogenic acid, oxidative stress, inflammation, antioxidants, carrageenan, green coffee

Abbreviations: CGA – chlorogenic acid, CGN – carrageenan, GSH – reduced glutathione, GSSG – oxidized glutathione, MDA – malondialdehyde, ROS – reactive oxygen species.

Introduction

Carrageenan is an irritant polysaccharide that may produce inflammation, largely used in experiments to initiate paw edema to test the anti-inflammatory effects of different substances, and to study the mechanisms involved. The cascade of chemical events is initiated by the release in the lesion area of histamine, serotonin, bradykinin, prostaglandins, and in addition, different complement fractions, coagulation products, and numerous lymphokines released by the sensitized T lymphocytes. Under their action, macrophages become activated, initiate phagocytosis of the tissue debris and release mediators that recruit the circulatory inflammatory cells, maintaining the local reaction. The decrease of paw edema in mice is constantly accompanied by the inhibition of monocyte migration into the inflammatory zone (Iqbal et al., 2016).

During inflammation, monocytes and neutrophils produce high levels of reactive oxygen and nitrogen species, leading to local oxidative stress (Mittal et al., 2014). The reactive species induce the oxidation of proteins, lipids, carbohydrates and nucleic acids, intervene in cellular membrane and organelle destruction, initiating cancerous cell development and proliferation, and also enzyme alterations.

Antioxidants, endogenous and exogenous substances with important roles in the protection of biological systems against the noxious action of excessive oxidation products may block the enzymatic reactions, inflammatory agent synthesis and the cleansing of oxidative products. Inflammation and oxidative stress can be reduced by

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numerous natural substances such as curcumin (Bidian et al., 2020), quercetin (Bidian et al., 2019) or resveratrol (Mitrea et al., 2017).

Chlorogenic acid (CGA) is a biologically active polyphenol identified in tea or green coffee beans (Naveed el al., 2018). It has numerous favorable effects: antioxidant, anti-inflammatory, antibacterial, anti-diabetic, anti-obesity, it may protect the heart, liver, kidneys, etc. (Kim & Park, 2019).

In the present research, indomethacin was used as a positive control. It is known that oral administration of high doses of this non-steroidal anti-inflammatory drug can activate oxidative stress in the stomach (Mureșan et al., 2008), but in our experiments we used a low dose that was proved to be safe for the gastric mucosa (Uçar et al., 1998) and able to block the synthesis of prostaglandins, eicosanoids involved in edema development in the carrageenan injection area (Necas & Bartosikova, 2013).

In our study, we investigated the antioxidant and anti-inflammatory effects of chlorogenic acid, in comparison with indomethacin, a well-known anti-inflammatory drug, in a rat model of carrageenan-induced paw edema.

Hypothesis

This experimental study was designed to assess the hypothesis that chlorogenic acid may decrease paw edema after carrageenan injection and can protect against the oxidative stress generated by inflammation.

Material and methods

Research protocol

a) Place of the research

The research was performed in the Experimental Research Laboratory of the Physiology

Department, at "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca.

Adult male albino Wistar rats were used for the experiments, being provided by the University Animal Facility. The animals were hosted in plastic lab cages in standard conditions: environmental temperature 22 ± 2ºC, relative humidity 45-50% and day-night cycle of 12 hours, with a 1-week environmental accommodation period before the experiments.

All procedures were performed with the approval of the Ethics Committee of "Iuliu Haţieganu" UMPh Cluj-Napoca (No 15/14.11.2016), in accordance with Directive 86/609/EEC.

b) Experimental design

Adult male albino Wistar rats with weights between 180 and 200 g were used, which were randomly allocated to 4 groups, with six rats per group (n=6). All groups of rats received for 7 days, between 8 a.m. - 9 a.m., physiological saline, 0.5 ml/day by oral gavage. On the eighth day of the experiment, the rats were intraplantarly injected, in the posterior right paw, with 0.2 ml fresh prepared carrageenan solution 1%. One hour after carrageenan (CGN) injection, the rats received, by oral gavage, 0.5 ml physiological saline or medication solution.

According to the treatment administered by oral gavage after the CGN injection, the groups of rats were: *C* (*Control)*, which received physiological saline; *I (Indomethacin)*, which received indomethacin 1 mg/kg; *II (CGA 100)*, receiving chlorogenic acid 100 mg/kg, and *III (CGA 150*), receiving chlorogenic acid 150 mg/kg.

Carrageenan, indomethacin and chlorogenic acid were purchased from Sigma-Aldrich Co. LLC, Germany. At the time of the treatment administration, they were dissolved in physiological saline.

c) Tests applied

The plethysmometer test was performed using a plethysmometer UGO BASILE North America 7140. The measurements for all rats were done before carrageenan injection and at 2, 6 and 24 hours after CGN administration.

The differences in the rat paw volume (dv) were calculated using the formula:

dv (mL) = inflamed paw volume (mL) – basal volume of the same paw (mL)

The basal paw volume represented the paw volume before carrageenan injection.

To establish the efficiency of chlorogenic acid administration, antiedematous activity (AE) was calculated at every measurement using the formula:

$$\%AE = \frac{control\ group\ average\ dv - test\ group\ average\ dv}{control\ group\ average\ dv} \text{ x } 100$$

If the difference between the *control group average dv* and the *test group average dv* gave a negative result, the antiedematous activity was considered zero (Stepanovic-Petrovic et al., 2012).

d) Biochemical determination

After the last measurement of paw edema, the rats were humanely euthanized under general anesthesia. To investigate oxidative stress, blood, paw skin and kidney samples were collected to determine malondialdehyde (MDA), glutathione (GSH) and oxidized glutathione (GSSG). Malondialdehyde (MDA) was determined using Conti's method (Conti et al., 1991), reduced glutathione (GSH) using the method developed by Hu (Hu, 1994), and oxidized glutathione (GSSG) by the method described by Vats (Vats et al., 2008). The GSH/GSSG ratio was calculated as a valuable indicator for oxidative stress.

e) Statistical processing

The oxidative stress parameters were statistically analyzed using GraphPad Prism version 5.03 for Windows, GraphPad Software (San Diego California USA), one-way ANOVA followed by the Tukey post-test. The paw volume variations over 24 hours after carrageenan injection were observed and were statistically analyzed by two-way ANOVA followed by Bonferroni post-tests. The threshold significance level was set at $p<0.05$ (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Results

a) Antiedematous activity

The volume of the inflamed paws was measured at 2, 6 and 24 hours after carrageenan injection. At 2 hours after CGN injection (1 hour after treatment administration), all groups that received medication presented significant decreases of the paw volume compared to the control group ($p<0.001$). At 6 hours after CGN injection, compared to group C, the volume of inflamed paws was significantly decreased in the rats receiving indomethacin (group I; $p<0.001$) and in those

receiving chlorogenic acid, 100 mg/kg (group II, $p<0.05$). At 24 hours after rat posterior paw inflammation, all rats that received medication presented significant decreases of the paw volume compared to the control group (group I, $p<0.001$; groups II and III, $p<0.01$) (Fig. 1a).

Chlorogenic acid administered in a 100 mg/kg dose had similar antiedematous effects to those of indomethacin, but only at 2 hours after carrageenan injection. The administration of 150 mg/kg of CGA presented a reduced antiedematous activity along the entire experiment (Fig. 1b).

b) Oxidative stress evaluation

- In *inflamed skin*, compared to the control group (C group), malondialdehyde (MDA) level was significantly decreased in all treated groups (group I, $p<0.001$; groups II and III, $p<0.05$) Chlorogenic acid inhibited lipid peroxidation but had a lower efficiency than that of indomethacin: MDA levels were significantly higher ($p<0.05$) compared to group I (Fig. 2a). Compared to the carrageenan group (group C), all the rats that received medication had significantly increased levels of reduced glutathione (group II, $p<0.001$; groups I and III, $p<0.01$) (Fig. 2b) and significantly decreased concentrations of glutathione disulphide (groups I and III, $p<0.001$; group II $p<0.01$) (Fig. 2c). Indomethacin and chlorogenic acid significantly increased the antioxidant protection investigated based on the GSH/GSSG ratio ($p<0.001$), compared to the control group (Fig. 2d).

- In *serum*, compared to the carrageenan group (group C), the administration of 100 mg/kg chlorogenic acid significantly reduced MDA concentration, an effect that was similar to the indomethacin effect ($p<0.001$).

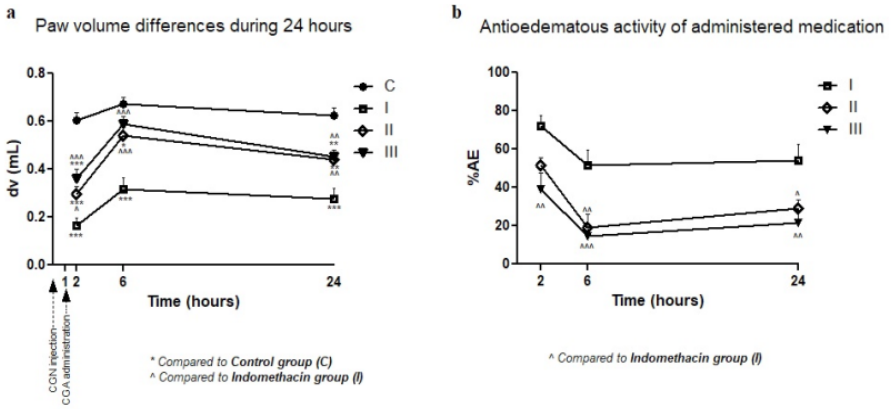

Fig. 1 – (a) Temporal course of changes in the paw volume of rats with or without medication. The graph represents the paw volume differences (dv) between inflamed and basal paw volume (volume of the same paw before carrageenan injection, indicated by arrow. (b) Temporal course of the local antiedematous effect of indomethacin and chlorogenic acid (100 mg/kg; 150 mg/kg).

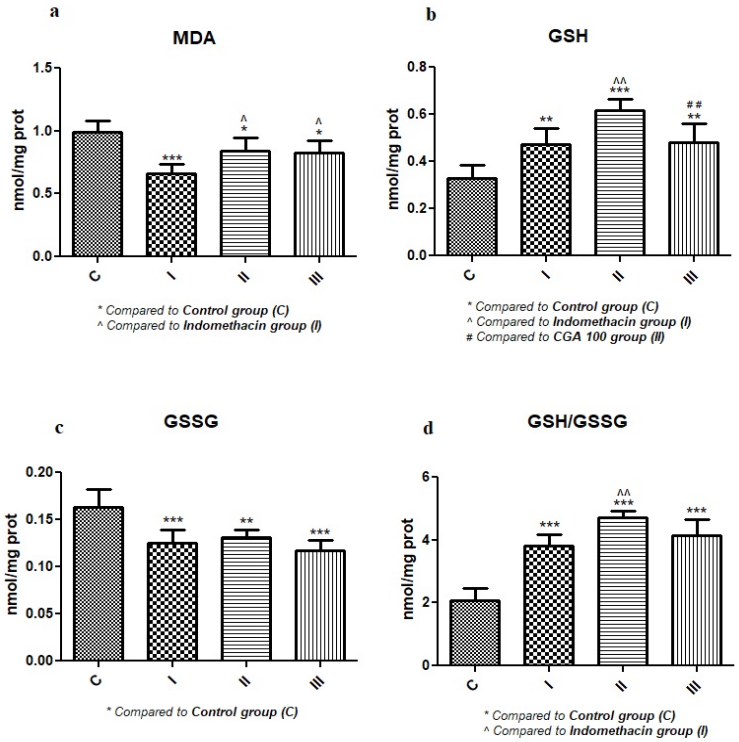

Fig. 2 – Oxidative stress parameters in inflamed rat paw. Modifications of malondialdehyde (a), reduced glutathione (b), oxidized glutathione (c) and GSH/GSSG ratio (d) in inflamed paws. Data are expressed as mean ± SEM (n=6).

Chlorogenic acid administered in a dose of 150 mg/kg had a lower effect on malondialdehyde decrease ($p<0.01$) (Fig. 3a). Compared to the control group, all the treated rats presented significant increases of GSH, (groups I and III, $p<0.001$; group II, $p<0.01$) (Fig. 3b), but did not exhibit any significant modifications of GSSG concentration (Fig. 3c). The best antioxidant protection in the serum was provided by indomethacinadministration($p<0.001$),whileCGAoralgavage showed a dose-dependent effect (group II, $p<0.05$; group III, $p<0.01$) compared to the carrageenan group (Fig. 3d).

- In *kidneys*, lipid peroxidation was significantly decreased in all treated rats ($p<0.001$) compared to the control group (Fig. 4a). The reduced glutathione levels were significantly increased by chlorogenic acid (group II, $p<0.01$; group III, $p<0.001$) compared to group C (Fig. 4b). The glutathione disulphide levels did not present significant variations compared to the control group (Fig. 4c). The best antioxidant protection was provided by the administration of 100 mg/kg chlorogenic acid (group II, $p<0.001$), but significant increases of the GSH/GSSG ratio were also recorded in the other treated groups of rats (group I, $p<0.05$; group III, $p<0.01$), compared to the control group (Fig. 4d).

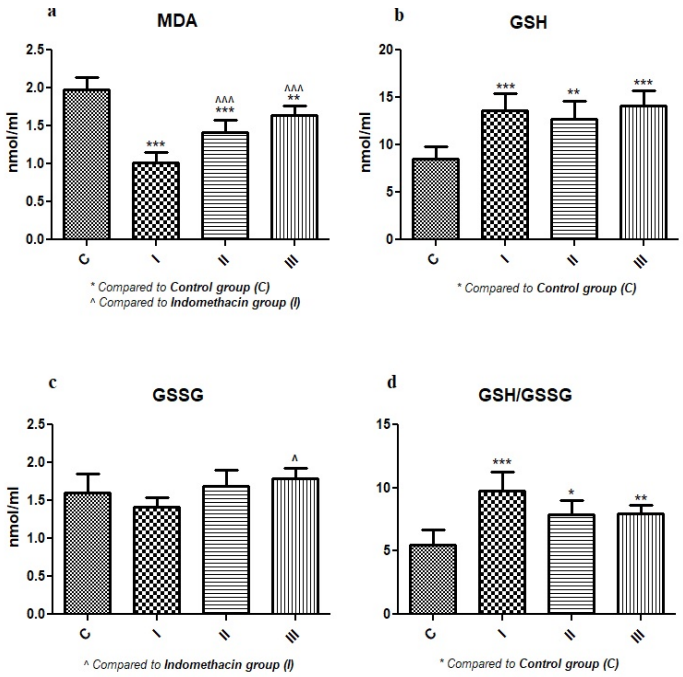

Fig. 3 – Oxidative stress parameters in the serum of rats with carrageenan-induced paw inflammation: modifications of malondialdehyde (a), reduced glutathione (b), oxidized glutathione (c) and GSH/GSSG ratio (d). Data are expressed as mean ± SEM (n=6).

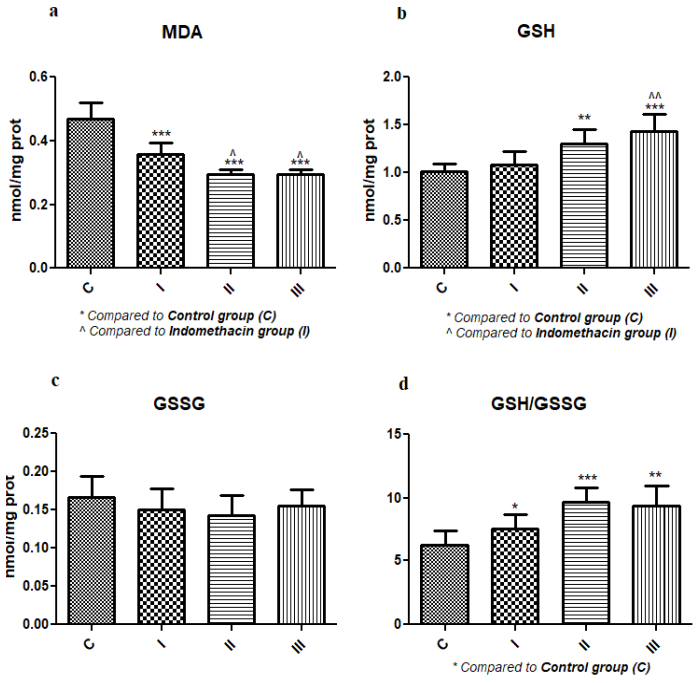

Fig. 4 – Oxidative stress parameters in the kidneys of rats with carrageenan-induced paw inflammation: modifications of malondialdehyde (a), reduced glutathione (b), oxidized glutathione (c) and GSH/GSSG ratio (d). Data are expressed as mean ± SEM (n=6).

Discussion

In the present study, the administration of a single dose of chlorogenic acid (CGA) showed antiedematous and antioxidant effects on the carrageenan-induced paw edema model of inflammation.

Carrageenan is a natural compound that may initiate reactive oxygen species production when injected in skin tissue, leading to edema development (Di Rosa, 1972; Posadas et al, 2004).

In the present study, indomethacin was used as a positive control, in 1 mg/kg administration, a dose that was demonstrated to reduce edema without causing gastrointestinal lesions, even in long-term administration (Uçar et al., 1998).

Chlorogenic acid, a phenolic compound from herbs, fruits and vegetables, showed important antioxidant (Bonita et al., 2007) and anti-inflammatory (Dos-Santos et al., 2006) effects in previous studies.

The present experiments investigated the CGA effects in oral administration to rats with inflamed paw and the results were concordant with those of other researchers that investigated this polyphenol in carrageenan-induced inflammation. The *plethysmometer test* performed at two hours after carrageenan injection presented significant decreases of the edema in all the rats that received medication, with the best effects in the indomethacin group (I). The oral administration of CGA, in a single dose, one hour after CGN injection, reduced significantly the paw edema, an effect that was also observed at 24 hours after inflammation. Our results were in accordance with those presented by Azza et al. in their study with CGA administered by intraperitoneal injection at two hours post-carrageenan injection (Azza et al., 2011), showing the efficiency of the same doses (100 and 150 mg/kg) of chlorogenic acid in both types of administration: oral and intraperitoneal. Chagas-Paula et al. showed in their study that chlorogenic acid (100 and 150 mg/kg) administered orally 30 minutes before carrageenan injections presented antiedematogenic effects and inhibited oxidative stress (Chagas-Paula et al., 2011). The same effects were shown by our study with chlorogenic acid that was administered 1 hour after carrageenan injection.

Chlorogenic acid is absorbed and metabolized through complex and dynamic processes and has an apparent bioavailability of 33 ± 23%, with plasma peaks at 0.5–8 hours after oral ingestion (Farah et al., 2008). These mechanisms can explain our results: the effects of CGA administration were much more favorable during the first hour. Carrageenan inflammation evolves in three phases: the first phase with histamine and serotonin release (0-1.5 hours), the second phase that involves kinins (1.5-2.5 hours), and the third phase with prostaglandin synthesis (2.5-6 hours) (Di Rosa et al., 1971). The results of the present research are in agreement with those of previous studies that showed the lower significant effects of oral CGA at 6 hours after CGN injection, even in prolonged administration of chlorogenic acid (Mitrea et al., 2020).

Indomethacin, a non-steroid inflammatory drug used as positive control, maintained its anti-inflammatory effects throughout the experiment and significantly decreased the rat paw edema, results that are concordant with those found in the literature (Ferreira & Vane, 1974).

Carrageenan injection induces acute inflammation by the release of bradykinin, serotonin, histamine, reactive oxygen and nitrogen species. Neutrophil recruitment may also produce reactive species that exacerbate the inflammation (Morris, 2003). Our study investigated *oxidative stress* in oral CGA administration to rats with carrageenan-induced paw edema.

In *inflamed skin*, oxidative stress was significantly reduced by both doses of chlorogenic acid. *Lipid peroxidation* was significantly decreased by CGA, but indomethacin had the best effects. The results of our study are in agreement with those presented in the literature (Bonita et al., 2007; Azza et al., 2011). Bagdas et al. reported in their study a decrease of MDA concentration in the lesion area in CGA administration, and an acceleration of wound healing (Bagdas et al., 2015), an effect that was also visible in our groups of rats. Tsai et al. demonstrated that chlorogenic acid inhibited ROS synthesis and decreased the oxidation of low density proteins in their study performed on endothelial cells that were pretreated with CGA (Tsai et al., 2018). The same effects on lipid peroxidation were shown by our study. Chlorogenic acid, in our study, significantly increased the concentration of *reduced glutathione (GSH)* in inflamed skin, the administration of 100 mg/kg providing better protection against the carrageenan-induced oxidative stress. These results are concordant with those presented by Azza et al. with the same dose (100 mg/kg) of chlorogenic acid, but in intraperitoneal administration (Azza et al., 2011). In our study, *glutathione disulphide (GSSG)*, the oxidized form of GSH, was significantly reduced in the rats that received medication, compared to the control group. The oxidation of GSH was reduced significantly in all treated rats, the best results being shown in the groups that received indomethacin or 150 mg/kg of CGA. Antioxidant protection (investigated through the *GSH/GSSG* ratio) was significantly increased in the inflamed skin in all rats that received medication, but administration of 100 mg/kg of CGA presented the best effect. These results are in agreement with those presented by Park et al. in hepatic tissue in their study on a colorectal cancer model where the administration of CGA significantly increased the GSH/SSG ratio (Park et al., 2010).

In our study, the *serum* determinations of oxidative stress parameters showed that a single dose of chlorogenic acid, orally administered, had significant antioxidant effects. *Lipid peroxidation* was reduced significantly in all treated groups, with the best effects in the rats that received indomethacin. Compared to the control group, *reduced glutathione (GSH)* levels were increased significantly in the serum of rats that received 150 mg/kg chlorogenic acid or indomethacin, while *GSSG* did not present significant variations. These results are in accordance with the results presented by Budryn et al. in their study performed in rats that received a modified diet with bread supplemented with CGA (Budryn et al., 2017). In the literature, chlorogenic acid is presented as a potent scavenger of superoxide anion ($O_2^{\cdot -}$) (Cha et al., 2014) and of hydroxyl radical ($HO^{\cdot}$) (Zhang et al., 2003), and in our study, these effects were

indirectly observed by the significantly increased GSH concentration in the serum. The potentiality of CGA to reduce the concentration of these reactive oxygen species left the glutathione unused (Fiser et al., 2013), a mechanism that may explain the insignificant GSSG modifications. The best antioxidant protection in serum was provided by indomethacin even if it was administered in a low dose (1 mg/kg), our results being in accordance with the literature data. Chao et al. demonstrated in their study that similar low doses of indomethacin can reduce the release of IL-1β (Chao et al., 2012). During carrageenan inflammation processes, this non-steroidal anti-inflammatory drug may block the production of prostaglandin E_2 (Hwang et al., 2008), diminishing in this way the activation of macrophages that can produce IL-1β. The study performed by Hammad et al. on humans showed increases of this mediator in serum during inflammatory processes (Hammad et al., 2015). In our experiments, 1 mg/kg indomethacin decreased rat paw inflammation, possibly by reducing the level of serum pro-inflammatory cytokines, and had an antioxidant effect.

In the scientific literature, chlorogenic acid is described as a renoprotective natural polyphenol (Domitrović et al., 2014) and our study presented the antioxidant effects of oral CGA administration on *kidney* tissue. The end-product of lipid peroxidation, *malondialdehyde*, was reduced significantly in the rat kidneys by both types of medication (chlorogenic acid and indomethacin), compared to the carrageenan group. Different animal-based models were presented by Liang and Kitts in a review that showed the CGA antioxidant protective effects in the brain or gastrointestinal tract, with significant decreases of MDA concentration (Liang & Kitts, 2015). Basivireddy et al. showed that high doses of indomethacin (20 mg/kg/day) cause renal damage (Basivireddy et al., 2004), effects that appear to be related to the administered dose because, in our research, 1 mg/kg of this anti-inflammatory drug decreased lipid peroxidation. In kidney tissue, the level of *reduced glutathione* was increased significantly by chlorogenic acid, with the highest concentration in the group of rats that received 150 mg/kg CGA. The antioxidant protection investigated through the *GSH/GSSG* ratio increased significantly in all the treated rats, with the best results in the group that received 100 mg/kg CGA, results that are in accordance with the literature data (Domitrović et al., 2014; Feng et al., 2016).

The present study demonstrated the antioxidant and antiedematous effects of a single dose of chlorogenic acid, orally administered, in the treatment of carrageenan-induced paw inflammation. The study had expected outcomes, but also unexpected results regarding the effectiveness of a single oral dose of chlorogenic acid. Many studies present the weak antioxidant effects of oral CGA administration in relation to the low detectable levels of CGA in the blood (Rodriguez-Mateos et al., 2014). After its ingestion, chlorogenic acid is poorly absorbed in the small intestine, most of the chlorogenic acid reaches the colon, is metabolized and its metabolites are absorbed, conjugated in the liver and transported to the tissues (Stalmach et al., 2009). After the filtration of CGA conjugates, they can be identified in urine (Fumeaux et al., 2010).

The present study showed that chlorogenic acid can exert antioxidant effects through its conjugates in the inflamed skin, in kidneys as well as in the serum, and this polyphenol can be considered as an agent with therapeutic effects.

Conclusions

1. The oral administration of a single dose of chlorogenic acid at one hour after carrageenan injection showed antiedematous and antioxidant effects in inflamed skin. The protection against oxidative stress in the serum and kidneys presents this polyphenol as a potent antioxidant substance.

2. Oral indomethacin administration had antioxidant and antiedematous effects, even in a low single dose.

Conflict of interest

The authors declare that they have no conflict of interest.

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