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Chlorogenic acid modifies plasma and liver concentrations of: cholesterol, triacylglycerol, and minerals in (*fa/fa)* Zucker rats

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Abstract

Chlorogenic acid, a phenolic compound found ubiquitously in plants, is an *in vitro* antioxidant and metal chelator. Some derivatives of chlorogenic acid are hypoglycemic agents and may affect lipid metabolism. Concentrations of cholesterol and triacylglycerols are of interest due to their association with diseases such as non-insulin-dependent-diabetes- mellitus and obese insulin resistance. As little is known about the effects of chlorogenic acid *in vivo*, studies using obese, hyperlipidemic, and insulin resistant *(fa/fa)* Zucker rats were conducted to test the effect of chlorogenic acid on fasting plasma glucose, plasma and liver triacylglycerols and cholesterol concentrations. Aditionally, the effects of chlorogenic acid on selected mineral concentrations in plasma, spleen, and liver were determined. Rats were implanted with jugular vein catheters. Chlorogenic acid was infused (5 mg/Kg body weight/day) for 3 weeks via intravenous infusion. Chlorogenic acid did not promote sustained hypoglycemia and significantly lowered the postprandial peak response to a glucose challenge when compared to the same group of rats before Chlorogenic acid treatment. In Chlorogenic acid-treated rats, fasting plasma cholesterol and triacylglycerols concentrations significantly decreased by 44% and 58% respectively, as did in liver triacylglycerols concentrations (24%). We did not find differences ($p > 0.05$) in adipose triacylglycerols concentration. Significant differences ($p < 0.05$) in the plasma, liver, and spleen concentration of selected minerals were found in chlorogenic acid-treated rats. *In vivo*, chlorogenic acid was found to improve glucose tolerance, decreased some plasma and liver lipids, and improve mineral pool distribution under the conditions of this study.

Keywords: Improved magnesium concentrations; Hypolipidemic effect; Chlorogenic acid; Triacylglycerols; Glucose tolerance; Zucker *(fa/fa)* rats

1. Introduction

Obesity; insulin resistance (IR); high levels of plasma cholesterol (CHOL), and/or triacylglycerols (TG); and electrolyte disturbances are associated with a high risk for cardiovascular diseases (CVD) and are complications found in non-insulin-dependent-diabetes- mellitus (type 2 diabetes) [1,2]. Reduced risk for CVD is often attributed to phytochemicals lowering excessive serum glucose, CHOL, and/or TG concentrations [3]. Phenolics, such as chlorogenic acid (CGA) formed by esterification of caffeic (CFA) and quinic acids, have been claimed to modulate the activity of glucose-6-phosphatase (G-6-PASE; EC 3.1.3.9) involved in glucose metabolism [4] and to reduce the risk of CVD by decreasing oxidation of low density lipoproteins (LDL) CHOL and total CHOL [5,6].

Research conducted to investigate the absorption and metabolism of CGA exist. Intravenous (IV) or intraperitoneal administration of CGA to rats resulted in very low concentrations of CGA in the urine [7]. The authors suggested that CGA was picked up by the liver [7,8]. Chlorogenic acid was found to enter human hepatoma cells but noted that CGA incubated with human plasma or liver extracts was not hydrolyzed, due to lack of esterase activity, in the plasma and liver [9].

If CGA reduces blood glucose, CHOL, or TG concentrations *in vivo*, the effect may be due to more than a direct inhibition of G-6-PASE activity. Phenolic acids might regulate hepatic function by modulating, for example, the concentrations of CHOL and/or TG caused by an increase insulin sensitivity associated with an improved distribution of mineral. Electrolyte disturbances such as reduced concentration of serum and liver magnesium are found in IR, obese type 2 diabetes and CVD [1,2,10,11]. Magnesium

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ions in plasma exists in free, complexed, and/or protein bound forms [12]. Chlorogenic acid has antioxidant activity due to its cation chelation properties [13]. Chlorogenic acid infuse via IV might chelate minerals forming complexes that could be picked up by soft tissue.

Metformin, an insulin sensitizer improves CHOL and/or TG plasma profiles in obese type 2 diabetics and increases the concentration of magnesium in liver of diabetic rats consuming the same non-magnesium supplemented diet as controls [11,14]. The increase was associated with an improved insulin action and magnesium tissue distribution. The genetically obese and insulin resistant Zucker (*fa/fa*) rat has increased serum CHOL and/or TG, and low serum magnesium concentrations [11], thus, it is a good model to study the *in vivo* effect of CGA as a metal chelator and a novel hypolipidemic agent. The purpose of this research was to determine the effects of IV infused CGA to Zucker (*fa/fa*) rats on blood glucose concentrations; and on plasma TG, CHOL, and mineral concentrations; and on liver and spleen mineral concentration.

2. Methods and materials

2.1. Animals

Approval for care and treatment of animals was obtained from the Institutional Animal Care and Use Committee of North Dakota State University. Male Sprague-Dawley Zucker (*fa-fa)* rats (9 weeks old), obese, insulin resistant, hyperlipidemic, and type 2 diabetic weighing between 315 and 327 g, were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and housed in hang wire bottomed stainless steel cages. The room housing the rats was temperature controlled (22°C, 40–60% relative humidity) and kept under a daily 12-hr light/dark cycle. Water and AIN-93M Purified Rodent Powder Diet (Dytes ® Inc., Bethlehem, PA) were supplied *ad libitum.* The animals (n = 12) were allowed to acclimatize for one week before they were subjected to surgery. A flow chart of the experimental design for this research is shown (Fig. 1).

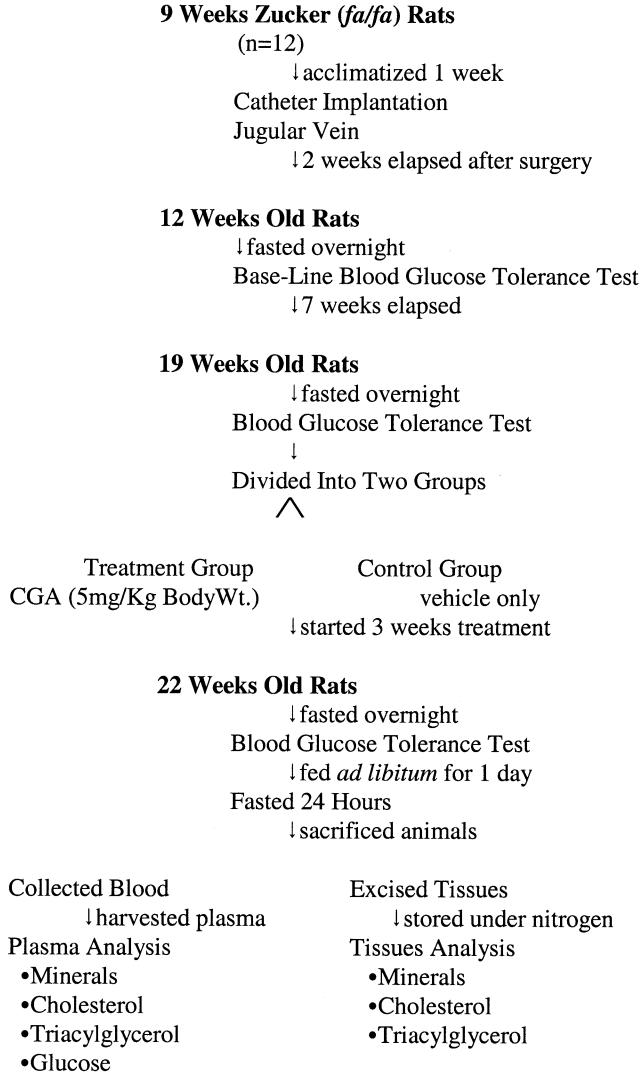

Fig. 1. Schematic representation of the timeline and design to study the effects of intravenous infusion of chlorogenic acid (CGA) in Zucker (*fa/fa*) rats.

2.2. Animal surgery

Distilled water was sterilized using a Barnstead® Sterilizer (Barnstead Thermolyne Co. Dubuque, IW). Saline (0.9% NaCl) and heparinized saline solutions were prepared in sterile distilled water and filter sterilized using a 0.2 μm Syrfil® Disposable Filter Unit. Pentobarbital and the gentamicin sulfate antibiotic Garacin® solutions were filter sterilized using a 0.2 μm Syrfil® Disposable Filter Unit before used.

A week after arrival, groups of up to 6 rats per day were anesthetized by intraperitoneal injection of pentobarbital (60 mg/Kg body weight) and implanted with indwelling jugular vein catheters [15]. Briefly, the animal surgery procedure was as follows: an incision was made above the animal's right jugular vein and a section of this vein was isolated. A silastic catheter was inserted into the vein approximately 4–5 cm, flushed with a heparinized saline solution (5 units/ml), and secured with nylon absorbable sutures and cyanoacrylate glue. The catheter was then passed subcutaneously to the back of the neck and affixed to a right angle pedestal that had been secured to the skull by using skull screws and dental cement. The incisions made on the animal above the right jugular vein area and on the skin protecting the skull were sutured using monofilament nylon non-absorbable sutures. The body temperature was monitored using a rectal probe thermometer and the temperature was maintained at 37°C by using a heated surgical plate [15].

Immediately after surgery, rats were treated via subcutaneous injection with 5 mg/Kg of a gentamicin sulfate antibiotic, Garacin®. Post surgical care for the animals included a daily treatment with the antibiotic for the next

week and monitoring of body temperature [15]. To maintain flow, the catheters were flushed every other day with 0.1 ml heparinized saline solution (5 units/ml) [15]. The animals recovered completely, regained the weight lost due to surgery, and appeared to be healthy gaining weight and eating well post the surgical period. Two weeks elapsed after surgery and before the experimental period was started [15]. The catheter implanted and secured on a pedestal in the head facilitated IV treatment and the withdrawal of blood for the measurement of blood glucose before and during glucose tolerance tests.

The rats (12 weeks old) were bled to determine base-line fasting blood glucose concentrations (see section 2.3 for methodology). This was immediately followed by an oral glucose tolerance test [16]. After the base-line fasting blood glucose concentrations, a total of seven weeks elapsed before the experimental period was started. This approach was followed in an effort to understand the response to a glucose challenge at different ages (12, 19, and 22 weeks old) prior to and after CGA treatment.

The rats (19 weeks old) were divided randomly into two groups (n = 6): control and treatment. Prior to CGA intervention, fasting blood glucose and glucose tolerance test were repeated (see section 2.3 for methodology). Thereafter, for a period of 3 weeks, the treatment rats were given IV infusion of CGA (5 mg per Kg body weight per day) in a 5 mM phosphate buffer pH 7.4 (vehicle). The control rats were given IV infusion of the vehicle only. The administration of CGA or the vehicle continued until the rats (22 weeks old) were sacrificed. Food consumed [grams (g)] and body weights (g) were recorded daily. These results, as averages over the experimental period, were estimated.

The rats (22 weeks old) were fasted for 24 hr, subjected to cervical dislocation, and exsanguinated by heart puncture. Blood was collected using heparinized syringes and transferred into heparinized tubes. Plasma was harvest from blood by centrifugation at 4°C for 10 min at 2000 × g and immediately stored on ice and glucose, CHOL, and TG concentrations were determined. The liver and spleen were removed, weighed, and rinsed with ice cold isotonic saline solution. Portions of sliced liver were removed; pulverized in liquid nitrogen; stored at -80°C; used for determination of hepatic lipids, water, CHOL, TG. Sections of epididymal fat tissues were removed and frozen at -80°C under liquid nitrogen and later used for determination of TG.

Separate portions of plasma and of pulverized liver and spleen were stored at -80°C, and freeze-dried at 70 millitorr at -80°C using a Dura-Dry Freezer (FTS Systems, Inc. Stone Ridge, NY). Freeze-dried plasma and tissue samples were used to determine the plasma concentration of iron, copper, zinc, calcium, magnesium manganese, potassium, sodium, and phosphorous. The de-ionized distilled water used to prepare reagents was 18 mΩ purity, collected from a 4-ModuleE-pure® purification system (Barnstead Thermolyne Co. Dubuque, IW).

2.3. Blood analysis and glucose tolerance test

After an overnight fast, rats were bled from the implanted catheter using heparinized syringes, and blood was transferred into heparinized tubes. A 32 μl aliquot of blood was used to determine glucose concentrations using a Reflotron ® System (Mannheim Corp. Indianapolis, IN).

The determination of fasting blood glucose concentrations was immediately followed by stomach gavage with a glucose solution (3 g glucose/Kg body weight) [16] to determine glucose tolerance. Blood glucose concentrations were determined at 30 min after glucose administration and, thereafter, at 30 min intervals up to 120 min. Blood glucose concentrations (mg/dl) were plotted versus time (min) and the plot used to determine the blood glucose peak time.

When the rats were 22 weeks old, fasting blood glucose and glucose tolerance tests were repeated for the treatment group only. Although catheters were flushed daily with 0.1 ml heparinized saline solution (5 units/ml), blood could not be drawn from the control rats as the catheters were plugged by 22 weeks.

The fasting plasma harvested from blood after euthanization was used for determination of glucose, TG, and CHOL concentrations for both controls and CGA-treated rats. Separately aliquots (32 μl each) of fasting plasma were used to determine either glucose, TG and CHOL concentration using a Reflotron ® System (Mannheim Corp. Indianapolis, IN).

2.4. Plasma, liver, and spleen concentrations of minerals

Freeze-dried plasma samples were reconstituted in deionized distilled water to obtain a final concentration of 4 mg/ml and used for determination of iron, copper, zinc, calcium, magnesium, manganese, potassium, sodium, and phosphorous concentrations. The determination of plasma minerals was performed at the USDA laboratories facilities in Grand Forks, ND, using an atomic absorption 3300DV Inductively Coupled Argon Atomic Emission Spectrometer (Perkin Elmer Co, Norwalk, CT) versus the appropriate external standards.

Freeze-dried portions of spleen (20 ± 1.0 mg) and of liver (40 ± 1.0 mg) were used for the determination of iron, copper, zinc, calcium, magnesium, manganese, potassium, sodium, and phosphorous concentrations. The spleen and liver samples were reduced to ash using a Furnace Oven (Perkin Elmer Co, Norwalk, CT) at 450°C for 16 hr. The ash from liver and spleen was reconstituted in 2 N hydrochloric acid (HCl) to a final volume of 10.0 ml. The determination of liver and spleen minerals was conducted at the USDA laboratories facilities in Grand Forks, ND, using a 4110ZL Zeeman Graphite Furnace Atomic absorption Spectrometer (Perkin Elmer Co, Norwalk, CT).

2.5. Liver and epididymal fat tissues analysis

Total lipid from frozen liver and epididymal fat tissue (2.000 ± 0.003 g) was extracted with 250 ml of hexane using a Soxhlet apparatus [17]. After 16 hr of extraction, the hexane was removed by distillation and a nitrogen flow was applied to the flasks until the lipid was completely dry. The dried lipids were kept frozen at -20°C until analyzed for TG and CHOL.

Separately, the dried lipids obtained from liver and epididymal fat tissues were reconstituted in 10 ml ethanol at 50°C and transferred into test tubes [18]. The dissolved lipids were dried under nitrogen to a final volume of 1.0 ml and used for CHOL and/or TG determination [18].

Liver cholesterol was determined using an INFINITY™ Cholesterol kit (SIGMA® Chemical Company, St. Louis, MO) based on the cholesterol oxidase UV method at $\lambda =$ 500 nm. The absorbance was read against a blank and a cholesterol calibrator (200 mg/dl) using a Beckman Du®-50 Spectrophotometer (Beckman Instruments, Inc. Irvine, CA). The absorbance was proportional to the CHOL concentration.

The TG concentration was determined using a TRIGLYCERIDE Kit (SIGMA® Chemical Company, St. Louis, MO) based on the final production of NADH. The decrease in absorbance of NADH was measured at $\lambda =$ 340 nm using a Beckman Du®-50 Spectrophotometer (Beckman Instruments Inc. Irvine, CA) was proportional to the concentration of TG.

Total lipids were extracted from epididymal fat tissue as explained for the liver tissue. The TG concentrations were determined using the same kit used for liver samples.

2.6. Statistical analysis

All results are expressed as means ± SEM (n = 6). The data were analyzed by analysis of variance (ANOVA) using SAS software and Duncan's multiple range model at an $\alpha =$ 0.05 [19].

3. Results and discussion

3.1. Effects of CGA treatment on food intake and body weight

There was no significant difference in the mean daily food intake of CGA-treated rats when compared to controls (Table 1). Therefore, supplementation of CGA did not suppress food intake in the obese Zucker (*fa/fa*) rats.

No significant difference was found in the initial or final body weights of the CGA-treated group versus the control group (Table 1). Within groups: in the CGA group there was no significant difference between their initial and their final body weights; however, for the control group, a significant difference ($p < 0.05$) was found between their initial and their final body weights. During the 3 weeks of treatment, the CGA-treated rats gained an average of 0.88 ± 0.3 g/day. Whereas the control group body weight gain averaged 1.78 ± 0.1 g/day. The higher rate of weight gain, could be due to increased lipid synthesis which is often associated with obesity.

Table 1
In vivo effect of cholorogtenic acid on body weight and food intake in Zucker (*fa/fa*) rats

	Control	[1]CGA Treated Rats
Number of Rats	6	6
Initial [2]BW (g)	577.3 ± 22.8[a]	538.2 ± 21.8[a]
Final BW(g)	614.7 ± 23.1[a]	556.7 ± 26.0[a]
Food Intake (g/day)	23.39 ± 1.6[a]	22.33 ± 2.2[a]
Daily Food Intake/BW	0.038 ± 0.003[a]	0.040 ± 0.004[a]

Values are means ± SEM.

([1] CGA = chlorogenic acid, injected intravenously every day for 3 weeks at 5 mg/Kg body weight. [2] BW = rat body weight).

Means in the same row and with different letters are significantly different ($p < 0.05$).

3.2. Plasma concentration of minerals

Electrolyte disturbances are found in serum of obese type 2 diabetics and/or insulin resistant individuals and hypertense patients [1,2]. The concentration of some minerals were determine in plasma of CGA and control rats. Prior to freeze-drying, the plasma pH was measured and no CGA-induced changes in pH were observed when compared to control rats.

In the CGA-treated rats, the tendency was toward an elevated concentration of iron, zinc, and manganese; and a reduced concentration of calcium, but when compared to the control group no significant ($p > 0.05$) differences were found (Table 2). However, a significant increase in the concentration of copper, magnesium, sodium, and potas-

Table 2
In vivo effect of chlorogenic acid after 3 weeks intervention on plasma concentration of some minerals of Zucker (*fa/fa*) rats

	Controls	[1]CGA Treated Rats
Number of Rats	6	6
Plasma Iron (μg/g)	68.36 ± 8.39[a]	86.74 ± 25.51[a]
Plasma Copper (μg/g)	22.04 ± 0.96[a]	34.37 ± 2.92[b]
Plasma Magnesium (μg/g)	351.44 ± 8.96[a]	400.05 ± 14.81[b]
Plasma Calcium (μg/g)	901.74 ± 85.66[a]	831.49 ± 18.73[a]
Plasma Managanese (μg/g)	0.846 ± 0.50[a]	0.934 ± 0.85[a]
Plasma Zinc (μg/g)	20.01 ± 0.82[a]	18.09 ± 1.14[a]
Plasma Sodium (μg/g)	42553.00 ± 3175.60[a]	59520.01 ± 5604.07[b]
Plasma Potassium (μg/g)	5078.50 ± 161.94[a]	5384.30 ± 174.96[b]
Plasma Phosphorous (μg/g)	3145.30 ± 440.97[a]	2394.60 ± 728.01[b]

Values are means ± SEM.

([1] CGA = chlorogenic acid, injected intravenously every day for 3 weeks at 5 mg/Kg body weight.)

Means in the same row and with different letters are significantly different ($p < 0.05$).

sium was found in the plasma of CGA-treated rats (Table 2). The concentration of phosphorus was significantly ($p < 0.05$) lower in the CGA-treated rats.

As the plasma magnesium concentration increased significantly ($p < 0.05$) in the CGA-treated Zucker (*fa/fa*) rats, the tendency towards a reduced calcium concentration was evident while the opposite was true for the controls rats (Table 2). In rats, the tendency toward an elevated calcium concentrations in the plasma, occurs during the early stages of magnesium deficiency [20]. Increased concentrations of calcium in the serum are associated with CVD due to the risk of plaque formations [1]. A 7.8% increase in plasma calcium concentration (2.77 ± 0.07 μmol/ml) was found in obese Zucker (*fa/fa*) rats, 18 weeks old, when compared to plasma calcium concentration (2.57 ± 0.02 μmol/ml) of the lean Zucker (*Fa/fa*) rats of the same age [21]. The control rats, 22 weeks old, had an 8.4% increase in plasma calcium concentration which is in close agreement with results from the previous report [21]. Additionally, an 18% decrease in plasma magnesium concentration was found in 16 week old obese Zucker (*fa/fa*) rats when compared to lean Zucker (*Fa/fa*) rats of the same age [10]. In this investigation, a decrease in plasma magnesium (12%) for the control obese Zucker(*fa/fa*) rats at the age of 22 weeks is in agreement with previous findings [10]. The decreased plasma magnesium concentration and the increased (8.4%) calcium concentration might, in part, explain why the catheters of the control group, at the age of 22 weeks, were plugged.

Low serum concentrations of magnesium, elevation of calcium concentrations in the serum, and a high ratio of calcium/magnesium are found in humans and in animals with IR, diabetes mellitus, and CVD [10,22]. The hyperlipidemic characteristic of the obese Zucker (*fa/fa*) rat, together with the disturbance in magnesium and calcium concentrations can promote atherogenesis in these rats [10,21, 23]. Thus, is possible that the control rats used under the conditions of this experiment were undergoing atherogenesis. This suggestion is supported by observations that obese Zucker (*fa/fa*) develop vascular complications over time due to obesity [24].

In addition to magnesium, serum concentrations of potassium and sodium are decreased in obese type 2 diabetics individuals suffering from CVD, and in animal models of these diseases [10,23,25]. Clinical studies have shown that the use of magnesium and potassium salts via IV lead to an improved arrhythmia in patients with myocardial infarction [25]. Although not addressed in this preliminary research, CGA might have acted as a modulator of the electrolyte pool distribution by improving the plasma concentration of magnesium, potassium, and sodium, thus, preventing arrhythmia in the Zucker (*fa/fa*) rats (Table 2).

A significant decrease ($p < 0.05$) in the serum phosphorous concentration for the CGA-treated rats was found when compared to controls. The obese Zucker (*fa/fa*) rat has characteristic disturbances in electrolyte concentrations such as those found in type 2 diabetes. For example, the concentration of calcium and phosphorous in the blood of obese Zucker (*fa/fa*) rat is reported to be higher than either of two lean rats breeds, Zucker (*Fa/fa*) and Wistar rats [21]. Additionally, the obese Zucker (*fa/fa*) rat develops renal disease early in life and 90% of these rats die of end-stage renal disease [25]. Thus, these rats are considered an animal model for chronic renal failure [26]. Electrolyte disorders such as elevated serum phosphorous and calcium are found in patients with renal failure [22]. Thus, phosphorus binders are often used to manage chronic renal insufficiency [27]. While the renal characteristics of the Zucker (*fa/fa*) rats used in this experiments were not investigated, CGA acid might have acted as a phosphorous binder, decreasing the plasma phosphorous concentration in the treated Zucker (*fa/fa*) rats. If this is the case, CGA might be beneficial in preventing renal failure.

A significant increase in the plasma copper concentrations (Table 2) was found in the CGA-treated Zucker (*fa/fa*) rats when compared to controls. An increased of 66% in the plasma concentration of copper was found in obese Zucker (*fa/fa*) rats when compared to lean Zucker (*Fa/fa*) rats [21]. Caffeic acid has been shown to form a complex with copper due to its chelating activity [5]. In this investigation, CGA was administered IV, and possibly formed a complex with copper, which was not excreted via the biliary duct, but rather deposited in the liver or left in circulation. The increased copper levels is supported by work reporting that biliary excretion of copper became depressed after prolonged feeding of ascorbate to rats [28]. The post-absorptive administration of ascorbate is known to enhance copper transfer into cells for tissue utilization [29]. Thus, it is possible that CGA, in the same manner as ascorbate enhanced the transfer of excess copper in the plasma to cells thus improving copper retention in the obese Zucker (*fa/fa*) rat.

3.3. Mineral concentrations in spleen and liver

Liver and spleen weight was calculated per 100 g of body weight and a 3.2 fold increase ($p < 0.05$) was found in the spleen weight of CGA-treated rats. There was a 1.20-, 1.10-, and 1.30-fold increase ($p < 0.05$) in the magnesium, potassium, and phosphorous levels in the spleen of CGA-treated rats respectively (Table 3). Increased concentrations of minerals, including magnesium, have been reported in the spleen of rats consuming a black tea decoction [30]. Teas contain phenolics compounds including CGA [31]. The increased spleen weight and mineral concentrations found in the spleen might have been due to the formation of CGA complexes which were deposited in the spleen (Table 3).

Soft tissue concentration of minerals are altered in CVD, obese type 2 diabetes and/or IR in humans and some genetically obese animals models of these diseases [1,2,10]. The concentration of some minerals in the spleen and liver of CGA-treated rats and controls were determined.

Plasma calcium and phosphorous were lower in CGA-treated rats. It might be that the excess minerals in the blood

Table 3
In vivo effect of chlorogenic acid after 3 weeks intervention on the spleen concentration of some minerals in Zucker (*fa/fa*) rats

	Controls	[1]CGA Treated Rats
Number of Rats	6	6
Spleen Iron (μg/g)	2876.40 ± 541.13[a]	1051.60 ± 64.81[b]
Spleen Copper (μg/g)	9.92 ± 0.84[a]	11.23 ± 1.09[a]
Spleen Magnesium (μg/g)	1312.06 ± 32.63[a]	1629.06 ± 51.87[b]
Spleen Calcium (μg/g)	288.10 ± 17.27[a]	222.34 ± 13.39[b]
Spleen Managanese (μg/g)	3.05 ± 0.49[a]	2.01 ± 0.12[a]
Spleen Zinc (μg/g)	132.51 ± 1.50[a]	138.90 ± 4.36[a]
Spleen Sodium (μg/g)	7558.70 ± 135.82[a]	7522.60 ± 197.11[a]
Spleen Potassium (μg/g)	16411.20 ± 358.76[a]	18331.70 ± 396.10[b]
Spleen Phosphorous (μg/g)	14965.60 ± 440.97[a]	19350.50 ± 728.01[b]

Values are means ± SEM.
([1] CGA = chlorogenic acid, injected intravenously every day for 3 weeks at 5 mg/Kg body weight.)
Means in the same row and with different letters are significantly different ($p < 0.05$).

were chelated by CGA and transported by erythrocytes and stored in the spleen thus, explaining the increased mineral concentrations in the spleen of CGA-treated rats (Table 3). This suggestion is supported by work reporting the accumulation of erythrocyte waste, including metal complexes, in the spleen of rats [32].

There was no significant ($p > 0.05$) difference in the concentration of iron in the liver between groups, while tissue distribution of copper, magnesium, zinc, calcium, manganese, sodium, potassium, and phosphorus were significantly increased ($p < 0.05$) in the CGA-treated rats (Table 4). *In vivo*, it is possible that CGA chelates metals and that, CGA-metal complexes are deposited in the liver. This suggestion is supported by early work in which it was reported that CFA chelated metal ions *in vitro* and *in vivo*, thus reducing the oxidation of LDL particles [5,6]. Chelation of zinc by CGA and CFA has been reported in a study using male Wistar rats [33] and may account for higher zinc levels in the liver.

Table 4
In vivo effect of chlorogenic acid after 3 weeks intervention on liver concentration of some minerals of Zucker (*fa/fa*) rats

	Controls	[1]CGA Treated Rats
Number of Rats	6	6
Liver Iron (μg/g)	322.09 ± 70.82[a]	280.75 ± 9.16[a]
Liver Copper (μg/g)	9.33 ± 0.31[a]	16.36 ± 1.22[b]
Liver Magnesium (μg/g)	562.90 ± 31.78[a]	790.36 ± 18.26[b]
Liver Calcium (μg/g)	153.06 ± 9.31[a]	104.66 ± 4.46[b]
Liver Managanese (μg/g)	3.30 ± 0.20[a]	4.60 ± 0.19[b]
Liver Zinc (μg/g)	63.17 ± 3.07[a]	97.66 ± 3.60[b]
Liver Sodium (μg/g)	3460.04 ± 50.96[a]	3940.97 ± 29.21[b]
Liver Potassium (μg/g)	6237.10 ± 151.98[a]	7809.80 ± 172.45[b]
Liver Phosphorous (μg/g)	5535.00 ± 325.46[a]	8468.60 ± 224.55[b]

Values are means ± SEM.
([1] CGA = chlorogenic acid injected intravenously every day for 3 weeks at 5 mg/Kg body weight.)
Means in the same row and with different letters are significantly different ($p < 0.05$).

The control of magnesium homeostasis by insulin in rat liver has been documented [11,14,20]. In IR and in insulin dependent diabetes mellitus (type 1 diabetes) and type 2 diabetes, a disturbance in distribution of zinc, copper, and magnesium was found [1,34,35]. For instance, in spontaneously type 1 diabetic BB Wistar rats, the decrease in insulin, is correlated with increased zinc and copper concentrations in the liver [35,36]. While in streptozotocin-induced diabetic rats, an increase in serum and liver magnesium concentrations is associated with improved insulin action or decreased peripheral IR [11,14]. The obese Zucker (*fa/fa*) rat, a model for peripheral IR, has reduced serum and liver magnesium concentrations [10,23].

One of the indicators for cardiovascular complications associated with type 2 diabetes is the elevated tissue zinc/copper ratio [34]. In the CGA Zucker (*fa/fa*) rats, a significant increase ($p < 0.05$) in the concentration of magnesium, zinc, and copper in the liver was found compared to controls. Decreased copper content in the serum and increased deposition of iron in the liver and spleen were found in the control group. Increased iron deposition and, decreased copper and zinc in serum; and an increased zinc/copper ratio in soft tissues have been associated with glucose intolerance, type 2 diabetes, and/or deficiency of ceruloplasmin ferroxidase [34,37,38].

Compared to the controls, the CGA-treated rats had a decreased zinc/copper ratio in the plasma and decreased zinc/copper ratio in the liver, while in plasma, the concentration for copper was increased significantly ($p < 0.05$). The zinc/copper ratio of plasma and liver in the control group increased ($p < 0.05$), 41% and 12% respectively, over CGA-treated rats, and the tendency was to an elevated deposition of iron in the liver. Increased peripheral IR has been associated with the early deposition of iron and later with the progression of β-cell dysfunction [37,39]. The male obese, insulin resistant, and type 2 diabetic Zucker (*fa/fa*) rat has been found to evolve β-cell dysfunction [39]. Metformin, used in the management of type 2 diabetes, improves mineral distribution and potentiates insulin action in animals and humans [11,14,38]. Chlorogenic acid might have acted as an insulin sensitizer by improving tissue mineral distribution in the treated Zucker (*fa/fa*) rats and decreasing peripheral IR.

3.4. Plasma and liver cholesterol and triacylglycerol concentrations

Chlorogenic acid was infused daily for a period of 3 weeks in an attempt to understand its effect on some lipid metabolites in hyperlipidemic Zucker (*fa/fa*) rats. Specifically, CHOL and TG concentration were determined in fasting plasma and liver tissue.

The fasting plasma CHOL and TG concentrations in CGA-treated Zucker (*fa/fa*) rats decreased significantly

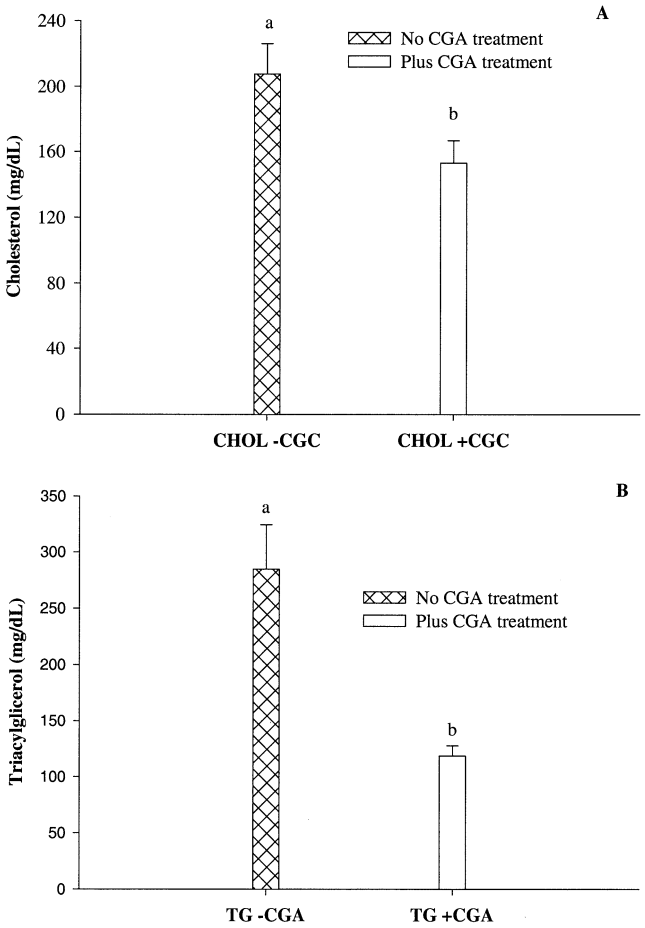

Fig. 2. Effect of chlorogenic acid (CGA) treatment in Zucker (*fa/fa*) rats for 3 weeks on fasting plasma cholesterol (CHOL) and triacylglycerol (TG). A. Twenty-four hour fasting concentration of CHOL. B. Twenty-four hour fasting concentration of TG. [a,b]($p < 0.05$). Values are means ± SEM (n = 6).

($p < 0.05$) by 44% and 58%, respectively (Figs. 2A and 2B). A disturbance in electrolyte distribution has been correlated with an increased lipogenesis and plasma lipid concentrations leading to CVD [1,23]. Reduced magnesium concentrations in serum have been associated with increased CHOL and/or TG concentrations [1,20]. Type 2 diabetic rats, including the obese Zucker (*fa/fa*) rats, have low serum and soft tissue magnesium concentrations and elevated concentrations of CHOL and TG [10,14].

Under the condition of this study, the magnesium concentration in the plasma and liver (Tables 2 and 4) of CGA-treated Zucker *(fa/fa)* rats increased significantly ($p < 0.05$). Furthermore, the zinc/copper ratio in the plasma and in the liver of CGA-treated Zucker *(fa/fa)* rats significantly decreased ($p < 0.05$) when compared to controls. Thus, the improved pool distribution of minerals in the CGA-treated Zucker *(fa/fa)* rats might have been due to an increased insulin sensitivity leading to a reduction of plasma CHOL and TG concentrations. A decreased concentration of circulating CHOL and TG in Zucker *(fa/fa)* rats treated with insulin sensitizers supports this suggestion [40].

In the liver, a significant decrease ($p < 0.05$) of 24% in TG was found in the CGA-treated rats when compared to controls. Furthermore, a significant difference ($p < 0.05$) was found in the ratio of total TG per g liver weight per 100 g body weight between the CGA-treated group (0.066 ± 0.006) and the control group (0.088 ± 0.007). Therefore, CGA intervention in the obese type 2 diabetic Zucker (*fa/fa*) rat had an *in vivo* effect on liver TG synthesis probably due to an increased insulin sensitivity as a result of increased insulin clearance by the liver, and/or increased glucose uptake by other tissue such as muscle. This suggestion is supported by others [41] reporting a decrease in hepatic insulin, mediated diversion of glucose to hepatic lipid synthesis in insulin resistant *cp/cp* rats using blenfluorex treatment, a hypolipidemic and anti-hyperglycemic agent. Additionally, reduced hepatic glucose utilization, for process such as glycogen synthesis, due to IR, contributes to the hypertriglyceridemia associated with increased hepatic lipogenesis in the Zucker *(fa/fa)* rat [42]. Furthermore, it has been shown that in the peripheral IR of the Zucker *(fa/fa)* rat, great quantities of glucose are shunted toward hepatic fatty acid synthesis causing both TG accumulation in the liver and elevated serum concentrations of TG [42]. The Zucker *(fa/fa)* rat has elevated serum fatty acids and TG, both peaking at the age of 24 weeks [43]. Thus, it is possible in the experiments reported here, that the 22 weeks old control rats, had increased hepatic fatty acid synthesis and the resultant TGs were released into the blood. This suggestion is supported by others [43], who investigated age related changes in obese Zucker *(fa/fa)* rats versus lean Zucker *(Fa/fa)* rats. In the obese Zucker *(fa/fa)* rats, but not in their lean littermate, TG concentration in the bloodstream increased as the rats aged, peaking at about 30 weeks [43]. Compared to lean Zucker *(Fa/fa)* rats, CHOL in the blood of obese Zucker *(fa/fa)* rats began rising early in life and continued to accumulate until the rats were 58 weeks old [43]. In agreement with work by others [43], the results of this investigation indicate no significant ($p > 0.05$) differences in TG concentration of epididymal fat between CGA-treated and control Zucker *(fa/fa)* rats. However, since CGA decreased liver and plasma TG concentrations it acted as an *in vivo* hypolipidemic agent.

Hepatic steatosis, an increased synthesis of hepatic TG together with an increased serum TG concentration, has been associated with obesity and IR, and its incidence in type 2 diabetes is as high as 78% [37,44]. The obese Zucker (*fa/fa*) rat is an animal model for hepatic steatosis [41,45]. The results found under the conditions of this research indicate an improvement of hepatic function since plasma TG concentrations decreased. This may indicate that CGA is an antihyperglycemic agent. Lipid-lowering and insulin-sensitivity effects of antihyperglycemic agents such as metformin and thiazolidinedione have been reported in Zucker (*fa/fa*) rats [40].

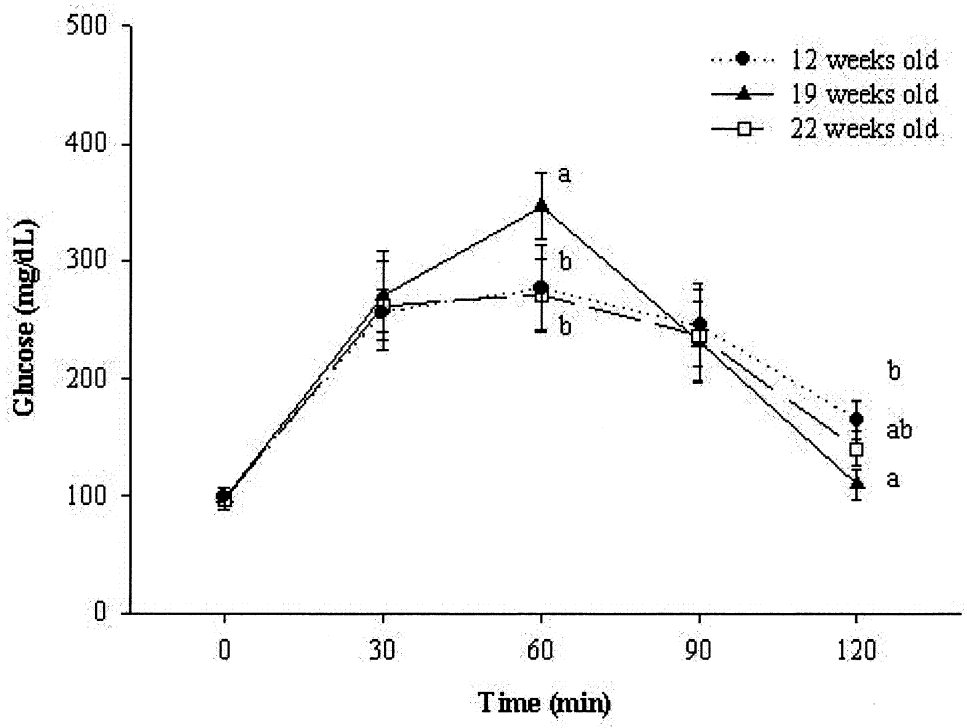

Fig. 3. Blood glucose (mg/dl) for Zucker (*fa/fa*) rats at 12 and 19 week old without chlorogenic acid treatment, and at 22 week old with chlorogenic acid treatment. [a] [b]($p < 0.05$). Values are means ± SEM (n = 6).

3.5. Fasting blood glucose concentrations and oral glucose tolerance test

Insulin resistance is an impaired biological response to insulin by one or more of its target tissues such as the muscle, adipose, or the liver; leading to reduction of glucose clearance [41]. In the liver specifically, IR leads to increased glucose output resulting in peripheral hyperinsulinemia due to a decrease of insulin clearance [41,42,45]. The Zucker (*fa/fa*) rat is characterized as severely insulin resistant [43, 46]. Thus, it is possible that the insensitivity to insulin by the control group in this study caused the glucose and lipid metabolic alterations found when compared to the CGA-treated rats.

Previously, elevated fasting blood glucose concentrations were used as an indication that a patient might be diabetic. One of the newest criteria to screen for the early development and/or management of type 2 diabetes and its cardiovascular complications in humans is not based solely in fasting blood glucose concentrations but also on elevated postprandial blood glucose concentrations [47].

In humans, postprandial hyperglycemia is defined as a blood glucose concentration of more than 200 mg/dl two hours after a challenge with oral glucose or after a meal [47]. Sustained postprandial hyperglycemia is considered one of the risk factors indicative of CVD associated with obese type 2 diabetes [47].

It has been shown that Zucker (*fa/fa*) rats develop an inability, with age, to maintain serum glucose following a glucose challenge or a meal [43]. A similar response, with age, was found as shown by the significant higher peak glucose response at 19 weeks old (Fig. 3).

After 3 weeks intervention with CGA, the Zucker (*fa/fa*) rats, age 22 weeks, had a significant ($p < 0.05$) decrease in the peak blood glucose concentration after one hour when compared to the same group of rats at 19 weeks of age (Fig. 3). Moreover, the CGA-treated rats maintained the same peak blood glucose concentration found when the rats were 12 weeks old before CGA intervention and before they showed postprandial hyperglycemia (Fig. 3).

The normal average for fasting blood glucose concentrations found in the insulin resistant and obese Zucker (*fa/fa*) rats after 13 weeks of age is 100 ± 10 mg/dl [46]. Chlorogenic acid did not lower fasting blood glucose concentrations in the 22 weeks old Zucker (*fa/fa*) rats (97.94 ± 6.33 mg/dl), when compared to the same group of rats before CGA treatment at either 12 or 19 weeks of age (98.46 ± 9.57 and 99.25 ± 7.33) mg/dl, respectively. Furthermore, for the control group (22 weeks old) the blood glucose concentration (97.89 ± 6.68 mg/dl) did not differ from the CGA-treated group. This findings lead to the suggestion that CGA did not stimulate insulin release, thus it is not possible for CGA to cause sustained hypoglycemia. Chlorogenic acid might have acted as an antihyperglycemic agent, a therapeutic action known of insulin sensitizers such as metformin which does not increase insulin release [44,48]. Further research is needed to explore the long term effect(s) of CGA as a novel antihyperglycemic agent using insulin

resistant obese and non-obese type 2 diabetic animals at different ages and stages of the disease, and non-insulin resistant non-diabetic animals.

As shown in Fig. 3, IV infusion of CGA significantly lowered the peak response to a glucose load when compared to the same group of rats before CGA treatment. This decrease may be indicative of an improved sensitivity to insulin. Thus, it is possible that during the 3 weeks of CGA intervention, peripheral hepatic hyperinsulinemia decreased due to an increased hepatic clearance of insulin. Consequently, a decrease in insulin and glucose concentrations might explain the decreased synthesis of hepatic lipids and therefore, the decreased secretion of TG by the liver into the blood in the CGA-treated group.

Under the conditions of this study, significant differences were found in plasma and tissue minerals; plasma and liver TG and CHOL; and postprandial glucose concentrations in CGA-treated rats. Therefore, the null hypothesis: "infusion of CGA via IV to genetically obese, IR, and hyperlipidemia Zucker (*fa/fa*) rats will not: chelate minerals, decrease concentrations of plasma and/or liver CHOL and/or TG, and improve glucose tolerance" was rejected.

Overall, the findings under the conditions of this investigation suggest that insulin sensitivity might have been improved significantly in the liver of CGA-treated rats. Although this research was conducted using an animal model, the results indicate additional studies are needed to clarify the mechanisms involved and the regulatory functions of CGA on lipid and mineral metabolism. Future research in this area may lead to a new therapeutic agent for use in treatment for insulin-resistant and/or type 2 diabetic individuals. Furthermore, this study adds to the evidence for the possible beneficial health effects of phytochemicals such as CGA.

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