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## Original paper

# Hepatoprotective effects of chlorogenic acid under hyperglycemic conditions

HATTF BAZOOL FARHOOD<sup>1,2#</sup>, MIHAELA BALAS<sup>2\*#</sup>, DANIELA GRADINARU<sup>3</sup>,  
DENISA MARGINĂ<sup>3</sup>, ANCA DINISCHIOTU<sup>2</sup>

<sup>1</sup>University of Thi-Qar, College of Sciences, Department of Chemistry, Thi-Qar, Iraq

<sup>2</sup>University of Bucharest, Department of Biochemistry and Molecular Biology, Bucharest, Romania

<sup>3</sup>Department of Biochemistry, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania

# Authors with equal contribution

### Abstract

Chlorogenic acids (CGAs) are a family of polyphenol compounds with antioxidant, anti-inflammatory and anticarcinogenic activities, that exerts hypoglycemic and hypolipidemic effects. In this study we focused on the investigation of the chlorogenic acid (3-O-Caffeoylquinic acid, CGA) effect on human hepatocytes cells under hyperglycemic (HG) condition. HepG2 (human liver carcinoma) cells grown in normoglycemic (NG, 5.5 mM glucose) and HG (25 mM and 35 mM glucose) culture media were pre- and post-treated with different doses of CGA (0 = control, 5, 10 and 50  $\mu$ M) for 24 and 48 h. The cellular metabolic activity and level of reactive oxygen species (ROS) were evaluated. Exposure to HG media caused an increase in cellular metabolic activity in a dose and time-dependent manner. In cells pre- or post-treated with CGA the cellular metabolic activity decreased in a CGA dose-dependent manner, reaching after 48 h a similar level with that of NG cells. Under HG condition, intracellular ROS were generated in higher amounts compared to NG ones. Both treatments reduced the excess of ROS produced in HG cells up to that of NG cells, revealing a pronounced antioxidant effect. Our findings demonstrate the potential of CGA to mitigate the damaging effects induced by HG conditions in both pre- and post- treatment of HepG2 cells.

### Keywords

Chlorogenic acid, hyperglycemia, hepatocytes, reactive oxygen species

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\*Corresponding author: MIHAELA BALAS, <sup>2</sup>University of Bucharest, Department of Biochemistry and Molecular Biology, Bucharest, Romania; Department of Biochemistry, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania  
E-mail: [radu\\_mihaella@yahoo.com](mailto:radu_mihaella@yahoo.com), [mihaela.radu@bio.unibuc.ro](mailto:mihaela.radu@bio.unibuc.ro)

## Introduction

Nowadays, diabetes mellitus characterized by chronic hyperglycemia, is a growing health concern. Beside the well-known cardiovascular, renal, and ophthalmologic complications of diabetes, liver injuries often occurs through several mechanisms. Changes induced by hyperglycemia in the liver's cellular environment have been extensively studied in *in vitro* and *in vivo* models (A. DEY & K. CHANDRASEKARAN [1]). Some of the effects induced by HG conditions include: increased levels of oxidative and nitrosative stress (P.R. LING & al. [2]; Z. MADAR & al. [3]), activation of stress-related signaling pathways (M. NAWANO & al. [4]; H. KANETO & al. [5]), high cytokine levels (M. CARMIEL-HAGGAI & al. [6]), impairment of protective mechanisms such as the expression of molecular chaperones and proteasome activity (M. PORTERO-OTIN & al. [7]; C. SWIECKI & al. [8]) and dysregulation of glucose and lipid metabolism (K.J. BROCKLEHURST & al. [9]; X. HOU & al. [10]).

Liver is involved in glucose homeostasis and it plays the most important role together with skeletal muscles and brain in glucose metabolism and regulation. The hepatocytes use glucose as a fuel and store it as glycogen and also synthesize it from non-carbohydrate precursors via gluconeogenesis.

Epidemiological studies revealed that a healthy diet rich in vegetables and fruit protects the liver against different injuries and this is mainly due to the content in antioxidant compounds such as: polyphenols, that can be of flavonoid, hydroxybenzoic acids, stilbenes, lignans and hydroxycinnamic acids (P. VITAGLIONE & al. [11]).

Chlorogenic acid (3-*O*-Caffeoylquinic acid, CGA) is an ester formed between caffeic acid and the 3-hydroxyl group of quinic acid that belong to the group of hydroxycinnamic acids and is found alongside isochlorogenic acids in coffee beans and different forms of coffee being easily absorbed through small intestine (N. LIANG & D.D. KITTS [12]). A series of health benefits have been associated with the consumption of CGA (N. TAJIK & al. [13]), such as: reduction of the relative risk of several diseases such as the cardiovascular one (T. RANHEIM & B. HALVORSEN [14]), type 2 diabetes (E. SALAZAR-MARTINEZ & al. [15]) and Alzheimer's diseases (J. LINDSAY & al. [16]). The mechanisms for specific health benefits attributed to CGA involve mitigation of oxidative stress and related adverse effects associated with anti-inflammatory activities by modulating a number of important metabolic pathways (N. LIANG & D.D. KITTS [12]; M.D. SANTOS & al. [17]), inhibitory effect against the growth of the microorganisms (S.A.A. FARAH & al. [18]) and protecting effects on cells and tissues against carcinogenesis and carcinogenic metabolites through the activation of the Nrf2/ARE pathway (U. BOETTLER & al. [19]).

In the last few years, several studies have demonstrated that CGA also plays a role in glucose and lipid metabolism *in vivo* (S. MENG & al. [20]) being involved in the controlled release of glucose into the bloodstream and probably delaying the development of diabetes. A unique dose of 50 mg/b.w. of CGA was found to be sufficient to decrease glycaemia in diabetic rats (A.W. HERLING & al. [21]). This polyphenol compound also inhibited fat absorption and activated the fat metabolism in liver. The experiment of H. SHIMODA & al. [22] revealed that oral administration of different doses between 30 and 60 mg/kg/day CGA for 14 days significantly reduced the level of hepatic triglycerides in mice.

Coffee consumption has been associated with elevated activity of liver enzymes and a reduced risk of advanced liver disease and its complications (A.A. MODI & al. [23]; M. WADHAWAN & A.C. ANAND [24]). A recent study showed that CGA suppressed liver fibrogenesis and carcinogenesis by reducing oxidative stress and counteracting steatogenesis through the modulation of glucose and lipid homeostasis in the liver (F. SALOMONE & al. [25]). However, the mechanisms of action underlying these effects are not fully understood.

In this study we focused on the investigation of the CGA protective effect on human hepatocytes cells under hyperglycemic condition.

## Materials and Methods

### Cell culture and treatment

HepG<sub>2</sub> cell line (purchased from the American Type Culture Collection, HB-8065) derived from a hepatocellular carcinoma of a 15 years-old male was chosen for this study due to their ability to easily grow in both low- and high-glucose media. HepG<sub>2</sub> cells are a well-established model for the study of the effects of glucose on cellular metabolism and response to different stimuli (R. DOMENIS & al. [26]). The cells were cultured in NG (5.55 mM glucose) Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotic-antimycotic solution (A5955, Sigma) and 10% fetal bovine serum at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. The cells were seeded at a density of 5×10<sup>5</sup> cells/75 cm<sup>2</sup> flask. A 20 mM stock solution of chlorogenic acid (CGA) (C3878, Sigma) was prepared in PBS and sterilized before the cell treatment. Two different variants of treatments were designed for this study: (A) *Post-treatment with CGA* – the cells were first incubated in HG medium (25 mM and 35 mM glucose) and after 24 h, different concentrations of CGA (5, 10 and 50 μM) were added for 24 h and 48 h in fresh NG medium and (B) *Pre-treatment with CGA* - the cells were incubated with CGA (5, 10 and 50 μM) for 24 h and then the medium was removed and replaced by medium supplemented with glucose (final concentrations in the medium 25 mM and 35 mM) and incubated for another 24 h and 48 h. Cells cultured in NG medium and without chlorogenic acid were used as controls.

### **MTT assay**

The MTT reduction assay was used to determine the level of metabolic activity in human hepatocytes after treatment (J. MOSMANN [27]). HepG2 cells were seeded at a density of  $5 \times 10^4$  cells/well in a 24-well microplate. After the treatment periods, the medium from each well was removed by aspiration, then a volume of 500  $\mu$ L of 1 mg/mL 3-(4, 5-dimethylthiazo 1-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) solution (M2128, Sigma), was added to each well. After 2 h of incubation at 37°C in the dark, the MTT solution was removed and a volume of 300  $\mu$ L isopropanol was added to solubilize the formazan crystals. The optical density (OD) for each sample was recorded at 595 nm.

### **Measurement of intracellular ROS**

For the quantification of intracellular ROS it was used the cell-permeant reagent 2', 7'-dichlorofluorescein diacetate (DCFDA, D6883, Sigma). After the pre- and post-treatment with CGA, the cells seeded in a 96-well microplates were immediately incubated for 30 min with 100  $\mu$ L DCFDA (50  $\mu$ M) in the dark at 37°C. The fluorescence of the resulted compound dichlorofluorescein (DCF) was spectrofluorimetrically recorded at ex. 485 nm/em. 535 nm in each sample and expressed in relative fluorescence units (RFU).

### **Statistical analysis**

Data were expressed in terms of mean  $\pm$  standard deviation (SD) of triplicate measurements from three independent experiments. Student's *t*-tests were used to analyze the differences between the samples untreated and treated with CGA in hyperglycemic conditions. *p* values  $< 0.05$  were considered significant.

## **Results and discussion**

### **CGA effect on cell metabolic activity**

HepG2 cells cultured in NG and HG conditions were pre- and post-treated with CGA in order to study their efficiency to counteract the hyperglycemia-induced effects in human hepatocytes. An important number of studies, highlighted that high glucose exposure induced toxicity in cultured cells (S. KAWAHITO & al. [28]; A.A. FAROOQUI [29]). Some of the major pathways that could be affected by hyperglycemia in liver cells included the increase of oxidative stress, dysregulation of fatty acid metabolism and impaired cellular repair machinery (A. DEY & K. CHANDRASEKARAN [1]).

In this study, we assessed the cell metabolic activity by measuring the capacity of mitochondrial succinate dehydrogenase to reduce the MTT to formazan in HepG2 cells. This assay also gives information about proliferation rate and viability of the tested cells.

After exposure to HG medium (25 and 35 mM glucose) insignificant increases of HepG2 cell metabolism by 0.2 and 0.3 fold respectively after 24 h and by 0.1 and

0.2 fold respectively after 48 h compared with ones grown in NG medium (Figure 1) were noticed. After the post-treatment with CGA, cell metabolic activity decreased significantly compared to untreated HG cells in a CGA dose-dependent manner reaching the level of NG controls. No significant difference was observed between the metabolic activity of HepG2 cells incubated in media with 25 mM glucose and 35 mM glucose respectively. However, the cells treated with CGA were less metabolically active after 48 h compared with those incubated in NG medium.

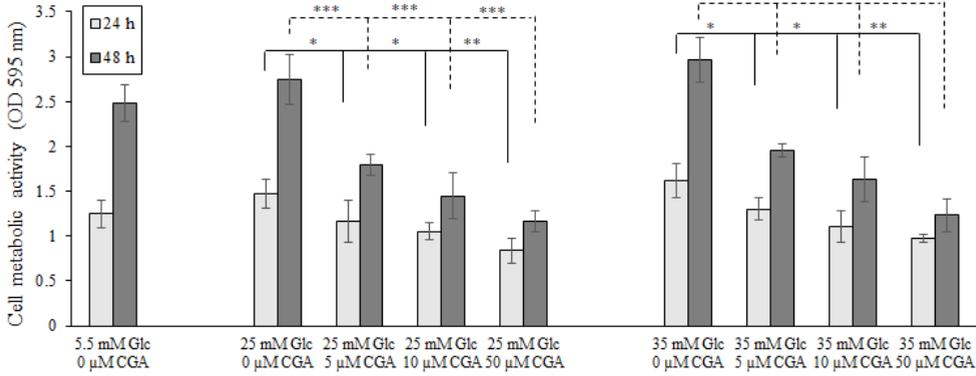
When HepG2 cells were pre-treated with CGA and then exposed to HG medium for 24 and 48 h respectively, the effects on cell metabolism were less pronounced. A significant decrease of cell proliferation was registered only in the cells incubated in medium with 25 mM glucose and 50  $\mu$ M CGA (Figure 2). The metabolic activity of those incubated in medium with 35 mM glucose was not affected by CGA pre-treatment but was higher than that of cells incubated in NG medium especially after 48 h.

Probably due to its lipophilic and weak-acidic nature, CGA could pass through the lipid layer of the inner mitochondrial membrane and enter mitochondria (C. SANDOVAL-ACUÑA & al. [30]) where it could bind to proteins via non-covalent and/or covalent bonds generating conformation transitions that are changing the tridimensional structure and as a consequence their biological activity (K. BRUDZYNSKI & L. MALDONADO-ALVAREZ [31]). Also HG-induced oxidative stress could damage the mitochondrial inner membrane (W.J. SIVITZ & M.A. YOREK [32]). Once entered the mitochondria, CGA could inhibit complex II (succinate CoQ reductase) of the mitochondrial electron transport chain, probably due to the presence of catechol ring, as previously it was suggested (C.SANDOVAL-ACUÑA & al. [30]) as well as complexes I and III, and as a result, the activities of mitochondrial oxidoreductases decreased.

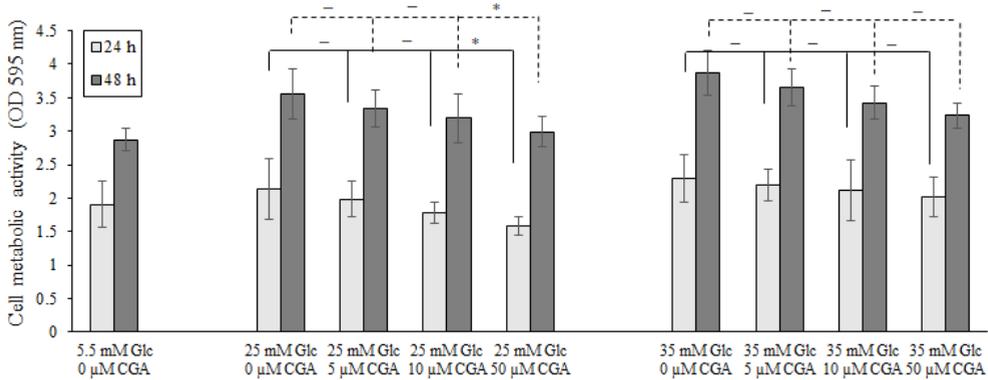
### **CGA effect on ROS production**

Glucose is commonly used in cell culture experiments as an inducer of oxidative stress, (F.A. MATOUGH & al. [33]) taking into account that hyperglycemia induces free radicals and impairs the endogenous antioxidant defense system in patients with diabetes. Exposure for long periods to high levels of glucose causes progressive alterations in gene expression and reduction of oxidative metabolism which generates an increase in ROS production, creating a vicious metabolic cycle that leads to irreversible tissue damage in subjects with diabetes (D.M. ANSLEY & B.WANG [34]).

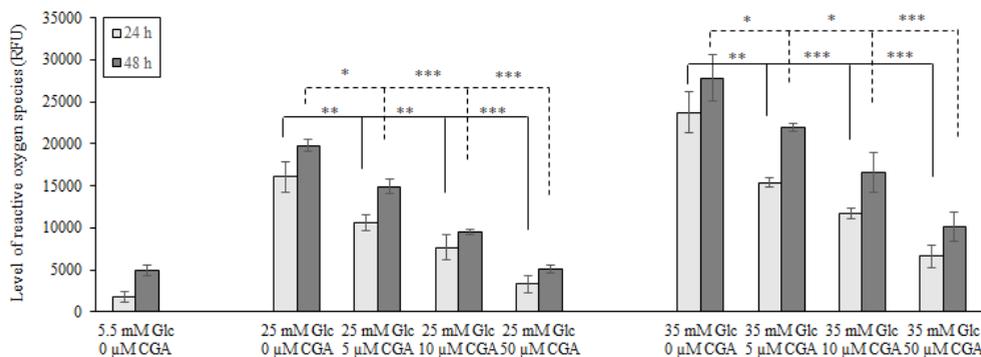
As expected, after incubation in HG medium, intracellular ROS levels increased significantly in HepG2 cells in a glucose concentration dependent manner. The ROS production increased rapidly after 24 h by 9.1 and 13.5 fold in cells exposed to 25 and 35 mM glucose respectively, in comparison with NG control. After 48 h of exposure, the production of ROS was higher only by 4 and respectively 5.6 fold than NG control (Figure 3).



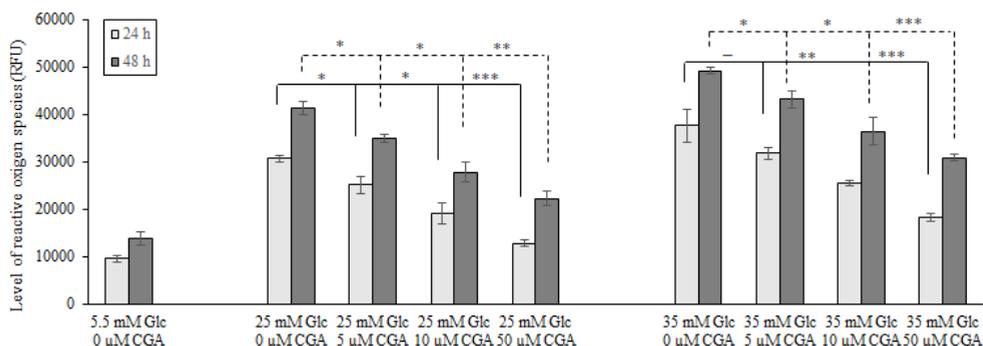
**Figure 1.** Level of cell metabolic activity after post-treatment with CGA. HepG2 cells incubated in HG medium (25 and 35 mM glucose) for 24 h were exposed to different concentrations of CGA (0, 5, 10 and 50 μM) for 24 h and 48 h. Untreated cells were used as controls. Values are expressed in mean absorbance at 595 nm ± SD. CGA-treated samples were compared with HG controls. The results were statistically significant when  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*)



**Figure 2.** Level of cell metabolic activity after 24 h pre-treatment with CGA (0, 5, 10 and 50 μM). HepG2 cells were then exposed for 24 and 48 h to high glucose concentrations (25 and 35 mM glucose). Untreated cells were used as controls. Values are expressed in mean absorbance at 595 nm ± SD. CGA-treated samples were compared with HG controls. The results were statistically significant when  $p < 0.05$  (\*), a value  $> 0.05$  was considered not significant (-)



**Figure 3.** Level of ROS production after post-treatment with CGA. HepG2 cells incubated in HG medium (25 and 35 mM glucose) for 24 h were exposed to different concentration of CGA (0, 5, 10 and 50 μM) for 24 h and 48 h. Untreated cells were used as controls. Values are expressed in mean RFU ± SD. CGA-treated samples were compared with HG controls. The results were statistical significant when  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*)



**Figure 4.** Level of ROS production after 24 h pre-treatment with CGA (0, 5, 10 and 50 μM). HepG2 cells were then exposed for 24 and 48 h to high glucose concentrations (25 and 35 mM glucose). Untreated cells were used as controls. Values are expressed in mean RFU ± SD. CGA-treat samples were compared with HG controls. The results were statistically significant when  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) a value  $> 0.05$  was considered not significant (-).

Hyperglycemia has been found to stimulate ROS production in liver through protein kinase C dependent activation of NADPH oxidase, and induced MAPK phosphorylation subsequent to proliferation and type I collagen production (R. SUGIMOTO & al. [35]). Also, ROS overproduction induced by exposure to high glucose concentrations was associated with mitochondrial morphology changes which can be targeted to control acute and chronic production of ROS in hyperglycemia-associated disorders (T. YU & al. [36]).

In our study, the increase of ROS level in HepG2 cells was highly correlated with the enhanced mitochondrial enzymes activity and proliferation of cells in HG medium.

In the same manner, C.M. PALMEIRA & al., [37] demonstrated that prolonged hyperglycemia-induced ROS overproduction caused an increase in cell proliferation of HepG2 cells.

After post-treatment with CGA, the ROS level decreased significantly in a dose-dependent manner. As shown in Figure 3, the level of HepG2 cells treated with a dose of 50 μM CGA almost reached the ROS level registered in NG cells. Our study suggests that CGA treatment could diminish the ROS production in hepatocytes under HG conditions.

Previous studies showed that CGAs possess *in vitro* and *in vivo* antioxidant capacity (Y. SATO & al. [38]) that

inhibit ROS production by donating hydrogen atoms to reduce free radicals and to inhibit oxidation reactions. After donating hydrogen atoms, CGAs are oxidized to respective phenoxyl radicals and these are quickly stabilized by resonance stabilization (N. LIANG & D.D. KITTS [12]). F. SALOMONE & al. [25] showed that CGA suppresses liver fibrogenesis and carcinogenesis by reducing oxidative stress and counteract steatogenesis through the modulation of glucose and lipid homeostasis in the liver. Recently, it was shown that CGA protects murine osteoblasts against oxidative damage by decreasing ROS, MDA and NO production, as well as by increasing GSH content. Furthermore, it was demonstrated that CGA augmented the cellular antioxidant defense capacity by enhancing Nrf2 nuclear translocation and upregulating hem oxygenase (HO-1) induction. In these events, PI3K/Akt signaling pathway played a central role. (D. HAN & al. [39]).

By comparison, the pre-treatment of hepatocytes with CGA had less pronounced effect on ROS production especially for cells incubated in medium with 35 mM glucose (Figure 4). The ROS levels decreased in the same CGA concentration dependent manner but did not reach the one from NG cells.

## Conclusion

Many studies tried to investigate natural compounds, especially polyphenols, which have been found to inhibit the production of ROS. In this study we investigated the role of CGA in protecting hepatocytes before and after hyperglycemia exposure up to 48 h. Our results indicated that the pre- and post-treatment with CGA reduced the cell metabolic activity and counteracted the oxidative stress induced by HG conditions in human hepatocytes, diminishing, therefore, cell damage and enabling the cells to cope in better conditions with further oxidative insults.

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