

MATERNAL AND FETAL HEPATIC INJURY IN GESTATIONAL DIABETIC RATS: PROTECTIVE ROLE OF CINNAMALDEHYDE AND ELLAGIC ACID

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Received: 05 February 2018, Revised and Accepted: 28 March 2018

ABSTRACT

Objective: This study aims to determine the protective effect of cinnamaldehyde (Ci) and ellagic acid (EA) on maternal and fetal hepatic injury caused by fatty-sucrosed-diet/streptozotocin (FSD/STZ)-rat model of gestational diabetes (GD).

Methods: Female Wistar rats were allocated into four groups. Group I fed with a normal diet, while Groups II, III, and IV fed with FSD for 8 weeks (five pre-gestational and three gestational). One week before mating onward, Group III and IV administered daily oral dose of 20 mg/kg Ci or 50 mg/kg EA, respectively. At the 7th day of gestation, FSD-fed groups injected intraperitoneally with STZ (25 mg/kg b.wt.) to induce GD mellitus. At the end of gestation, maternal and fetal blood, pancreas and liver samples were taken for the subsequent analysis.

Results: Ci and EA potentially protect from the elevated glucose, triglycerides, total cholesterol, liver transaminases, and the depleted serum insulin, high-density lipoprotein-cholesterol, total proteins and albumin of GD-rats. As well, tested agents alleviated the altered fetal glucose and insulin levels. Alongside, Ci and EA reduced the maternal and fetal liver lipid peroxidation and increased their total thiols and total peroxidases. Furthermore, Ci and EA kept the normal intact of maternal and fetal pancreatic islets and preserve their livers away from the pathological changes observed in GD group that included fatty depositions, inflammation, hepatocytic necrosis and fibrosis, congested veins, diminutive storable glycogen and proteins, and the numerous proliferating cell nuclear antigen nuclei.

Conclusion: Ci and EA have potent hepatoprotective effects through their insulin-sensitizing action and activating the antioxidant defenses.

Keywords: Diabetes, Oxidative stress, Lipid profile, Liver architecture, Hepatocytes proliferation, Natural products.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is the most frequent issue facing mothers during pregnancy. The International Diabetes Federation reported that GDM develops in one of 25 pregnancies worldwide, with increasing prevalence in the population with low- to middle-income countries [1]. GDM is defined as the impaired glucose tolerance that affecting pregnant women who have never before had diabetes [2]. It represents the failure to adapt the metabolic demands of pregnancy through both inadequate insulin secretion and insulin resistance which mimicking the pathophysiological characteristics of type 2 diabetes. GDM is associated with potential complications for the health of mothers and their babies concerning the future risk of metabolic syndrome, type 2 diabetes, and cardiovascular disorders (CVDs) [3]. The liver is the focal organ of carbohydrate metabolism, blood glucose homeostasis, and detoxifying processes. Until now, the link between GDM and risk of hepatic injury is still unclear. Recent epidemiological studies suggested that GDM is an early marker for the development of non-alcoholic fatty liver disease (NAFLD) [4]. NAFLD is the hepatic indicator of the metabolic syndrome, and it covers a disease spectrum ranging from steatosis to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [5].

Insulin is the commonly used therapeutic agent for GDM management. But rather than its inadequacy for many pregnant women [6], it may increase the risk of acquiring viral hepatitis because of the contact with needles. On the other hand, however, pharmacologic intervention with oral synthetic antidiabetic drugs (mainly metformin and glyburide) seemed to be tempting, hepatotoxicity of these drugs emerged common clinical complications [7] besides the paucity of data on the exposure of fetuses to them during pregnancy. As opposed to conventional

medications, antioxidants from plant-based and natural products are emerging as a future therapy and an alternative way to prevent and treat life-threatening disease. Cinnamaldehyde (Ci) and ellagic acid (EA) are bioactive natural compounds extracted from *Cinnamomum zeylanicum* bark and *Padina boryana* Thivy, respectively. Recent studies reported that both of them have potent antioxidant and anti-inflammatory properties besides their hypoglycemic action in type 1 and type 2 diabetic rats [8-11]. To date, there is still a lack of data describing their protective effect on the GD liver injury. Hence, the present study was conducted using a rat model of GDM induced by fatty-sucrosed diet (FSD) combined with low dose streptozotocin (STZ) to investigate the possible protective activities of Ci and EA on the maternal and fetal hepatic tissues and explore their possible mechanisms using different biochemical, histopathological, and immunohistochemical examinations.

METHODS

Plant materials collection and extract preparation

Bark of *C. zeylanicum* was collected from the botanical garden of Cairo University, Egypt, while *P. boryana* algae were gathered from the Red Sea, Safaga province, eastern coast, Egypt. Samples were authenticated by the Taxonomy Division, Botany Department, Faculty of Science, Beni-Suef University, Egypt. Ci and EA were extracted according to Subash-Babu *et al.* [9] and Abu El-Soud *et al.* [12] phytochemical investigation's, respectively.

Chemicals and reagents

Glucose, triglycerides (TG), total cholesterol, and high-density lipoprotein-cholesterol (HDL-cholesterol) kits were procured

from Spinreact Co. (Spain). Rat insulin ELISA kit was obtained from BioSource Europe S.A. (Belgium). Total proteins, albumin, alanine transaminase (ALT), and aspartate transaminase (AST) kits were obtained from Human Diagnostics (Germany). STZ, ethyl acetate, n-hexane, 5,5'-dithiobis-2-nitrobenzoic acid, 1,1,3,3-tetramethoxypropane, and thiobarbituric acid were purchased from Sigma Chemical Co. (USA), while pyrogallol was procured from Fluka analytical (Germany). Diet ingredients were purchased from Oxford Laboratories, India. All other chemicals were obtained from commercial suppliers.

Experimental animals

Female albino Wistar rats (*Rattus norvegicus*) weighing about 100-120 g were obtained from the animal house of VACSERA Co., Helwan city, Egypt. Rats were housed individually in standard cages and maintained in an air-conditioned atmosphere, set at 25°C with alternatively 12 h light and dark cycles for a week before the beginning of the experiment for acclimatization. Rats fed with two types of diets either normal diet (ND) or FSD and allowed to drink water *ad libitum*. The composition of both diets was illustrated elsewhere [13]. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Beni-Suef University, Egypt (BSU/FS/2015/11).

Experimental design

The experimental design is described in Fig. 1. A total of 60 albino Wistar rats were randomly divided into four groups (15 animals/group) as follows:

Group I (normal pregnant [NP]): Fed on ND. Four weeks after dietary manipulation, rats have received 0.5% dimethyl sulfoxide (DMSO) as a vehicle by oral gavage daily to the end of the experiment.

Group II (GD): Fed on FSD. Four weeks after dietary manipulation, rats have received the vehicle by oral gavage daily to the end of the experiment.

Group III (GD pre-treated with Ci [GD+Ci]): Fed on FSD. Four weeks after dietary manipulation, rats have received Ci (20 mg/kg b.wt. dissolved in 0.5% DMSO) by oral gavage [9] daily to the end of the experiment.

Group IV (GD pre-treated with EA [GD+EA]): Fed on FSD. Four weeks after dietary manipulation, rats have received EA (50 mg/kg b.wt. dissolved in 0.5% DMSO) by oral gavage [10] daily to the end of the experiment.

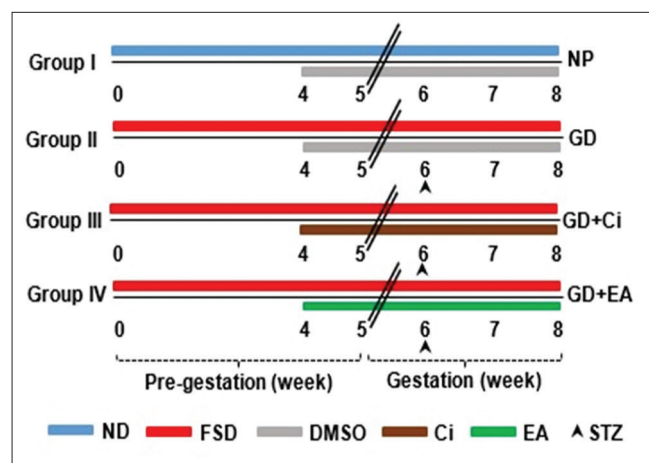


Fig. 1: *In vivo* experimental design. ND: Normal diet, FSD: Fatty-sucroed diet, DMSO: Dimethyl sulfoxide, Ci: Cinnamaldehyde, EA: Ellagic acid, STZ: Streptozotocin, NP: Normal pregnant, GD: Gestational diabetic, GD+Ci: Gestational diabetic pre-treated with cinnamaldehyde, GD+EA: gestational diabetic pre-treated with ellagic acid

After 5 weeks from the onset of the experiment, rats of all groups were mated overnight with males. Positive vaginal smear checked in the morning indicated the zero day of the gestation (rats with negative vaginal smear were excluded). The time before mating indicated the pre-gestational period while that after mating indicated the gestational period. At the 7th day of pregnancy (end of the 6th week), dams were fasted for 16 h and those of Groups II, III, and IV were injected with STZ intraperitoneally (25 mg/kg b.wt. in citrate buffer; pH 4.5) [13], while those of Group I was injected only with the citrate buffer. Blood samples were collected at the 20th day of gestation (8th week; post-STZ) from the lateral tail vein of each dam for estimation of fasting glucose and insulin levels to evaluate the development of GDM. At the 21st day of gestation, overnight fasted dams were sacrificed under light diethyl ether anesthesia.

Samples preparations

Blood was collected from the jugular vein of each dam, centrifuged, and sera were kept at -23°C for future biochemical analyses. As well, fetuses of each dam were delivered and blood pool was collected from the jugular veins where sera were separated for measurement of glucose and insulin concentrations. After dissection of dams and their fetuses, pancreas and liver samples were fixed in 10% neutral-buffered formalin for histopathological processing. Another liver samples were homogenized in phosphate buffer saline (PBS) at 4°C, centrifuged at 4000 rpm for 10 min and the clear homogenates were separated and stored at -23°C for subsequent determination of antioxidants and oxidative stress markers.

Biochemical assays

Both maternal and fetal serum glucose and insulin levels were determined using the corresponding reagent kits, while maternal TG, total cholesterol, HDL-cholesterol, total proteins, albumin, ALT, and AST concentrations were also detected. Lipid peroxidation (LPO) of maternal and fetal liver homogenates was assessed by measuring malondialdehyde (MDA) concentrations according to the method of Yagi [14], while total thiols (as non-enzymatic antioxidants) and total peroxidases (as enzymatic antioxidants) were estimated according to the methods of Koster *et al.* [15] and Kar and Mishra [16], respectively.

Histological and histochemical staining

Fixed pancreas and liver tissues were dehydrated, cleared, paraffin-embedded, and sectioned (5 µm thick) using Leica rotary microtome (Germany). After deparaffinization and rehydration, maternal pancreatic sections were stained with the modified aldehyde fuchsin-method [17], while those of fetuses were stained with hematoxylin and eosin (H and E) [18]. Liver sections were stained with H and E, periodic acid-Schiff (PAS) reaction [19], and mercuric bromophenol blue method [20] for the demonstration of any histopathological alterations, glycogen, and total proteins, respectively.

Immunohistochemical staining

For immunohistochemical localization of proliferating cell nuclear antigen (PCNA), liver sections (5 µm) were deparaffinized, rehydrated, and immunostained by the peroxidase-antiperoxidase method. In brief, high-temperature antigen unmasking technique was employed in 0.01 M citrate buffer (pH 6) in a microwave oven twice for 5 min each. Blocking of the non-specific reaction was performed with 1% normal goat serum, 3% non-fat milk, and PCNA mouse monoclonal antibody (Novocastra NCL-L-PCNA; Leica Biosystems, UK) (1:100). After rinsing in phosphate-buffered saline (0.01 mol/L PBS, pH 7.4), the sections were incubated with secondary antiserum. They were then washed in PBS and incubated in avidin and biotin complex (ABC) reagents (ABC kit, Vector Laboratories, Inc.) and in peroxidase reaction (3,3'-diaminobenzidine tetrahydrochloride, Sigma) containing 0.01% H₂O₂ in PBS buffer. For the control reaction, some slides were processed by omitting the primary antibody and other slides through omitting the primary and secondary antibodies (Bhan, 1995) [21].

Statistical analysis

Data were presented as means±standard error of the mean of ten rats. Measured variables were analyzed using one-way ANOVA test followed by Tukey–Kramer test for *post hoc* analysis. $p < 0.05$ were considered statistically significant. All statistical analyses were performed using the SPSS v.22 software (SPSS Inc., Chicago, USA).

RESULTS

Effect of Ci and EA on maternal and fetal glycemic state

GD-dams showed significant elevation of blood glucose level with a marked decrease in their serum insulin as compared to NP ones (Table 1). Similarly, GD raised the fetal glucose and insulin serum concentrations markedly as compared to those of NP-dams. Oral administration of Ci (20 mg/kg b.wt.) and EA (50 mg/kg bwt) resulted in an obvious improvement of these parameters, whereby Ci achieved more glycemic control than EA.

Effect of Ci and EA on maternal lipid and protein profiles

Maternal data in Table 1 clarified significant increase ($p < 0.05$) in serum TG and total cholesterol levels of GD-dams than NP-dams with noticeable depletion of their HDL-cholesterol, total proteins, and albumin serum ratios. Both of Ci and EA revealed profound amelioration of these parameters with an interest in their abilities to increase HDL-cholesterol concentrations than normal rats.

Effect of Ci and EA on maternal liver function enzymes

Levels of serum ALT and AST displayed marked increase in GD-rats in comparison to those of normal ones. Ci administration seemed to be more potent in reducing these enzymes serum concentration than EA.

Effect of Ci and EA on maternal and fetal liver oxidative and antioxidant parameters

Our results revealed significant elevation in MDA concentration in maternal and fetal liver samples of GD-group with a concomitant reduction of total thiols content and total peroxidase activity than those of normal group (Table 1). Otherwise, notable attenuation of MDA production parallel to the marked increase in total thiols concentration and peroxidases activity was achieved by both treatment agents with preferability to Ci.

Histopathological assessments

Light microscopical examination of modified aldehyde fuchsin-stained pancreatic sections from NP-rats demonstrated a normal architecture

of islets of Langerhans (as the endocrine portion) and pancreatic acini (as the exocrine portion) (inset in Fig. 2a). Three main types of islets cells are well recognized (Fig. 2a). Beta-cells are the most abundant and occupied the core of the islet, while alpha and delta cells are detected at the periphery. Delta cells are usually located adjacent to alpha cells and are somewhat large in size. In contrast, fatty-sucrose-diet-feeding/25 mg/kg b.wt streptozotocin induces variable structural alterations of pancreatic islet cells including pyknotic, irregular and/or hyperchromatic nuclei, vacuolated cytoplasm, and visible necrotic area. Significant reduction in total islets volume and cells number was also noticed (Fig. 2b). On the other hand, Fig. 2c and d, respectively, illustrated great protection and restoration of the normal islets configuration under Ci and EA administration where alpha, beta, and delta cells looked more intact. Ci seemed to stimulate a marked increase in the total islets volume than EA (inset in Fig. 2c).

Fig. 3a depicted the normal histological structure of fetal pancreatic section from NP-group stained with H and E. It consists of acinar exocrine lobules separated by septa and diffused with typical endocrine islets. Instead, fetal pancreatic islets of GD rats showed numerous vacuolations and its cells appeared hyperplastic at both islets core and periphery (Fig. 3b). Maternal manipulation of Ci made the fetal pancreatic islets more organized with a lesser extent of cells hyperplasia that existed only at the islets periphery (Fig. 3c). Furthermore, EA administration nearly restored the islets integrity except for the appearance of some vacuolations within (Fig. 3d).

H and E stained liver section from NP-group demonstrated the normal hepatic microarchitecture (Fig. 4a), where a typical lobule appeared with a central vein and hepatocytes plates radiating from it toward the periphery. Blood sinusoids and Kupffer cells are located between these plates. Conversely, liver of GD rats showed obvious histopathological alterations that include dilatation and congestion of portal veins (Fig. 4b), obscured sinusoids and distorted cell plates (Fig. 4d), appearance of signet ring and/or ballooning hepatic cells (that characterize the accumulation of fat droplet in liver tissue; inset in Fig. 4b and Fig. 4d), besides inflammatory infiltration of leukocytes into the liver parenchyma (Fig. 4c). Moreover, many hepatocytes underwent cytoplasmic vacuolar degeneration in addition to the appearance of the necrotic and fibrotic area (Fig. 4d and inset in Fig. 4d). Oral administration of Ci and EA 1 week before mating onward caused marked recovery of the normal structure of the liver where the

Table 1: Effect of Ci and EA on maternal and fetal biomarkers at term pregnancy

Parameter	Group			
	NP	GD	GD+Ci	GD+EA
Maternal parameters				
Fasting glucose (mg/dl)	64.7±2.11	267.44±9.53 ^a	71.83±6.43 ^b	119.12±0.9 ^{a,b}
Fasting insulin (μU/ml)	27.12±0.46	21.25±0.24 ^a	26.30±0.27 ^b	24.77±0.26 ^{a,b}
Triglycerides (mg/dl)	58.72±1.44	189.64±1.34 ^a	60.90±3.70 ^b	59.28±1.230 ^b
Total cholesterol (mg/dl)	81.12±1.45	185.13±2.51 ^a	130.08±0.88 ^{a,b}	118.58±1.51 ^{a,b}
HDL cholesterol (mg/dl)	58.06±1.54	52.52±1.18 ^a	89.76±1.12 ^{a,b}	76.04±2.12 ^{a,b}
Total proteins (g/dl)	7.48±0.08	5.88±0.19 ^a	7.32±0.14 ^b	6.92±0.10 ^{a,b}
Albumin (g/dl)	4.76±0.12	3.86±0.09 ^a	4.42±0.11 ^b	4.32±0.22 ^b
ALT (U/l)	36.44±1.05	95.58±1.88 ^a	41.98±0.95 ^b	70.60±0.86 ^{a,b}
AST (U/l)	160.60±3.48	241.50±1.75 ^a	161.10±1.45 ^b	209.16±2.04 ^{a,b}
MDA (nmol/g tissue)	54.63±1.64	120.93±2.18 ^a	80.85±2.4 ^{a,b}	89.29±1.91 ^{a,b}
Total thiols (nmol/100 mg tissue)	769.57±6.86	558.85±4.55 ^a	711.73±5.01 ^{a,b}	644.95±7.24 ^{a,b}
Total peroxidase (U/g tissue)	124.48±2.70	76.06±1.82 ^a	110.58±4.97 ^{a,b}	94.90±4.86 ^{a,b}
Fetal parameters				
Glucose (mg/dl)	42.94±1.46	161.50±4.12 ^a	52.10±2.18 ^{a,b}	86.44±2.60 ^{a,b}
Insulin (μU/ml)	3.60±0.14	6.20±0.16 ^a	4.01±0.19 ^{a,b}	4.98±0.12 ^{a,b}
MDA (nmol/g tissue)	19.03±0.81	45.00±2.37 ^a	24.91±1.54 ^{a,b}	29.76±1.22 ^{a,b}
Total thiols (nmol/100 mg tissue)	252.43±4.72	136.32±1.84 ^a	211.60±7.31 ^{a,b}	190.05±1.54 ^{a,b}
Total peroxidase (U/g tissue)	43.40±3.33	23.51±3.51 ^a	38.02±2.70 ^{a,b}	32.77±4.05 ^{a,b}

Results are presented as means±SEM of ten dams. ^a $p < 0.05$ versus NP, ^b $p < 0.05$ versus GD. NP: Normal pregnant, GD: Gestational diabetic, GD+EA: Gestational diabetic pre-treated with ellagic acid, HDL-cholesterol: High-density lipoprotein-cholesterol, ALT: Alanine transaminase, AST: Aspartate transaminase, MDA: Malondialdehyde, Ci: Cinnamaldehyde, SEM: Standard error of the mean

leukocytic infiltration, fatty accretion, vacuolated hepatocytes, and necrotic area are almost disappeared (Fig. 4e and f; respectively).

The fetal liver section from NP-group displayed a homogeneous mass of parenchymal hepatocytes that arranged in poorly defined lobules. Each lobule distinguished by a central vein and composed of irregular branched and interconnected hepatic strands which anastomose to

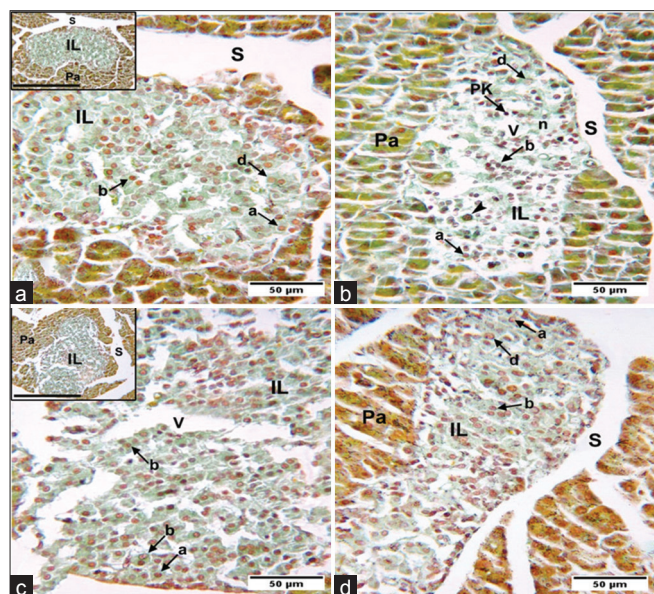


Fig. 2: Photomicrographs of modified aldehyde fuchsin-stained maternal pancreatic sections sampled from the experimental groups. (a) The normal configuration of pancreatic islets cells from normal pregnant-rats; a: Alpha, b: Beta, d: Delta cells, inset: The normal architecture of the pancreas which is subdivided by septa (S) into pancreatic lobules with pancreatic acini (Pa) and islets of Langerhans (IL) (scale bar=100 μ m). (b) Indicates disruption of the islets structure of gestational diabetic (GD)-rat's pancreas with reduced number of beta (b) cells, decreased total islets (IL) volume, appearance of vacuolation (V), pyknotic nuclei (PK), necrotic cells (n), and irregular hyperchromatic nuclei (arrowhead). (c) Reveals restoration of the normal islets cells with some vacuolations in GD+Cinnamaldehyde pancreatic section; inset: Displays a marked increase in the total islets (IL) volume (scale bar = 100 μ m). (d) Clarifies a marked increase in beta (b) cells number and normal intact alpha (a) and delta (d) cells of GD+ellagic acid pancreas

form a network enclosing a system of tortuous blood sinusoids (Fig. 4g). The fetal hepatic tissue of GD-rats revealed apparent changes. Central veins appeared dilated and engorged with blood (Fig. 4h), regions of necrosis and fibrosis were observed, and autolysis of some nuclei and dilated sinusoid were noticed (Fig. 4i and j). Treatment with Ci and EA induced significant protection of the normal fetal hepatic configuration against the GD stress (Fig. 4k and l, respectively). Meantime, some sinusoidal dilatations were still detected in their hepatic tissue.

Histochemical demonstration of glycogen and proteins content

A strong PAS-positive reaction was viewed in hepatocytes of maternal and fetal livers of NP-group indicating the presence of a large storable amount of glycogen (Fig. 5a and e). On the other hand, liver cells of GD-rats and their fetuses were almost depleted of glycogen (Fig. 5b and f). Ci and EA-treated dams revealed marked increase of their hepatic glycogen content which approximated that of the control ones (Fig. 5c and d, respectively). Moreover, the fetal liver of these both treated groups showed intensive glycogen deposition (Fig. 5g and h), but to a lesser extent in that of GD+EA-group than GD+Ci-one. By the same manner, a dense blue color was observed in the liver sections from NP-dams and fetuses illustrating an intensive reaction toward the protein granules which appeared randomly distributed in the ground cytoplasm of the hepatocytes (Fig. 5i and m). In contrast, both maternal and fetal livers from GD-group depicted a weak reactivity of bromophenol blue stain that indicated a significant diminution of the total proteins content (Fig. 5j and n). However, after treatment with Ci, a considerable amount of proteins was accumulated in the maternal and fetal hepatocytes (Fig. 5k and o). Liver section from EA-treated rats displayed a moderate amount of proteins (Fig. 5l), and liver of their fetuses showed a marked increase of proteins content (Fig. 5p) than that of the diabetic one.

Hepatic expression of PCNA

PCNA expression was immunohistochemically localized in maternal and fetal hepatic tissues (Fig. 6). In NP-group, a very weak expression of PCNA was founded in few hepatocytes of dam's livers but not in those of their fetuses (Fig. 6a and e). In contrast, parenchymal hepatocytes of GD-rats demonstrated strong PCNA-positive immunoreaction (Fig. 6b). As well as, fetal livers of this diabetic group displayed numerous PCNA-nuclei (Fig. 6f). Negative localization of PCNA in hepatic cells of GD+Ci rats was clearly observed (Fig. 6c), while their fetuses show pale brown color of PCNA-staining. In GD+EA group, maternal and fetal hepatic expression of PCNA was significantly weak when compared with that of the GD-group (Fig. 6d and h).

DISCUSSION

Our data indicated a marked elevation of maternal serum glucose level with diminished insulin secretion in GD-group. FSD-feeding stimulated

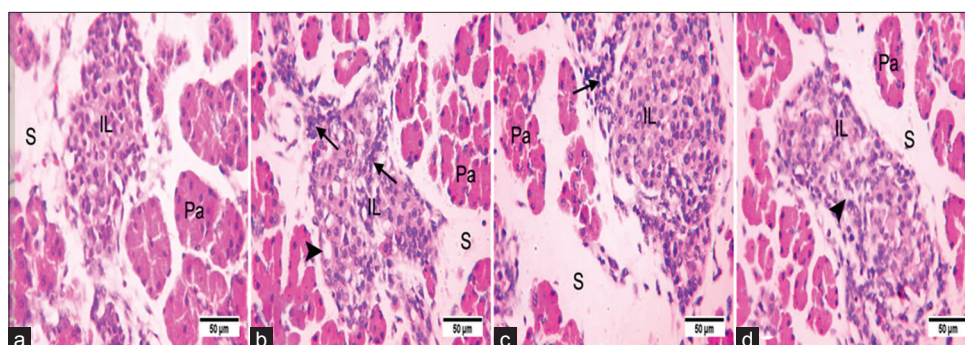


Fig. 3: Photomicrographs of H and E-stained fetal pancreatic sections sampled from the experimental groups. (a) Fetal pancreas from normal pregnant group with the normal histological features of the pancreatic lobules; S: Septa, Pa: Pancreatic acini, IL: Islets of Langerhans. (b) Depicts the fetal pancreatic cells hyperplasia at islets core and periphery (arrows) with numerous vacuolations (arrowhead) of gestational diabetic (GD)-group. (c) Fetal pancreas of GD+cinnamaldehyde-group appeared more organized with lesser extent of islets cells hyperplasia that existed only at the islets periphery (arrow). (d) Show the nearly restored integrity of fetal pancreatic islets of GD+ellagic acid-groups with the appearance of some vacuolations within (arrowhead)

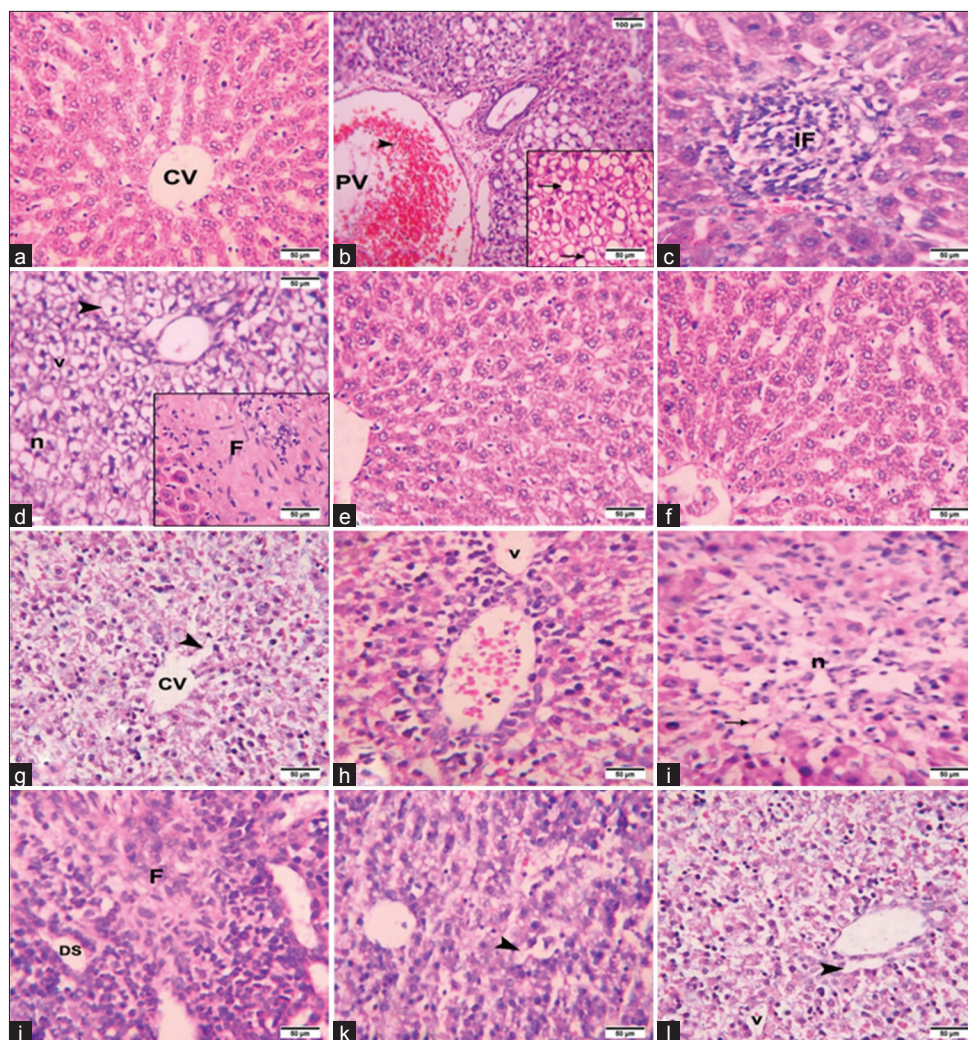


Fig. 4: Photomicrographs depict the histopathological changes in H and E-stained maternal and fetal liver sections sampled from the experimental groups. (a) Liver section of normal pregnant (NP)-rats illustrates the normal hepatic histological architecture; CV: Central vein. (b-d) Liver sections of gestational diabetic (GD)-rats. (b) Dilated and congested portal vein (PV) with mononuclear leukocytic infiltration (arrowhead); inset: Hepatocytes underwent fatty accretions (arrows). (c) Reveals mononuclear leukocytic infiltration (IF) in the hepatic parenchyma. (d) Displays vacuolar degeneration of the hepatocytes (v), necrotic area (n), and some ballooning hepatocytes (arrowhead); inset: Highlights liver fibrosis (F). (e and f) Liver sections of GD-rats pre-treated with Ci and EA, respectively, show great recovery and nearly normalized structure of the liver. (g) Fetal liver section from NP-group with the central vein (CV), sinusoids (arrowhead), and apparently healthy parenchyma. (h-j) Fetal liver sections from GD-group. (h) Demonstrates congested central vein (CV) and vacuolation (v). (i) Displays massive hepatocytic necrosis (n) and autolysis (arrow). (j) Reveals fibrotic area (F) and dilated sinusoids (DS). (k and l) Fetal liver sections of GD+cinnamaldehyde and GD+ellagic acid groups, respectively, showing restoration of the normal parenchymal structure with some dilated sinusoids that still present (arrowheads)

a state of glucose intolerance and insulin resistance through the glucose-fatty acid cycle [22]. In brief, feeding of diets rich in fats gives an increased source of fatty acids availability. The preferential utilization of these fatty acids for oxidation blunts the insulin-mediated reduction of liver glucose output and decreases glucose uptake by peripheral tissues which considered common features of insulin resistance. As well, the injected low dose of STZ resulted in morphological alterations for the pancreatic islets leading to subsequent suppression of insulin biosynthesis as a result of reactive oxygen species (ROS)-generation [23]. Oral administration of Ci achieved potent control of blood glucose level and stimulated more insulin secretion than EA through providing intense protection for pancreatic islets and increasing β -cell mass as seen from the histological screening of the maternal pancreas.

The persistent hyperglycemia of GD-rats considered, also, a principal mediator of ROS generation through the auto-oxidation of glucose molecules [24]. As the liver is the main organ of oxidative and detoxifying activities, ROS react with polyunsaturated fatty acids

of hepatocellular membranes leading to its peroxidation [23]. The increase of LPO impairs membrane function by decreasing its fluidity, increases its permeability, and changing activities of some bounded enzymes leading to cell death [25]. The present study hypothesized that the pre-treatment with Ci and EA significantly protects from MDA formation (main product of lipid peroxides) through their strong free radical scavenging action [8,26]. On the other hand, we observed changes in the hepatic enzymatic and non-enzymatic antioxidants in the different groups. Our results indicated depletion in total thiols concentration in the liver of GD-dams when compared to NP-ones. Thiols are organic compounds that have the sulphhydryl (SH)-group. Among all the body antioxidant's, thiols constitute the master portion of the body antioxidants that play a major role in defense against ROS. The functional consequences of liver total thiols losses in diabetic dams include protein misfolding, catalytic inactivation, and decreased antioxidative capacity [27]. Moreover, the decrease in activities of hepatic total peroxidases (main enzymes correlated to neutralization of hydrogen peroxide [28]) in GD-rats could be due to STZ-generated

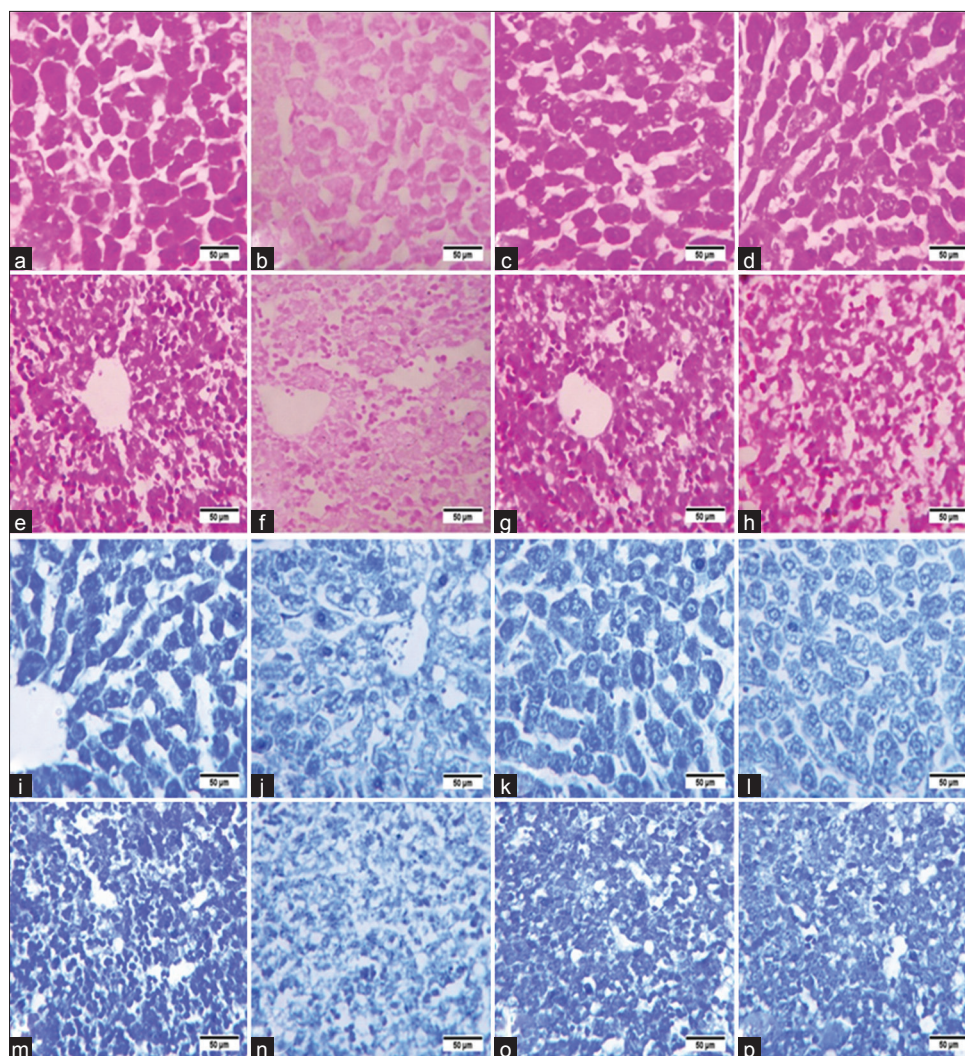


Fig. 5: Photomicrographs of periodic acid–Schiff (PAS) or bromophenol blue-stained maternal and fetal liver sections sampled from the experimental groups to demonstrate the cellular accumulation of glycogen or proteins, respectively. (a) Liver section of normal pregnant (NP)-rats reveals a strong PAS-positive reaction. (b) Depicts a marked depletion of glycogen content in the hepatocytes of gestational diabetic (GD)-rats. (c and d) Liver sections of GD-rats pre-treated with cinnamaldehyde (Ci) and ellagic acid (EA), respectively, show an obvious restoration of glycogen content as normal ones. (e) Fetal liver section from NP-group with an intense glycogen deposition. (f) Exposes little amount of glycogen stored in fetal liver of GD-group. (g and h) Respectively display a remarkable increase in the amount of glycogen deposited in the fetal livers of GD+Ci and GD+EA groups. (i) Maternal liver section of NP-group with an intensive amount of protein in the cytoplasm of hepatocytes. (j) Reveals a weak response toward bromophenol blue reaction in the liver of GD-rats. (k) Shows restoration of proteins in the hepatocytes of GD-rats pre-treated with Ci. (l) Liver section of GD+EA-rats illustrates moderate hepatic protein content. (m) Fetal liver from an NP-dam with considerable proteins deposition in its cells. (n) Shows marked diminution of the protein content of GD-group's fetal liver. (o and p) Fetal liver sections of GD+Ci and GD+EA groups, respectively, depict significant increase of protein elements

ROS [29]. Ci administration significantly reverted back these altered antioxidants more than EA as a result of its ability to provoke potent control of the glycaemic state.

Aberrations in the maternal serum lipid profile have been also reported in GD-group. GDM-dams showed significant increase in serum TG and total cholesterol concentrations with a diminished HDL-cholesterol level. Dietary manipulation of FSD increased the absorption of cholesterol and TG from the small intestine leading to hypercholesterolemia and hypertriglyceridemia. In addition, Raz *et al.* [30] have been linked the insulin insufficiency to the dysfunction of lipoprotein lipase (LPL) causing progressive impairment of TG catabolism and its uptake by the peripheral tissues. The antilipidemic action of Ci and EA reside in their ability to stimulate insulin secretion and action, stimulating LPL and increasing TG clearance. Interestingly, both treating agents increased serum HDL-cholesterol concentration

than normal dams. This potentially prevents the future development of CVDs [31,32]; commonly GDM complications.

A vital indicator in assessing hepatic injury is the serum level of liver transaminases. The observed increment of ALT and AST in the circulation of GD-dams may discuss the fatty changes in their livers. The excess free fatty acids as a result of FSD-feeding is known to be directly toxic to hepatocytes through mechanisms include cell membrane disruption, mitochondrial dysfunction, and toxin formation which decrease the liver cell membrane-stabilizing action, and consequently, the seep out of these enzymes from the liver cytosol to the circulation [33]. Another potential explanation for elevated serum transaminases is the hepatotoxic effect of STZ which generates ROS causing loss of the functional integrity of liver cell membranes and leakage of these enzymes into the bloodstream [34]. On the other hand, hepatocytes work exclusively to synthesize proteins and the

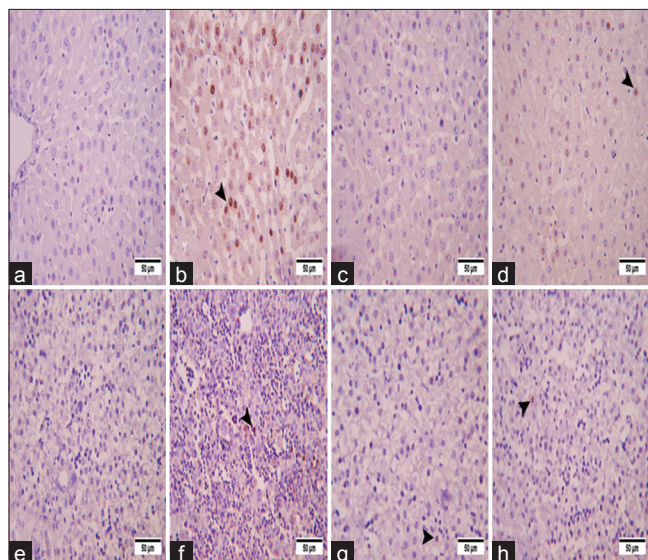


Fig. 6: Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in maternal and fetal liver sections sampled from the experimental groups. (a) Control of PCNA expression in the liver of normal pregnant (NP)-rats. (b) Reveals strong positive immunostaining of PCNA in the maternal liver of gestational diabetic (GD)-group (arrowhead). (c) Highlights negative localization of PCNA in hepatic cells of GD+cinnamaldehyde (Ci) rats. (d) Shows PCNA-weak immunoreactivity in parenchymal hepatocytes of GD+ellagic acid (EA) rats (arrowhead). (e) Fetal liver section from NP-group without any PCNA expression in its cells. (f) Demonstrates numerous PCNA-nuclei (arrowhead) in GD-group's fetal liver section. (g and h) Respectively display pale PCNA staining in the fetal livers of GD+Ci and GD+EA groups (arrowheads)

decline in their serum concentrations considered a sign of hepatic dysfunction. During normal pregnancy, the buildup of proteins by the fetus and mother would be expected for fetal growth and structural modifications of maternal tissues. Instead, proteins turnover from accretion to oxidation in GDM [35]. This may be due to the inhibition of insulin stimulation of protein synthesis at the translational level that involves the AKT, mammalian target of rapamycin, P70S6 kinase pathway [36]. Here, FSD/STZ administration induced liver injury which confirmed by declined serum albumin and total proteins levels, besides a marked depletion in the maternal and fetal proteins accumulation as seen by the histochemical screening. Interestingly, Ci administration significantly alleviated levels of circulating hepatic enzymes more than EA suggesting their potential membrane stabilizing action. The hepatoprotective effect of Ci and EA was further confirmed by the improved histological architecture of the liver and increased serum levels of albumin and total proteins.

The present study revealed prominent histopathological changes in the livers of GD-dams. The observed accretion of fats in hepatocytes of GD rats might be explained in this way. FSD-feeding increases the circulating fatty acids that will be taken up by the liver as an energy source secondary to insulin resistance. The accumulation of fatty acids disturbs the β -oxidation system in the hepatic mitochondria and leads to further infiltration of fats in the liver and the subsequent development of NAFLD as confirmed by the appearance of hepatocellular ballooning which considered a key feature of NAFLD [37]. As mentioned, NAFLD is an increasingly common cause of hepatic inflammation, necrosis, fibrosis, cirrhosis, and HCC. In the present study, the inflammatory reaction in the liver of GD-dams may be attributed to the imbalance of the systemic inflammatory milieu associated with insulin resistance in GDM [38]. As well, STZ may serve as haptens that when combining with normal cell membrane antigens it provides a focus for immunologically

directed hepatocellular injury. In this situation, a marked infiltration of lymphocytes occurs in both liver parenchyma and the portal triads [39]. On the other hand, the vasodilatation and the increase of vascular permeability (as a result of the inflammatory reaction) lead to loss of fluid from the blood, so the vessels appeared engorged with blood cells. Moreover, the reported degenerative structural may be due to LPO subsequent to free radical production as a result of insulin insufficiency, sustained hyperglycemia, and STZ-treatment [40]. The present cytoplasmic vacuolation of the hepatic cells may be attributed to a progressive ischemia, hypoxia, or hydropic production caused by excessive production of water in the metabolically distributed cells [41]. Treatment with Ci and EA preserved the normal hepatic structure and greatly attenuated the cellular changes observed in the diabetic liver.

Liver glycogen level is a sign of insulin action and considered a good marker for assessing the hypoglycemic activity of any drug. Maternal liver sections stained with PAS-reaction proved a critical depletion of glycogen content of GD-rats secondary to the impairment of insulin sensitivity. Both of Ci and EA restored the hepatic glycogen level near the normal level as a result of increased insulin secretion and action which stimulates the glycogen synthase system [42].

Monitoring of cell proliferation rate is of an interest since abnormal cell proliferation is a precursor of tumorigenesis. PCNA is a non-histone nuclear protein whose level of synthesis correlates directly with the rate of cellular proliferation and DNA synthesis [43]. Therefore, the accumulation of PCNA gene products in cycling hepatocytes of maternal and fetal GD-group indexing the higher degree of cellular proliferation and the expected development of HCC. In contrast, weak maternal and fetal expression of PCNA in liver cells of Ci- and EA-treated groups was a good marker of hepatocellular stability and preservation of the normal hepatic architecture.

Regarding fetal glycemia, Day *et al.* [44] reported that the placental transfer of glucose occurs by facilitated diffusion with the concentration gradient. As a result, maternal hyperglycemia of GD-rats caused marked elevation of serum glucose levels in their fetuses. As the development of the fetal pancreas is enhanced by the increase in blood glucose concentration, the percentage of its endocrine tissue increased because of hyperplasia and hypertrophy of the islets of Langerhans that causing subsequent hyperinsulinemia [45]. It is relevant here to mention that, STZ injected into the dams does not affect the fetal pancreas. Although STZ crosses the placenta, its maternal half-life is of the order of minutes and the amount reaching the fetal circulation does not damage fetal beta cells [46]. Higher fetal glucose levels in GD-group may indicate the decrease in responsiveness of the liver toward the insulin which considered a direct reflection of fetal insulin resistance. Furthermore, the fetal hyperglycemia could contribute directly to enhanced free radical synthesis and LPO and unmask the imbalance between oxidative stress and antioxidant status [45]. Our results indicated a significant increase in the MDA concentration with a marked depletion in total thiols and total peroxidases levels of GD-fetal livers. These also discuss the observed fetal liver histological changes of GD-group [47]. Maternal administration of Ci and EA cause fetal hypoglycemia, increase the antioxidant potentials, prevent progression of liver damage, attenuate hepatic cells proliferation, and normalize hepatic glycogen and protein contents.

CONCLUSION

Ci was founded to have a potent hepatoprotective effect than EA by ameliorating maternal and fetal glucose and insulin levels, alleviating serum lipids and proteins levels, decreasing serum transaminases concentration, activating the antioxidant defense system, restoring the normal islets and hepatic configuration, attenuating hepatic cells proliferation, and normalizing storable levels of liver glycogen and proteins.

ACKNOWLEDGMENT

The authors are grateful to BSU-research center, Beni-Suef University, Egypt, for providing the research grant.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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