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Genistein Produces Hepatoprotection through Modulating EGFR Expression and Phosphorylation in Experimental Fibrosis

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Abstract

Background and Objective: Liver disease chronicity leads to the appearance of fibrosis, cirrhosis, and eventually cancer. For this reason, it is important to research new fibrosis therapies. The use of genistein as a hepatoprotective agent has been studied, but its mechanism of action is unknown. The aim of this work was to evaluate the role of genistein as a fibrosis treatment and its possible mechanism of action through CCl4-induced inhibition of EGFR in rat specimens.

Methods: Hepatic fibrosis was brought about by chronic administration of CCl4 to rats. Animals with fibrosis were treated with 1 mg/kg genistein. To evaluate the hepatoprotection of genistein on liver fibrosis, we made a histopathological analysis using both H&E and trichrome staining, as well as an immunofluorescence analysis for α-SMA and an immunohistochemical analysis for PCNA. In order to find out the effects of genistein on EGFR expression and phosphorylation, we performed immunostaining for EGFR and dot blot analysis for two specific tyrosine residues: pY992 and pY1068. We also evaluated liver functionality.

Results: Genistein reduced liver fibrosis and improved liver architecture. α-SMA positive cells were lower in genistein-treated animals with fibrosis. Likewise we found a reduction in EGFR expression and phosphorylation of genistein-treated animals with fibrosis; PCNA positive cells were reduced in this group. We observed liver functionality improvement in those animals with fibrosis that were treated with genistein.

Conclusion: Genistein produces hepatoprotection through modulating the expression and phosphorylation of EGFR in experimental fibrosis.

Keywords: α-SMA; EGFR; Fibrosis; Hepatoprotection; PCNA

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide [1]. Epidemiological studies show that HCC is strongly associated with chronic liver diseases, including viral hepatitis B (HBV) and C (HCV) infections, and liver cirrhosis [2].

After tissue injury, the liver triggers a powerful defensive response mediated by a network of cytokines, mitogens, growth factors and their receptors in a coordinate multistep process [3]. However, when injury continues the response is associated with fibrosis development, as well as active proliferation and development of pre-neoplastic lesions. In that sense, the EGFR signaling system appears to be critically involved in both the hepatoprotective and hepatic regenerative responses. EGFR exert its function in the cellular environment mainly, if not exclusively, via its tyrosine kinase activities [4]. As a consequence, several intracellular signaling pathways are thus activated, which regulate a variety of transcription factors that initiate translation and regulate metabolic pathways [5]. An EGFR overexpression and its activation have been observed in all stages of liver disease, such as regeneration, fibrosis, cirrhosis and HCC. However, in some of those processes the role of EGFR is not clear [6,7]. Therefore, EGFR intervention through inhibitors might prove effective in the prevention and treatment of chronic liver disease.

Genistein (4,5,7-trihydroxyisoflavone) is an isoflavone primarily found in soy protein, and it possesses potent estrogenic activity [8]. Several studies have previously shown that genistein is a powerful inhibitor of protein-tyrosine kinases (PTKs) [9]. Previous in vitro studies have shown that genistein inhibits cell growth, increases apoptosis and DNA fragmentation, activates caspase-3, and arrests cells in the hepatocellular carcinoma G2/M phase (HepG2, Hep3B, Huh7, PLC and HA22T) [10,11]. It has also been demonstrated that genistein suppresses the activation of hepatic stellate cells and profibrogenic factors [12]. Several research groups have described the hepatoprotective role and the antioxidant profile of genistein in liver damage induced by chemicals, alcohol or diet [13,14]. Previous results by this groups have shown that genistein can modify liver fibrosis and improve liver function in rats in a model fibrosis induced by carbon tetrachloride and prolonged biliary obstruction in rats [15,16]. However, at present there are no reports regarding the effect of genistein on EGFR expression and phosphorylation in specific tyrosine residues and its role in the proliferative status in liver fibrosis. Therefore, the aim of the present study was to evaluate the effect of genistein on EGFR expression and phosphorylation of specific tyrosine

residues and its correlation with the hepatoprotective and antifibrotic effects on experimental liver fibrosis.

Material and Methods

Animals

Male Wistar rats were obtained from Harlan Laboratories Inc., Mexico. The animals were housed in a temperature and humidity controlled environment and were allowed food (Standard Purina Chow Diet, Mexico) and water ad libitum. The rats' diet consisted of approximately 200 g of PMI® Certified Rabbit Diet per day. High quality alfalfa hay cubes and tested tap water were offered ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the Veterinary Medical School at the National Autonomous University of Mexico. The experiments were conducted in accordance with the principles set forth in the Care and Use of Laboratory Animals Guide [17].

Induction of liver fibrosis

The rat model was established using the method described by Rojkind et al., [18]. In order to induce liver toxicity, 1.5 mL/Kg i.p. of CCl4 diluted in mineral oil (1:7, 1:6, 1:5 and 1:4 dilutions) was employed. Liver injury and fibrosis were produced by intraperitoneal administration of 0.15 mL solution of CCl4, 3 times a week for 8 weeks. Fibrosis grading and staging, as well the grading and staging of necrosis were performed based on METAVIR an Ishak Score criteria, as specified in paragraphs below.

Pharmacological treatment

Twenty-four Wistar rats (200–250 g) were randomly divided into four groups (six rats/group) as follows: Group 1, which served as normal control, received 1 mL of mineral oil/Kg body weight; Group 2, received a daily oral dose of 1 mg/Kg genistein (Enzo Life Science, Inc.) for four weeks, using an intragastric tubing; Group 3, Fibrosis received CCl4: mineral oil i.p. three times per week; Group 4, animals received Fibrosis + genistein, fibrosis was induced by administration of CCl4 as indicated previously and genistein was given at the same time as CCl4 during 4 weeks). Genistein (Enzo Life Science, Inc.) was dissolved in water and administered at a dose of 1 mg/Kg per rat through an intragastric tube. Administration of genistein began four weeks after induction of liver fibrosis and was continued for a further four weeks. After the treatments, animals were deprived of food, but not of water, for 12 h, and were sacrificed under light ether anesthesia. The rats' blood samples were centrifuged to separate serum, which was kept at −70°C until analysis. Liver tissues were collected from each animal and kept at -4°C for further studies.

Histopathological analysis

Slide evaluation was independently performed by two pathologists (INP, S.S.) with one investigator (FF, UAEM) blinded with regard to the length of storage. Overall interobserver difference was 5%. In case of differing results, consensus was reached by joint evaluation. Tissue sections were reviewed independently for the description of liver injuries for each treatment group. Fibrosis grading and staging was performed based on METAVIR criteria, where: F0 corresponded to no fibrosis; F1 corresponded to fibrous portal expansion; F2 corresponded to few bridges or septa; F3 corresponded to numerous bridges or septa; and F4 corresponded to numerous bridges with regeneration nodules (cirrhosis). The grading and staging of necrosis was performed in accordance with Ishak Score criteria, where: 0 corresponded to zero necrotic cells; 1 corresponded to focal confluent necrosis; 2 corresponded to Zone 3 necrosis in some areas; 3 corresponded to Zone 3 necrosis in most areas; 4 corresponded to Zone 3 necrosis + occasional portal central bridging; 5 corresponded to Zone 3 necrosis + multiple portal central bridging; and 6 corresponded to paracinar or multiacinar necrosis. In the case of inflammation the score was as follows: 0 corresponded to absence of portal inflammation; 1 corresponded to mild, some, or all-portal inflammation areas; 2 corresponded to moderate, some, or all-portal inflammation; 3 corresponded to moderate/marked all portal inflammation; and 4 corresponded to marked all portal inflammation [19].

Immunohistochemistry for activated HSC

The identification of activated HSC in liver tissue was made by immunohistochemistry using an α-SMA antibody. Analysis was developed in 2 μm-tick sections; tissue sections were deparaffinized and rehydrated, antigen retrieval was performed with target retrieval acetate solution (Dako Corporation, CA, and USA), endogenous peroxidase was inactivated with 0.9% H_2O_2, and washes were performed with distilled water. Then slides were allowed to stand for 5 min in phosphate-buffered saline (PBS). Tissue sections were incubated for 45 min with monoclonal anti α-SMA (Santa Cruz Biotechnology, CA, USA), diluted 1:100 in 1% bovine serum albumin in phosphate-buffered saline for 60 min at 37°C for 2h at RT. Bound antibodies were detected using anti-rat biotinylated (Jackson Laboratories Inc. Immuno Research, PA, USA) conjugated with 1:200-diluted streptavidin-peroxidase complex for 20 min, and the reaction was visualized using 3,3'-diaminobenzidine (Dako Cytomation Carpinteria, CA, USA) as substrate under a microscope. The tissues were counterstained with Lillie-Mayer's hematoxylin (Biocare Medical, CA, USA). Samples preincubated without immune mouse serum were used as negative controls.

Immunofluorescence for activated HSC

Unstained 2 μm-tick section were deparaffinized and rehydrated, antigen retrieval was performed with Target Retrieval citrate solution (Dako Corporation CA, USA), and endogenous peroxidase was inactivated with 0.9% H_2O_2. The slides were firstly wash with distilled water then with phosphate-buffered saline (PBS) 5 min. The tissues were incubated for 45 min with monoclonal anti α-SMA 1:100 dilutions (Santa Cruz Biotechnology CA.). Following incubation with the primary antibody, sections were incubated with FITC conjugated rabbit anti-mouse 1:200 dilution (Jackson Laboratories Inc. Immuno Research USA). Images were taken (40X) using an Olympus IX81 inverted fluorescence microscope (Olympus America Inc, Pennsylvania, US) using a ImagePro (Medi cybernetics, Inc.) program.

Dot blot assay for pY992 and pY106-EGFR

For the analysis of dot blot we follow the next procedure: 50 μg of proteins of liver lysates were spotted onto PVDF membrane and then allowed to dry. All samples of the groups were deposited in order. Non-specific sites were blocked with 5% BSA in TBS-T during1 h at RT. The membrane was incubate with primary antibody anti p Y992-EGFR or p- Y1068-EGFR 1:500 dilution (Cell Signaling, Technology Inc.) overnight at 4°C. The membrane was wash three times with TBS-T during 5 min. Secondary antibody anti-rabbit HRP (Cell signaling, Technology Inc.) 1:2000 dilution 2 h RT was used. Then the membrane

was wash three times with TBS-T during 5 min. Finally the membrane was exposing to reagent SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific Inc.) and visualized in a Bio-Rad Chemi Doc Imaging System by Quantity One program.

Immunohistochemical detection of EGFR and phosphorylation of tyrosine

The immunohistochemical stain of tissue samples was performed by using EGFR, pY992-EGFR and pY1068-EGFR antibodies. Analysis was performed in 2 µm-tick sections that were mounted on silanized/charged slides and dried for 1 h at RT, followed by 1h at 60°C. After deparaffinization and rehydration, antigen retrieval was performed with Target Retrieval acetate solution (Dako Corporation CA, USA), endogenous peroxidase was inactivated with 0.9% H_2O_2, and washes were performed with distilled water. Then slides were allowed to stand for 5 min in phosphate-buffered saline (PBS). The tissues were incubated for 45 min in a primary mouse anti-EGFR monoclonal 1:100 dilution (Santa Cruz Biotechnology, CA, USA). The secondary antibody used was 1:200-diluted rabbit biotinylated anti-mouse (Jackson Laboratories Inc. Immuno Research, PA, USA) for 30 min. In the case of EGFR-specific tyrosine residues phosphorylation, we used polyclonal anti-pY992-EGFR or pY1068-EGFR 1:50 dilution (Cell Signaling Technology Inc.) for 60 min. Tissue staining was visualized with a DAB substrate chromogen solution. Slides were counterstained with hematoxylin, dehydrated, and mounted. Skin tissue served as positive control to validate each staining as positive or negative.

Detection of proliferating cell nuclear antigen (PCNA)

Sections were cut at 4 pm, mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich Corporation; MO, USA), air-dried at room temperature, and heated at 60°C on a hot plate for a few seconds until the paraffin melted. After deparaffinization and rinses in 100% and 95% ethanol, the slides were incubated in 2% hydrogen peroxide diluted in methanol for 7 min, rehydrated in 95% ethanol, and rinsed again in PBS. When incubation was performed, the sections in 2 N HCl were incubated at room temperature for 30 min and subsequently washed three times in PBS at this point. This was followed by pre-incubation with 5% blocking serum diluted in PBS for 30 min and incubation with primary antibody diluted in PBS containing 1% bovine serum albumin at 4°C overnight. Samples were incubated for 45 min with monoclonal anti-PCNA 1:200 dilution (Santa Cruz Biotechnology, CA, USA) for 30 min at RT. The sections were then incubated with a biotinylated rabbit anti-mouse, diluted 1:200 in PBS with 1% bovine serum albumin at 42°C for 20 minutes. This was followed by incubation with avidin DH-biotinylated horseradish peroxidase H complex (Vectastain Elite ABC Kit; Vector Laboratories, CA, USA) for 20 min at RT. All steps were followed by appropriate washes in PBS. Finally, the sections were developed in a 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide substrate solution in 0.05M Tris-HC1 (pH 7.6) for 3 min, and then washed, lightly counterstained with hematoxylin, dehydrated in ethanol, and mounted. Control sections of representative tissues were prepared replacing the primary antibody with dilutions of normal mouse serum or omission of the primary antibody. As positive control tissue, we used a section of regenerating rat liver.

Liver function an biochemical parameters analysis

The enzymatic activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP) was measured using commercial kits and following the supplier's instructions (Bio Vision Inc., CA, USA); absorbance was then obtained with a Victor 3 Perkin Elmer (MTX Lab Systems, Inc., VA, USA) plate reader. Triglycerides, cholesterol and glucose levels were quantified by a kit based on enzymatic-colorimetric methods (Trinder Method) (ELI Tech, France) in serum from blood samples collected by centrifugation. Absorbance was then obtained with a Victor 3 Perkin Elmer (MTX Lab Systems, Inc., VA, and USA) plate reader.

Statistical analysis

The data were presented as the mean + standard deviation (SD). The statistical analysis was performed using SPSS 17 Real Stat software (Armonk, NY, USA). The statistical differences between groups were determined by ANOVA, followed by Tukey's test. A $p < 0.05$ values were considered as statistically significant.

Results

Histopathological analysis

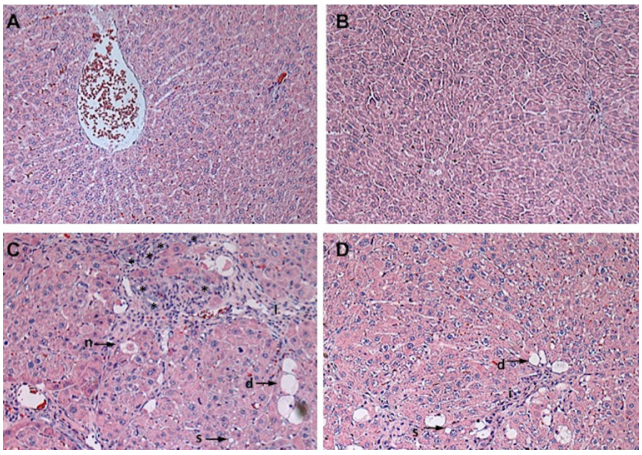

Figure 1: Histopathological analysis of liver sections from rats treated with genistein. Liver tissue sections from: A) Control rat; B) Animal treated with genistein; C) Animal with fibrosis, alterations in parenchymal, distortion of hepatic architecture with necrosis cells (n) inflammatory infiltrate (i), microvesicular steatosis (s) and biliar ducts proliferation (*) are observed; D) Animal with fibrosis treated with genistein, is observed a improvement in liver structure with less number of necrosis cells, inflammatory infiltrate and bile ducts. Liver sections were stained with H&E. A representative microphotograph of each group was choose, magnification 20X.

Liver samples were stained with H&E and Masson's trichrome to evaluate histopathological changes and fibrosis. The liver sections of the control and genistein groups showed a normal parenchymal morphology (Figures 1A and 1B). Hepatic tissue sections of rats with fibrosis showed many alterations and injuries in the parenchyma, including anisocytosis and anisonucleosis of hepatocytes, hydropic degeneration and steatosis, ballooning degeneration, dissociation of hepatic cords, and unicellular necrosis. There was a presence of inflammatory infiltrates consisting of lymphocytes, plasma cells, macrophages, and neutrophils in a lesser degree (Figure 1C). The presence of eosinophils and mast cells was noted in some portal tracts

and perivascular edema in some of the evaluated areas. In the same way, another set of higher-grade lesions and liver parenchyma conditions were observed, such as the proliferation of bile ducts, where up to 20 ducts in portal space were appreciated, as well as the proliferation of fibrous tissue with bridging between portal tracts and the presence of regenerative nodules (with hepatocytes in different degrees of degeneration, binucleation and 3-5 mitoses per field). Such alterations were severe, with multifocal or widespread distributions in the fibrosis group (Figure 1C). On the other hand, animals with fibrosis and treated with genistein showed a decrease of inflammation, necrosis, steatosis and fibrosis as compared with the fibrosis group, and the liver tissue morphology and structure improvements were evident (Figure 1D). Genistein also diminished the number of bile ducts in the portal space in animals with fibrosis. Necrosis was determined using Ishak scores (Table 1). The scores for animals with fibrosis were 3-4 in necrosis, and scores for portal inflammation areas were 1-2. However, animals with fibrosis treated with genistein diminished their necrosis score (2-3), and there was a presence of moderate inflammation in some portal areas scored 0-1.

Necrosis Score					
Groups	**Score (# of animals)**				
	F0	**F1**	**F2**	**F3**	**F4**
Control	6	0	0	0	0
Genistein	6	0	0	0	0
Fibrosis	0	0	0	2	4
Fibrosis+Genistein	0	0	4	2	0
Portal Inflammation score					
Control	6	0	0	0	0
Genistein	6	0	0	0	0
Fibrosis	0	3	3	0	0
Fibrosis+Genistein	0	6	0	0	0
Grading and stages of fibrosis					
Control	6	0	0	0	0
Genistein	6	0	0	0	0
Fibrosis	0	0	0	1	5
Fibrosis+Genistein	0	0	0	6	0

Table 1: Histopathological analysis of liver.

Values represent the number of animals showed necrosis corresponding to each score based on criteria of Ishak in experimental group. Values represent the number of animals showed fibrosis corresponding to each stage and grading based on criteria of Metavir in experimental group.

The Masson's trichrome staining of the liver was performed to assess collagen distribution (Figure 2). Animals treated with genistein showed the absence of fibrosis in parenchymal liver, and collagen was observed only surrounding vessels. However, animals with liver fibrosis exhibited extensive collagen depositions, which were surrounded by regenerative nodules and collagen bridges between portal spaces. Fibrosis was evaluated with Metavir criteria (Table 1). Animals with fibrosis had grading scores of F3 (1 animal) and F4 (4 animals). However, animals with fibrosis treated with genistein showed a significant reduction in the amount of collagen in the liver parenchyma, and the collagen bridges were thinner; a minor number of regenerative nodules was also observed. Treatment with genistein decreased the degree of fibrosis; according to Metavir criteria, the grading score was F3 for this group.

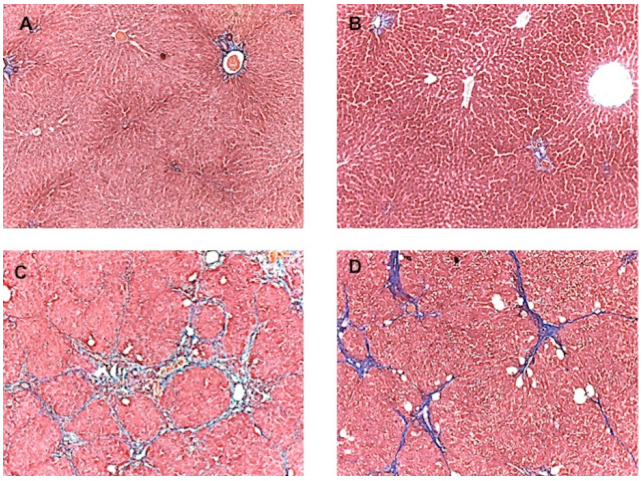

Figure 2: Effect of genistein on liver fibrosis. Liver tissue sections from: A) Control animal, is observed normal lobular architecture and a normal distribution of collagen (blue); B) Animal treated with genistein, normal distribution and deposition of collagen; C) Animal with fibrosis, is observed extensive collagen deposition in parenchyma and formation of bridges of collagen between portal space, is evident the presence of regenerative nodules; D) Animal with fibrosis and treated with genistein, is observed a less deposition of collagen, there is not the presence of regenerative nodules. Liver tissue was stained with Masson trichrome, collagen can be recognized by blue staining. A representative microphotograph of each group was choose, magnification 20X.

α-SMA expression as a marker of activated HSC

An immunofluorescent analysis of α-SMA was performed in order to identify activated hepatic stellate cells (HSC) (Figure 3). The α-SMA expression in liver tissue from animals treated with genistein was found in isolation within the parenchyma (Figures 3A and 3B). In animals with fibrosis, the immunoreactive cells were found mainly on collagen bridges. The presence of α-SMA positive cells in fibrosis areas indicated the presence of activated HSC (Figure 3C). In contrast, it was observed a minor number of positive cells on collagen bridges in animals with fibrosis treated with genistein, indicating the presence of a smaller number of activated HSC.

EGFR and PCNA expression and localization in the liver

In order to know whether both hepatoprotective and antifibrotic effects of genistein were also associated with signs of hepatic regeneration, we performed the analysis of EGFR and PCNA expressions in liver sections of animals. EGFR expression was observed in bile duct cells, and occasional and faint immunoreactive cells on

parenchyma in both control and genistein groups (Figures 4A and 4B). It is noteworthy that a positive reaction to EGFR was observed in the membrane and cytoplasm of immunopositive cells.

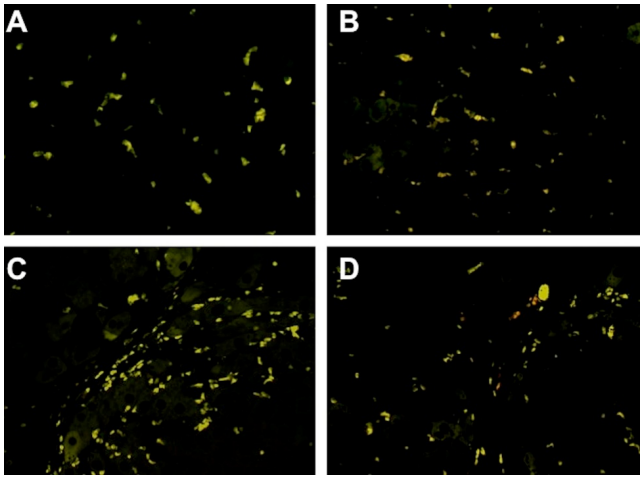

Figure 3: Effect of genistein on α-SMA expression and localization during experimental fibrosis. The localization of α-SMA was determining by immunofluorescence, positive cells fluorescence in green. A) In liver from control rat and B) animal treated with genistein; C) Localization of α-SMA in liver sample from animal with fibrosis, immunoreactive cells were observed in the bridges of collagen, indicating the presence of HSC activated; D) animal with fibrosis and treated with genistein, is observed a reduction of positive cells in the bridge of collagen. A representative microphotograph of each group was choose, magnification 40X.

On the other hand, the EGFR expression was more intense in hepatocytes located in regenerative nodules of animals with fibrosis. However, a high expression was evident in the periphery of these regenerative nodules, particularly in those hepatocytes joined to collagen bridges. Immunopositive cells were also observed on collagen bridges, suggesting that HSC activated also overexpressing EGFR (Figure 4C). Nevertheless, a lower number of immunopositive cells and less intensity of EGFR expression in the parenchyma and collagen bridges was found in the liver tissue from animals with fibrosis treated with genistein as compared with the fibrosis group (Figure 4D). An immunohistochemical analysis was also performed to determine whether genistein treatment induced changes in EGFR tyrosine phosphorylation at pY992 and pY1068 residues. Figure 5 shows the expression of tyrosine residues at pY992 and pY1068 from liver slices. As can be seen, genistein itself did not induce EGFR tyrosine phosphorylation. However, an important increase in EGFR tyrosine phosphorylation was observed in both tyrosine residues in animals with fibrosis (14-fold for pY1068-EGFR and 30-fold for pY992-EGFR) ($p<0.05$). Animals with fibrosis treated with genistein showed smaller amounts of positive cells for both tyrosine residues, but it was not statistically significant.

After analyzing EGFR expression and phosphorylation in the liver tissue, we evaluated PCNA expression in order to know the status of proliferative activity in the liver tissue (Figure 6). In the control and genistein groups, 4-5 PCNA-positive cells per field were observed (Figures 6A and 6B). In contrast, an increase in the number of PCNA-positive cells was observed in animals with fibrosis. Immunopositive cells were observed inside regenerative nodules (≈ 120 cells per field), as well as some cells on collagen bridges (≈ 30 cells per field). However, animals with fibrosis treated with genistein showed a smaller number of immunopositive cells and were only found in the parenchyma; non-positive cells were observed on collagen bridges.

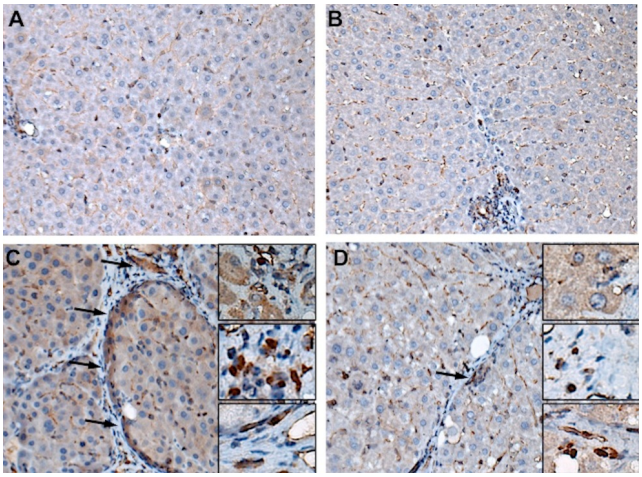

Figure 4: EGFR expression and localization in liver sections with fibrosis. The expression was determining by inmunohistochemistry, positive reaction is observed in brown (arrows). A and B) Livers from control and genistein groups respectively, stain revealed positive cell in bile ducts; C) EGFR localization in liver sample with fibrosis, many immunopositive cells were observed inside regenerative nodules and collagen fibers; D) Liver sample from animal with fibrosis and treated with genistein, there were occasional and faint immunoreactive hepatocytes in parenchymal and collagen fibers. A representative microphotograph of each group was choose, magnification 40X.

Liver function analysis

As is shown in Table 2, oral administration of genistein for 20 days in rats did not modify the liver function markers. The enzymatic activity of the liver was significantly higher in animals with fibrosis as compared with the control ($p < 0.05$). Glucose levels decreased, but triglycerides and cholesterol levels increased during fibrosis, whereas animals with fibrosis treated with genistein showed an important reduction in enzymatic levels as compared with fibrosis group ($p < 0.05$). It is important to mention that levels of glucose, triglycerides and cholesterol tended to normality, but differences were not statistically significant.

Discussion

The induction of hepatic fibrosis by administration of CCl4 is one of the most widely used models in the field of hepatology. The use and administration of this agent allows for a reproduction of the natural history of liver the disease in all stages, from hepatocellular carcinoma development to acute hepatitis. Because of this, it has been considered an important model for the study of new therapeutic agents and/or hepatoprotective agents. In this study we used a fibrosis model induced by chronic administration of CCl4 in rats, and subsequently treated the

subjects with genistein in order to study its effects on EGFR expression and phosphorylation, and its role on fibrosis development.

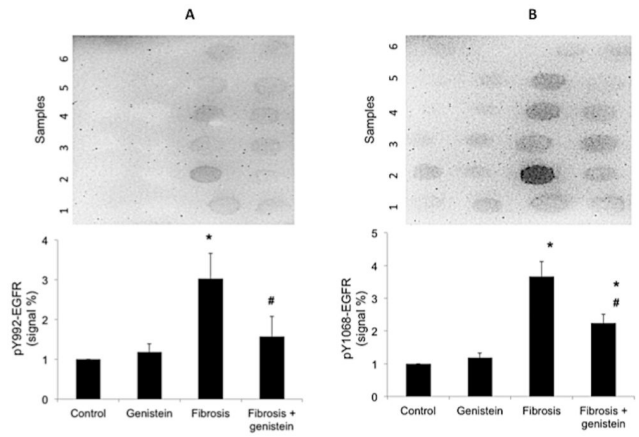

Figure 5: Dot blot analysis of phosphorylation of specific EGFR tyrosine residues in liver slices. (A) pY992-EGFR and (B) pY1068. EGFR, each point of dot blot represents one animal, all groups were included of the different group animals with fibrosis showed an important increase of immunopositive cells for both EGFR tyrosine residues. Animals with fibrosis and treated with genistein showed a minor number of immunoreactive cells in both phosphosites.

Hepatic fibrosis is the result of the wound-healing response of liver cells to chronic or iterative injury. After an acute liver injury (e.g., viral hepatitis), parenchymal cells regenerate and replace the necrotic or apoptotic cells. This process is associated with an inflammatory response and a limited deposition of ECM [19,20]. When disease is advancing, there is a progression from collagen bands to bridging fibrosis and finally to cirrhosis. The model of fibrosis used in the present study was characterized by the presence of several types of degeneration, steatosis, and inflammatory cells; as well as the presence of regenerative nodules and the presence of collagen fibers. The presence of cells expressing α-SMA in collagen bridges has been reported during fibrosis induced by CCl4 [21]. That occurrence is attributed mainly to the activation of HSC [20]. HSC activation entails a phenotype change; the cell acquires contractile capacity and produces α-SMA protein and large amounts of the main ECM components [22]. In the present results we found several of the changes mentioned above and an intense expression of α-SMA protein present on collagen bridges in animals with fibrosis. In contrast, animals with fibrosis and treated with genistein showed a significant decrease of α-SMA cells; indicating that in animals with fibrosis, genistein is able to reduce the amount of cells producing ECM, and for that reason there was a lesser amount of bridges of collagen and an improvement on liver architecture.

Genistein is an agent with protective properties and it has been studied in different models of liver injury [22]. Its effects have been attributed to its antioxidant and anti-inflammatory property [23]. However, it has been also reported that genistein is able to inhibit the proliferation of hepatic stellate cells in vitro conditions [13]. This group has previously reported that genistein modifies liver fibrosis and improve liver function by inducing uPA expression and proteolytic activity in experimental fibrosis [16]. Therefore, those results suggest that genistein might have a direct effect on HSC and hepatocytes and as consequence explain its hepatoprotective effects and its influences on hepatic regeneration during liver fibrosis.

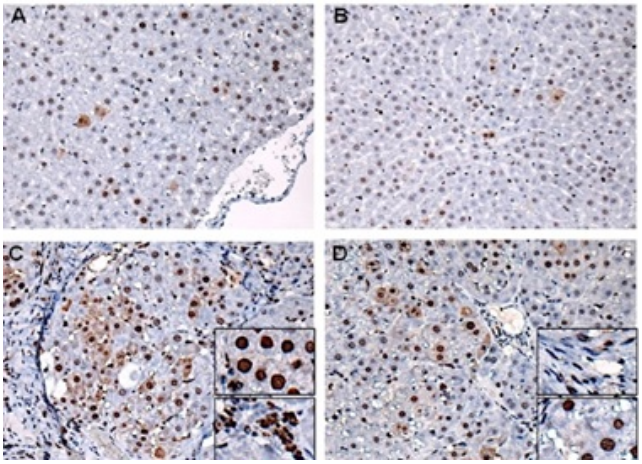

Figure 6: Effect of genistein on PCNA expression and localization in liver. The positive cells to PCNA were detected by inmunohistochemistry, positive reaction was observed in brown (nuclei). A and B) Liver from control and genistein groups respectively, there were observed scattered positive cell in parenchymal; C) PCNA localization in liver sample with fibrosis, is observed in the bridges of collagen, which might correspond to HSC activated, and inside regenerative nodules; D) Liver sample of animal with fibrosis and treated with genistein, where observed the positive cells in parenchymal and bridge of collagen, however, there were occasional and faint immunoreactive cells. A representative microphotograph of each group was choose, magnification 40X.

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase that is activated by several ligands leading to the activation of diverse signaling pathways controlling mainly proliferation, differentiation, and survival in several cells [24]. Upon ligand binding, EGFR can bind with other EGFR family members and with other receptor tyrosine kinases (RTK) and activates downstream signaling cascades [25]. Accumulated evidence from the last years indicates an indispensable role for EGFR in liver repair and regeneration [6]. The EGFR signaling plays an important role during liver regeneration and also during the processes of acute, chronic liver damage and the development of cirrhosis and hepatocellular carcinoma [7]. It has also reported that EGFR ligands like TGF-α, AR, HB-EGF and EREG induce strong mitogenic signals in cultured hepatocytes [26]. It was also previously shown that HB-EGF promotes HSC proliferation via activation of the EGFR and that HB-EGF is a potential therapeutic target in liver fibrosis [27].

The role of EGFR during chronic liver disease has been studied by investigating the effect of the EGFR inhibitor erlotinib on toxic fibrosis [28]. It has been demonstrated that erlotinib decreases hepatocyte proliferation and liver injury, and prevents the progression of cirrhosis and regressed fibrosis in some animals. It has been also demonstrated that EGFR is expressed on HSCs and in those cells erlotinib prevents disease progression by reducing EGFR phosphorylation in HSCs and by reducing the number of activated HSCs [29]. Having in mind those results, in the present study we evaluated the effect of genistein on liver fibrosis because has been shown that genistein has a potent inhibitory effect on protein tyrosine kinases (PTKs) [30]. Genistein modifies the

expression of some central genes for cell survival, cell cycle, and apoptosis through tyrosine kinases pathways. Specific effects of genistein on EGFR have not been previously reported. However, it has been reported that a synthetic genistein derivative inhibited EGFR phosphorylation in colon cancer cells [31].

In present work we evaluated the EGFR expression and phosphorylation of specific tyrosine residues (pY-992-EGFR and pY1068-EGFR) in animals with fibrosis. We found an increased expression and phosphorylation of EGFR inside of regenerative nodules in animals with fibrosis, which is consistent with previous reports about the role of EGFR in liver fibrosis development [32]. In addition to this, positive reaction was also found in some cells in collagen bridges, which might correspond to HSC activated. However, animals with fibrosis treated with genistein showed a decreased expression and phosphorylation of EGFR in parenchymal cells and in collagen bridges. The decreased in the number of positive cells to EGFR induced by genistein is attributable to the ability to inhibit this receptor with tyrosine kinase activity, as it has been previously reported [33].

The reduction in both number of immunopositive cells to tyrosine residues at pY992 and pY1062, and its effects on EGFR expression was correlated with a reduction in cell proliferation; tyrosine residues are associated with cell signaling pathways connected with proliferation, and a reduction in phosphorylation of tyrosine residues is indicative of an inhibition of cell proliferation. In present study, we determined by immunohistochemistry the expression of the proliferation cellular nuclear antigen (PCNA), which is synthesized in the early stages of cell proliferation G1 and S. This protein is responsible for recruiting proteins implicated in regulation of cell cycle; DNA synthesis and DNA repair [34].

The evaluation of PCNA in the group with fibrosis showed an increase in the number of immunopositive hepatocytes and cells within the collagen bridges. However, animals with fibrosis treated with genistein showed a decrease in the number of positive cells, indicating a reduction on proliferative activity. The correlation between EGFR and PCNA found in this work is important because it has been reported that EGFR phosphorylates PCNA, and gives stability to molecule for DNA replication and damage repair [35]. Besides of that, the presence of a minor number of positive cells to PCNA in liver parenchymal and in collagen bridges correlated directly with the findings for EGFR and their tyrosine residues, which suggest that genistein inhibits cell proliferation of hepatocytes and HSC by inhibiting the EGFR expression and phosphorylation.

In present results, we found that genistein inhibits the expression and phosphorylation of specific tyrosine sites that lead to cell proliferation. By histopathological and immunohistochemical analysis we demonstrated an improvement in liver morphology, a reduction of regenerative nodules, thinner collagen bridges, reduction of α-SMA, and reduction of cell proliferation, as well as an improvement on liver functionality. According with present results, genistein appear to exert its hepatoprotective effects and hepatic regenerative effects by inhibit the expression and phosphorylation of EGFR in liver fibrosis.

	Control	Genistein	Fibrosis	Fibrosis+ Genistein
ALT/SGPT (U/L)	116 ± 9.8	101 ± 19.4	342 ± 54.1*	207 ± 43.5*#
AST/SGOT (U/L)	228 ± 14.0	206 ± 17.0	510.1 ± 27.2*	408 ± 32.1*#
AP (U/L)	473 ± 144.1	522 ± 176.6	1276 ± 221.2*	890 ± 139.4*#
Glucose (mg/dL)	112 ± 16	124 ± 12	77.6 ± 9.11*	103 ± 18.5*
Triglycerides (mg/dL)	101 ± 22	69.8 ±15.5	189.3 ± 67*	133 ± 42.4*
Cholesterol (mg/dL)	70 ± 8.2	77.2 ± 19.3	146.9 ± 25.4*	108 ± 16*#
*p > 0.05 significant with respect control group				
#p > 0.05 significant with respect fibrosis group				
Values represent the mean ± SD (n=6)				

Table 2: Effect of Genistein on Liver Function.

This study demonstrated that genistein modifies liver fibrosis and improves liver function in experimental fibrosis. The mechanism of action of this drug is probably associated with an inhibition of the EGFR expression and phosphorylation in liver tissue. Genistein is an innovative and effective therapeutic agent in liver fibrosis.

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References

1. Attwa MH, El-Etreby SA (2015) Guide for diagnosis and treatment of hepatocellular carcinoma. World J Hepatol 7: 1632-1651.
2. Blachier M, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F (2013) The burden of liver disease in Europe: a review of available epidemiological data. J Hepatol 58: 593-608.
3. Lee YA, Wallace MC, Friedman SL (2015) Pathobiology of liver fibrosis: a translational success story. Gut 64: 830-841.
4. Friedman SL (2008) Mechanisms of hepatic fibrogenesis. Gastroenterology 134: 1655-1669.

5. Seshacharyulu P, Ponnusamy MP, Haridas D, Jain M, Ganti AK, et al. (2012) Targeting the EGFR signaling pathway in cancer therapy. Expert Opin Ther Targets 16: 15-31.
6. Komposch K, Sibilia M (2015) EGFR Signaling in Liver Diseases. Int J Mol Sci 17.
7. Tanabe KK, Lemoine A, Finkelstein DM, Kawasaki H, Fujii T, et al. (2008) Epidermal growth factor gene functional polymorphism and the risk of hepatocellular carcinoma in patients with cirrhosis. JAMA 299: 53-60.
8. Ganai AA, Farooqi H (2015) Bioactivity of genistein: A review of in vitro and in vivo studies. Biomed Pharmacother 76: 30-38.
9. Kim HJ, Ahn HS, Choi BH, Hahn SJ (2011) Inhibition of Kv4.3 by genistein via a tyrosine phosphorylation-independent mechanism. Am J Physiol Cell Physiol 300: C567-575.
10. Varinska L, Gal P, Mojzisova G, Mirossay L, Mojzis J (2015) Soy and breast cancer: focus on angiogenesis. Int J Mol Sci 16: 11728-11749.
11. Dastjerdi MN, Kavoosi F, Valiani A, Esfandiari E, Sanaei M, et al. (2015) Inhibitory Effect of Genistein on PLC/PRF5 Hepatocellular Carcinoma Cell Line. Int J Prev Med 6: 54.
12. Li Y, Luo Y, Zhang X, Lin X, He M, et al. (2013) Combined taurine, epigallocatechin gallate and genistein therapy reduces HSC-T6 cell proliferation and modulates the expression of fibrogenic factors. Int J Mol Sci 14: 20543-20554.
13. Saleh DO, Abdel Jaleel GA, El-Awdan SA, Oraby F, Badawi M (2014) Thioacetamide-induced liver injury: protective role of genistein. Can J Physiol Pharmacol 92: 965-973.
14. Huang Q, Huang R, Zhang S, Lin J, Wei L, et al. (2013) Protective effect of genistein isolated from Hydrocotyle sibthorpioides on hepatic injury and fibrosis induced by chronic alcohol in rats. Toxicol Lett 217: 102-110.
15. Salas AL, Ocampo G, Fariña GG, Reyes-Esparza J, RodrÃguez-Fragoso L (2007) Genistein decreases liver fibrosis and cholestasis induced by prolonged biliary obstruction in the rat. Ann Hepatol 6: 41-47.
16. Salas AL, Montezuma TD, Fariña GG, Reyes-Esparza J, Rodríguez-Fragoso L (2008) Genistein modifies liver fibrosis and improves liver function by inducing uPA expression and proteolytic activity in CCl4-treated rats. Pharmacology 81: 41-49.
17. Committee on Care and Use of Laboratory Animals of the Institute for Laboratory Animal Resources, Commission on Life Sciences,National Research Council (1996) Guide for the Care and Use of Laboratory Animals. Publication 86-23, Maryland US.
18. Rojkind M (1973) Inhibition of liver fibrosis by 1-azetidine-2-carboxylic acid in rats treated with carbon tetrachloride. J Clin Invest 52: 2451-2456.
19. Goodman ZD (2007) Grading and staging systems for inflammation and fibrosis in chronic liver diseases. J Hepatol 47: 598-607.
20. Hernandez-Gea V, Friedman SL (2011) Pathogenesis of liver fibrosis. Annu Rev Pathol 6: 425-456.
21. Fujii T, Fuchs BC, Yamada S, Lauwers GY, Kulu Y, et al. (2010) Mouse model of carbon tetrachloride induced liver fibrosis: histopathological changes and expression of CD133 and epidermal growth factor. BMC Gastroenterol 10: 79.
22. Yalniz M, Bahcecioglu IH, Kuzu N, Poyrazoglu OK, Bulmus O, et al. (2007) Preventive role of genistein in an experimental non-alcoholic steatohepatitis model. J Gastroenterol Hepatol 22: 2009-2014.
23. Kuzu N, Metin K, Dagli AF, Akdemir F, Orhan C, et al. (2007) Protective role of genistein in acute liver damage induced by carbon tetrachloride. Mediators Inflamm 2007: 36381.
24. Shi HY, Xu JW, Ren XX (2008) Effect of genistein on hepatic stellate cell proliferation and lipid peroxidation in vitro. Nan Fang Yi Ke Da Xue Xue Bao 28: 2066-2068.
25. Schneider MR, Wolf E (2009) The epidermal growth factor receptor ligands at a glance. J Cell Physiol 218: 460-466.
26. Almendro V, Garcia-Recio S, Gascon P (2010) Tyrosine kinase receptor transactivation associated to G protein-coupled receptors. Curr Drug Targets 11: 1169-1180.
27. Collin de L'hortet A, Gilgenkrantz H, Guidotti JE (2012) EGFR: A Master Piece in G1/S Phase Transition of Liver Regeneration. Int J Hepatol 2012: 476910.
28. Zhang D, Zhang J, Jiang X, Li X, Wang Y, et al. (2014) Heparin-binding epidermal growth factor-like growth factor: a hepatic stellate cell proliferation inducer via ErbB receptors. J Gastroenterol Hepatol 29: 623-632.
29. Fuchs BC, Hoshida Y, Fujii T, Wei L, Yamada S, et al. (2014) Epidermal growth factor receptor inhibition attenuates liver fibrosis and development of hepatocellular carcinoma. Hepatology 59: 1577-1590.
30. Berasain C, Perugorria MJ, Latasa MU, Castillo J, Goñi S, et al. (2009) The epidermal growth factor receptor: a link between inflammation and liver cancer. Exp Biol Med (Maywood) 234: 713-725.
31. Pavese JM, Farmer RL, Bergan RC (2010) Inhibition of cancer cell invasion and metastasis by genistein. Cancer Metastasis Rev 29: 465-482.
32. Gruca A, Krawczyk Z, Szeja W, Grynkiewicz G, Rusin A (2014) Synthetic genistein glycosides inhibiting EGFR phosphorylation enhance the effect of radiation in HCT 116 colon cancer cells. Molecules 19: 18558-18573.
33. Perugorria MJ, Latasa MU, Nicou A, Cartagena-Lirola H, Castillo J, et al. (2008) The epidermal growth factor receptor ligand amphiregulin participates in the development of mouse liver fibrosis. Hepatology 48: 1251-1261.
34. Moldovan GL, Pfander B, Jentsch S (2007) PCNA, the maestro of the replication fork. Cell 129: 665-679.
35. Wang SC, Nakajima Y, Yu YL, Xia W, Chen CT, et al. (2006) Tyrosine phosphorylation controls PCNA function through protein stability. Nat Cell Biol 8: 1359-1368.