

Gallic acid produces hepatoprotection by modulating EGFR expression and phosphorylation in induced preneoplastic liver foci in rats

Jorge Reyes-Esparza M.D. and Ph.D.¹, Rebeca Escutia-Gutiérrez M. Pharm.¹, Francisco Garcia-Vázquez Q.B.P.², Lourdes Rodríguez-Fragoso M.D. and Ph. D.¹

1Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Cuernavaca 62210, Mexico. 2 Instituto Nacional de Pediatría, Mexico City, Mexico.

ABSTRACT

The purpose of this study was to analyze the role of gallic acid as liver protector and identify its role in the regulation of EGFR expression and phosphorylation in induced preneoplastic liver lesions in rats. Male Wistar rats were randomly divided into four groups. (1) Control; (2) animals receiving gallic acid (AG) 50 mg/kg v.o. for 8 weeks; (3) animals with preneoplasia (P) induced by a single dose of diethylnitrosamine 200 mg/kg i.p. (DEN) and two weeks after a single dose of carbon tetrachloride 2 mL/kg i.p. (CCl₄); and (4) animals with preneoplasia treated with GA during 8 weeks. In order to evaluate GA hepatoprotection on preneoplastic lesions, we performed histological examination of liver tissue using H&E staining as well as an immunohistochemical analysis for PCNA. To evaluate the effect of GA on EGFR expression and phosphorylation, we performed an immunohistochemical and western blot analysis. The results indicated that GA significantly decreased EGFR expression and pY1068 EGFR phosphorylation in animals with preneoplastic lesions. GA significantly decreased PCNA expression in animals with preneoplastic lesions, suggesting it may work as an antiproliferative agent. Additionally, GA improved the architecture and organization of liver tissue and significantly decreased serum AST, ALT and FA, which are indicators of hepatocellular damage. By histopathological and immunohistochemical analysis we demonstrated an improvement in liver morphology, a reduction of preneoplastic liver foci and a reduction of cell proliferation, as well as an improvement on liver functionality. In conclusion, GA produces hepatoprotection by modulating EGFR expression and phosphorylation in preneoplastic lesions.

Keywords: antiproliferative, Gallic acid, preneoplastic lesions, EGFR, phosphorylation

*Correspondence to Author:

Lourdes Rodríguez-Fragoso, M.D. and Ph.D. Av. Universidad 1001, Col. Chamilpa. Cuernavaca. Morelos, Mexico

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Introduction

Statistical assessments of cancer indicate that hepatocellular carcinoma remains a cause of death in people suffering from chronic liver diseases [1]. Its geographical distribution is very heterogeneous and is closely related to the prevalence of the diverse risk factors associated with its development [2].

The early stages of hepatocarcinogenesis are identified by the appearance of preneoplastic lesions characterized by the presence of nodules with loss of organization, abnormal nuclear morphology, pleomorphism and mitosis [3]. The therapeutic options are determined by the stage in which the disease is diagnosed.

In that sense, the presence of preneoplastic lesions should be considered the ideal stage to start pharmacological treatment because at this stage there could be a better prognosis for the patient. Unfortunately and in most of cases, the diagnosis comes too late and therapeutic options include liver resection, transplantation, transarterial chemoembolization, and the use of new drugs as sorafenib, regorafenib, nivolumab and others [4-7]. However, these therapies have specific indications and contraindications, are considered high risk and are quite costly.

Current cancer therapies are targeted toward specific molecular targets located in different intracellular pathways that lead to tumor growth [8]. The epidermal growth factor receptor (EGFR) is part of the ErbB family of receptors with tyrosine kinase activity (RTK) [9]. The overexpression of EGFR occurs in 40-70% of cases of patients with HCC, so it has been linked to hepatocarcinogenesis [10]. EGFR activation can trigger different signaling pathways that can lead to cellular processes such as cell migration and proliferation, as well as survival and resistance to apoptosis [11]. For this reason, EGFR is considered a therapeutic target in the treatment of HCC.

Gallic acid is a polyphenol that has recently been associated with cell death caused by ROS-derived oxidative stress and mitochondrial

dysfunction [12]. Consequently, gallic acid could be used to reduce the viability of cancer cells, promoting apoptotic processes and inducing cytotoxicity in various types of human cancer cells [13,14]. Most *in vivo* studies with gallic acid highlight its antiproliferative property in different types of cancer [15,16]. At present, there are no reports regarding the association of gallic acid and EGFR in CHC in *in vivo* models. The purpose of the present study was to evaluate the effect of gallic acid on EGFR expression and its phosphorylation of specific tyrosine residues and its correlation with the hepatoprotective effects on altered hepatocytes foci and induced preneoplastic lesions in rats.

Material and Methods

Animal model.

The study used male Wistar rats weighing 120-140g. The animals were kept in cages in a controlled environment, with water and food ad libitum. They all had a week without manipulation for acclimatization. After this time, the animals were divided into 4 experimental groups: (1) animals who received 50 μ L of water and were considered as control; (2) animals who received 50 mg/kg of gallic acid per v.o. daily during 8 weeks; (3) animals treated with 200 mg/kg diethylnitrosamine (DEN) to induce preneoplastic lesions, , then treated two weeks later with 2 mL/kg of CCl₄ diluted in mineral oil (1:1), where both carcinogens were administered intraperitoneally in a single dose and animals were kept alive during 8 weeks; and the last group (4), animals with preneoplastic lesions and treated with gallic acid (50 mg/Kg) per v.o. daily, during 8 weeks after CCl₄ dose. Each group included 6 animals. Animals were sacrificed at the end of all treatments under anesthesia. To obtain blood samples, a cardiac puncture was performed and subsequently centrifuged at 3000 rpm for 15 min to obtain the sera. Liver samples were obtained, washed and weighed. Serum and liver samples were kept at -70°C for further analysis. The animals were treated in accordance with the Guide for the Care and Use for Laboratory Animals [17].

Western blot analysis for EGFR total and pY1068 EGFR

Liver tissues that were prepared by scraping with lysis buffer (20mM HEPES, 2mM EGTA, 50mM β -glycerophosphate, 5mM sodium fluoride, 50 μ M DTT, 100mM PMSF, 1% triton X-100, 10% glycerol and 0.1% protease inhibitor cocktails). The tissue lysates were centrifuged at 4600 rpm for 5 min and then the supernatant was separated from the precipitate, keeping the supernatant and centrifuging at 11 800 rpm for 10 min. Protein quantitation was carried out with the DC protein assay. For Western blot analysis, 125 μ g of protein were electrophoresed through 10% gels and transferred to PVDF membrane for 2 hours at 350 mA.

Then the membrane was washed with TBS-T, 3 times for 5 min. Immunoblot analysis was carried out using EGFR and pY1068 antibodies (1:1000) in 2.5% albumin in TBS-T overnight at 4°C. The membrane was washed with TBS-T and incubated with the secondary anti-mouse-HRP antibody (1:1500) in 5% casein in TBS-T. Specific protein was detected with a chemiluminescence kit (Perkin Elmer Life, Boston, MA). Finally, a semi-quantitative analysis of the bands corresponding to EGFR and actin was performed with the Quantity One program.

Immunohistochemical detection of EGFR and pY1068 in liver samples

The method used to determine the location and level of the EGFR and pY1068 EGFR proteins was through indirect immunohistochemistry, using a biotin/peroxidase/DAB (Sigma-Aldrich Corporation; MO, USA) free system, according with the Manual of Procedures for Molecular Pathology Laboratory of the National Institute of Pediatrics.

From the liver samples included in paraffin, slices of 4 μ m were cut and placed in positively charged slides. The slides were heated at 60 °C for 20 min and rehydrated (Xilol Absolute, Xylol-absolute alcohol, absolute ethanol, 80% ethanol and finally in distilled water). After the

rehydration process, the samples were subjected to the process of "epitope recovery", which is used to give a stable conformation to the proteins of interest. This epitope recovery process consisted of placing the slides with liver samples in containers with an antigen recovery solution (sodium citrate buffer: 10mM sodium citrate, 0.01 mol/L 0.05% Tween-20 to 0.05%, pH 6).

The containers with the slides were placed in an immunohistochemical capillary system and PBS-Tween was added. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in absolute methanol for 5 min. It was washed with distilled water and immediately the monoclonal antibody of EGFR and pY1068 (dilution 1:20, 1:50 and 1:50 with PBS-T, respectively for each protein) (EGF receptor antibody No. 2232 and Phospho-EGF receptor Tyr 1068 antibody No. 2236, Cell signaling Tech, Inc.) was added in 1% BSA-PBS to the corresponding slides and allowed to incubate for 45 min.

Once the incubation with the primary antibody was finished, the slide was washed with distilled water and the secondary antibody Mach 1 Mouse Probe Dropper Bottle (Biocare Medical, Inc.) was added and incubated for 40 min. Afterwards, it was washed with PBS-T, Mach 1 Universal HRP polymer dropper bottle (Biocare Medical, Inc.) was added and, once again, the slide [??] was washed with PBS-T. C 0.5 mg/mL 3,3'-diaminobenzidine tetrahydrochloride chromogen was subsequently added. The slides were contrasted with hematoxylin and then observed under a light microscope.

For EGFR, pY1045 and pY1068 EGFR proteins, only areas with immunopositive reactivity were examined. An Olympus IX81 inverted epifluorescence microscope was used to capture images. The quantitative analysis was made using an Image-Pro® Imaging Software (Media Cybernetics Inc.).

Detection and quantitation of proliferating cell nuclear antigen (PCNA).

Sections were cut at 4µm, mounted on slides coated with 3 aminopropyltriethoxysilane (Sigma-Aldrich Co.; 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. 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 liver pieces were then incubated with a biotinylated rabbit anti-mouse (dilution 1:200 in PBS) with 1% bovine serum albumin at 42°C for 20 min. After a time, they were incubated with avidin DH-biotinylated horseradish peroxidase H complex (Vectastain Elite ABC Kit; Vector Laboratories, CA, USA) for 20 min at RT. Finally, the slices were exposed with 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide substrate solution in 0.05M Tris-HCl (pH 7.6) for 3 min and then washed. Slices were counterstained with hematoxylin, dehydrated in ethanol, and mounted. As positive control tissue, we used a section of regenerating rat liver. The quantitative analysis was made using an Image-Pro® Imaging Software (Media Cybernetics Inc.).

Determination of serum liver enzymes activity.

The serum activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Alkaline Phosphatase (AP) activity was determined using commercial kits following the manufacturer's instructions (ELITech, Mexico). Briefly, 20 µL of serum was mixed with 200 µL of working solution. The reaction was allowed to run for 5 minutes, after which the absorbance of

AST and ALT was read at a wavelength of 307 nm and AP at 405 nm in a plate reader (Victor X3 Multimode Perkin Elmer plate) at 37°C using Workout 2.5 software. ALT, AST and AP values were expressed as international units per liter (U / L).

Histopathological analysis

Liver samples were fixed in 4% formaldehyde during 48 h and transferred in a cassette and immersed in paraffin a 60°C. Samples were processed and slices of 2 µm were cut using a microtome Microm (Carl Zeiss). Liver samples were stained with Hematoxylin-Eosin for further histological analysis.

Statistical Analysis

The results were expressed as the mean ± standard deviation (D.S.). The results obtained were analyzed using the statistical software SPSS Real Statistics v.19.0. We determined one-way ANOVA and the Tukey statistical test and considered the results statistically significant when $p < 0.05$.

Results

Figure 1 shows a representative microphotography of the liver sections from animals after treatment. Figure 1B shows a liver section with a preneoplastic lesion, which is morphologically characterized by a focus of small cell dysplasia. This had hepatocytes with decreased cytoplasmic volume, slight nuclear polymorphism and alterations in nucleus-cytoplasm ratio. A large number of atypical hepatocytes with large nuclei, nuclear pleiomorphism and prominent nucleoli were distributed through the liver parenchyma. The histological analysis showed a loss of architecture and organization of hepatocyte cords, alterations of the portal structure and presence of inflammatory cells.

The administration of gallic acid did not alter the architecture and organization of hepatocytes and presented the same morphological characteristics as the control group (Figure 1A). The characteristic morphology of a healthy liver

cut can be seen in the gallic acid group (Figure 1C).

Animals with preneoplastic lesions treated with gallic acid for 8 weeks showed an improvement in the organization and architecture of hepatocytes, as well as a decrease of

inflammatory cells. The gallic acid was able to reduce the appearance of dysplastic cells and restore the nucleus-cytoplasm ratio of hepatocytes (Figure 1D). The livers from animals of this group showed a microscopic morphology similar to the control group.

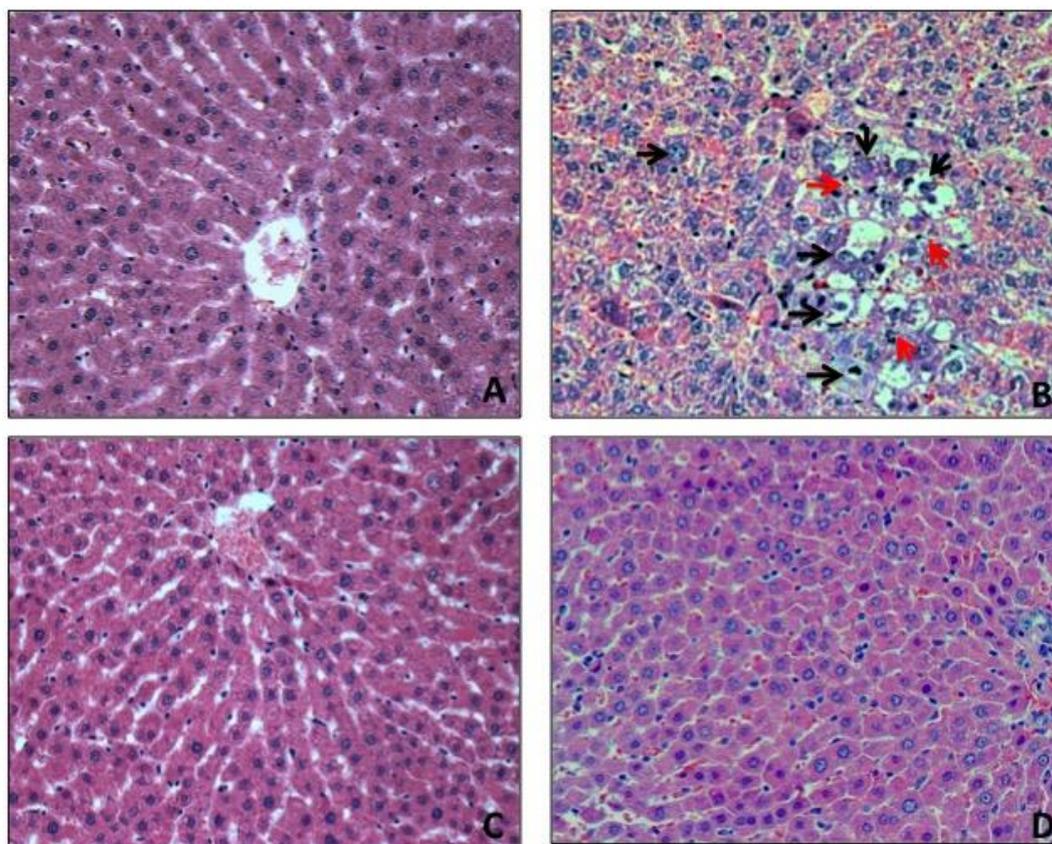


Figure 1. Representative microphotographs from liver sections from rats treated with gallic acid. Liver tissue from: A) Control rat; B) Animal with preneoplastic lesions. We can see a focus of small cell dysplasia, hepatocytes with nuclear pleiomorphism and prominent nucleoli of parenchymatous cells (black arrow), distortion of hepatic architecture with necrotic cells, and the presence of inflammatory infiltrate (red arrow); C) Animal treated with gallic acid; D) Animal with preneoplastic lesions treated with gallic acid. We can see an improvement in liver structure with fewer dysplastic hepatocytes, fewer necrotic cells, and reduced inflammatory infiltrate. Liver sections were stained with H&E, magnification 20X.

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP) enzymes were measured to establish the presence of functional damage. In the group with preneoplastic lesions, there was an increase in ALT (86%), AST (100%) and AP (200%) levels compared to control group ($p < 0.05$). The administration of gallic acid to

animals with preneoplastic lesions significantly decreased ALT (68%), AST (70%) and AP (73%) serum levels ($p < 0.05$) compared to animals with preneoplastic lesions (Table 1). The administration of gallic acid to healthy animals did not produce significant changes in ALT, AST and AP serum levels compared to control group.

Table 1 Biochemical findings

Group	Liver weight (g)	AST (U/L)	ALT (U/L)	AP (U/L)
Control	30 ± 10	50 ± 4	61 ± 8	144.3 ± 27
Gallic Acid	40 ± 5	55 ± 9	65 ± 9	135.1 ± 12
Preneoplasia	30 ± 7	158 ± 20 *	113 ± 12*	449.3 ± 86*
Preneoplasia + Gallic acid	38 ± 4	84 ± 23 *#	45 ± 11#	133 ± 18#

* p<0.05 as compared with control group # p<0.05 as compared with preneoplasia group

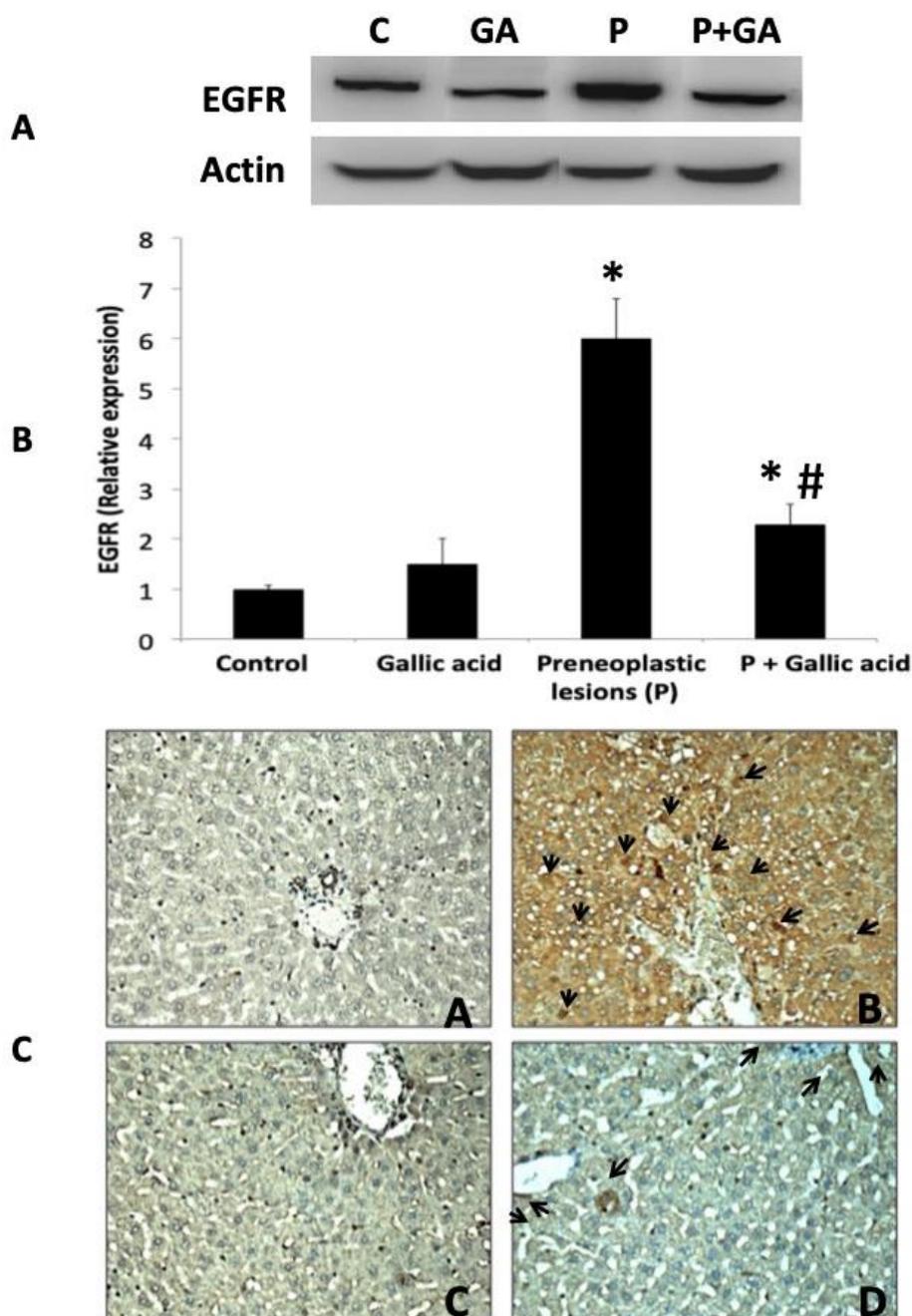


Figure 2. EGFR expression and localization in liver sections with preneoplastic lesions. A- B) An increase in the signal from liver with preneoplastic lesions was observed. Gallic acid significantly reduced EGFR expression. The analysis was determined using a Western blot. C) EGFR localization was determined by immunohistochemistry; positive reaction is shown in brown (arrows). We observed many immunopositive cells inside preneoplastic lesions and distributed across parenchyma. We chose a representative microphotograph of each group, magnification 40X. *p<0.05 as compared to control group; # p<0.05 as compared to preneoplasia group.

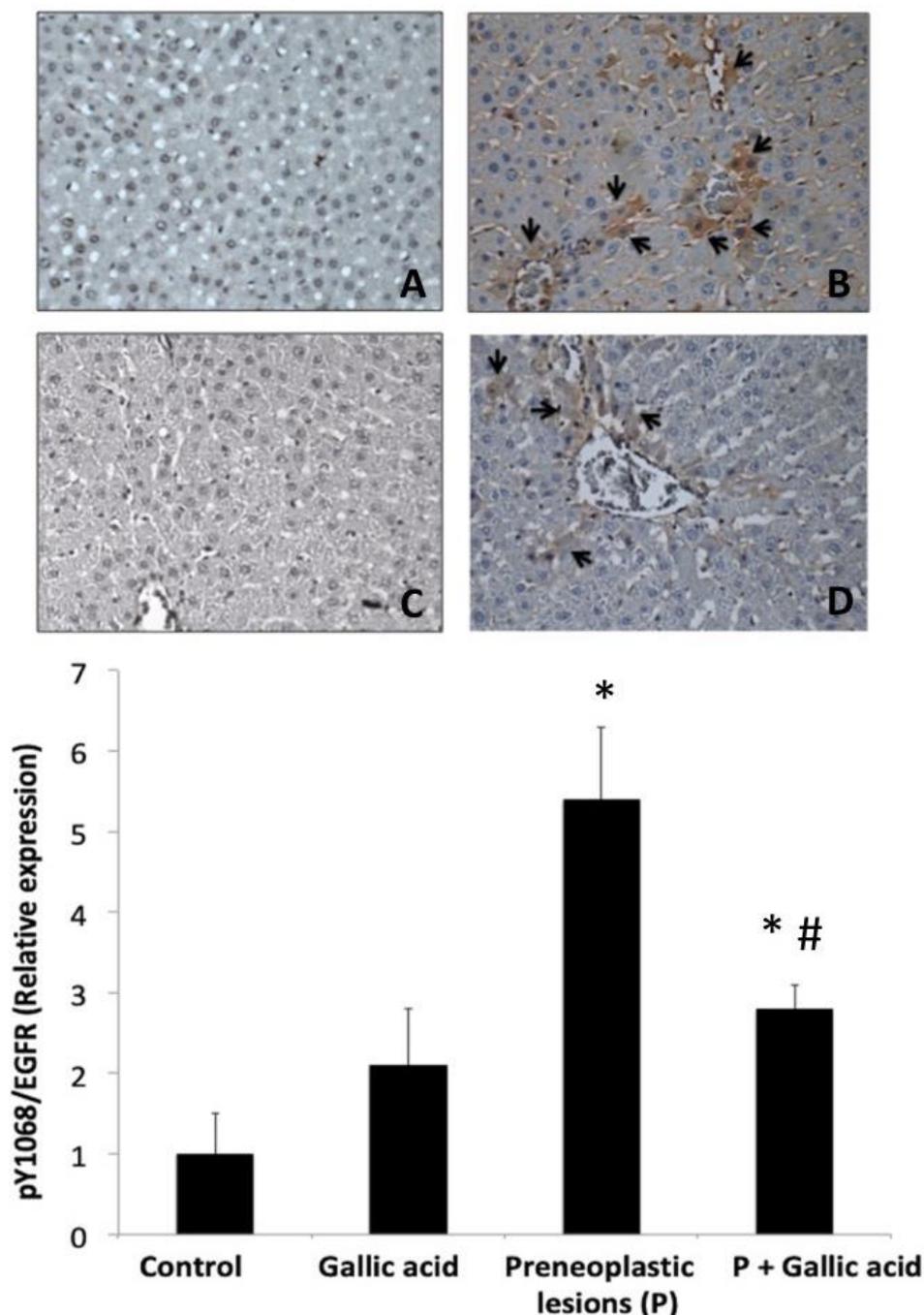


Figure 3. Immunohistochemical analysis of phosphorylation of specific EGFR tyrosine residue in liver slices. Positive reaction to pY1068 EGFR is shown in brown (arrows). We observed isolated immunopositive cells inside lesions and distributed across the parenchyma in the preneoplasia group. Animals with preneoplastic lesions and treated with gallic acid showed a lower number of immunoreactive cells. We observed immunopositive cells mainly around the portal vein and hepatic triad. We chose a representative microphotograph of each group, magnification 40X. * $p < 0.05$ as compared to control group; # $p < 0.05$ as compared to preneoplasia group.

Figure 2A,B shows the semiquantitative analysis of EGFR expression analyzed by Western blot. A significant increase in EGFR expression in animals with preneoplastic lesions (5.7-fold) ($p < 0.05$) was observed. Animals with lesions and treated with gallic acid showed a significant

decrease in EGFR expression in liver (1.2-fold) ($p < 0.05$). Animals treated with gallic acid showed no changes in EGFR expression. The immunohistochemical analysis revealed EGFR is expressed in very low levels in healthy liver tissue (Figure 2C). The group with preneoplastic

lesions showed different degrees of expression of immunopositive cells. EGFR expression was found throughout the liver parenchyma; we also found areas with a very marked immunoreactivity to EGFR (Figure 2C-a, black arrow). The preneoplasia group treated with

gallic acid showed a scattering of immunopositive cells and a low expression of EGFR protein when compared to the preneoplasia group. Animals merely treated with gallic acid also showed normal EGFR levels (Figure 2C,c).

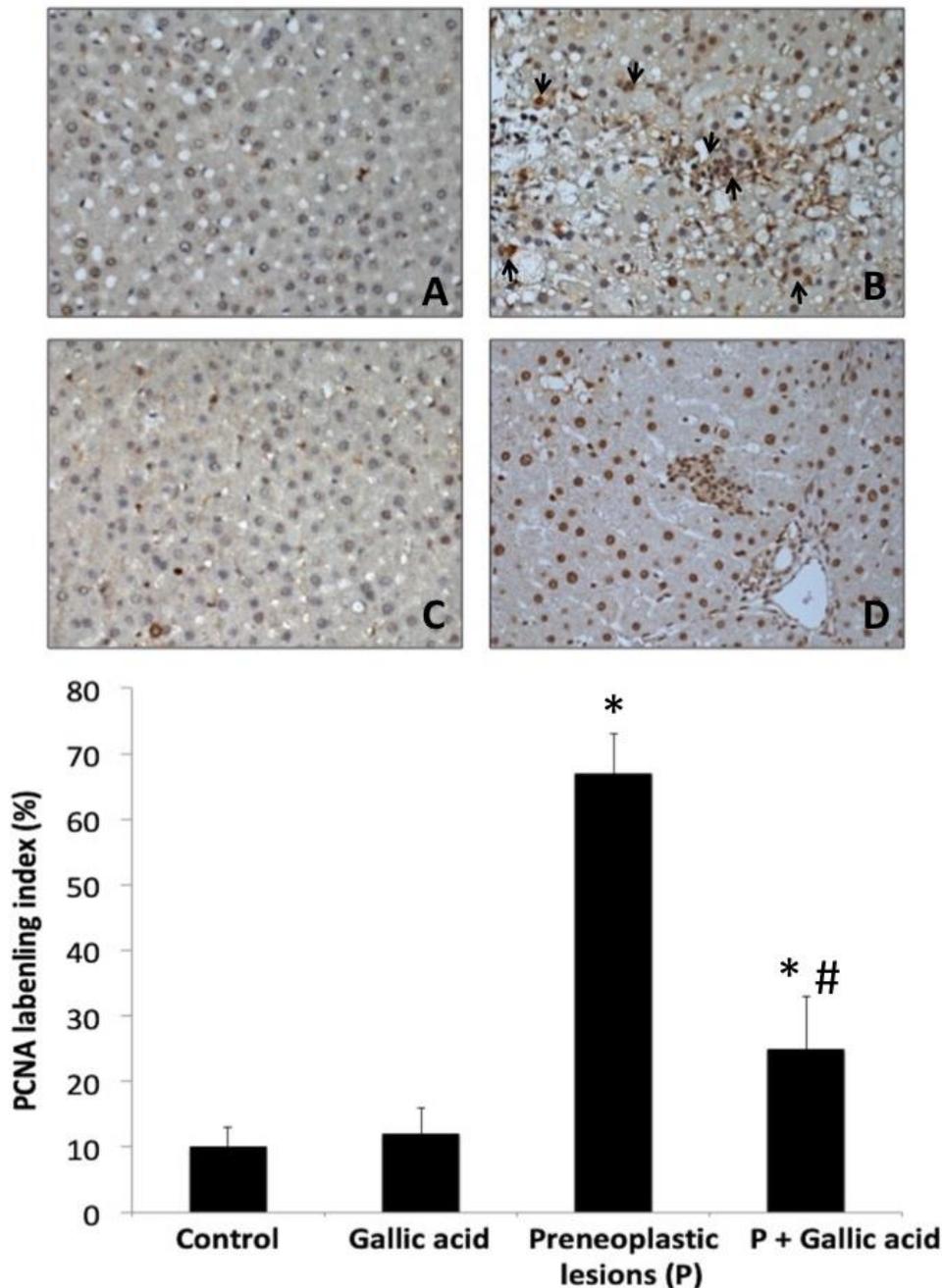


Figure 4. Effect of gallic acid on PCNA expression and localization in liver. PCNA-positive cells are shown in brown (nuclei). A and C) Liver from the control and gallic acid groups respectively, were we can see scattered positive cells across the parenchyma; B) PCNA localization in liver sample with lesions. We can see foci of small cell dysplasia as well as scattered positive cells across the parenchyma. D) Liver sample of animal with lesions and treated with gallic acid. We observed positive cells in parenchymal and inflammatory foci, was along with scattered and faint immunoreactive cells. We chose a representative microphotograph of each group, magnification 40X.

The preneoplasia group showed varying degrees of intensity in hepatocyte immunoreactivities to pY1068 EGFR phosphorylation in certain areas. These areas were all distributed in the hepatic parenchyma. It should be noted that most of the immunoreactive zones were found near portal structure, which may be related to the fact that the carcinogens we employed are hepatotoxins that arrive from the general bloodstream via the portal vein. The sub-cellular location of the protein of interest seems to be found in the cytoplasm (Figure 3a). On the other hand, the preneoplasia group that received gallic acid showed a limited area of immunoreactive hepatocyte to pY1068 EGFR (Figure 3d). The level of pY1068 EGFR phosphorylation for animals treated with gallic acid was similar to that of the healthy rats.

Figure 4 shows the PCNA levels in all groups. In the control and gallic acid groups, only isolated hepatocytes showed positive nuclei to the PCNA marker, indicating that only some hepatocytes were found in cell proliferation (Figure 4a,c). The preneoplasia group showed an increased number of nuclei positive to the PCNA marker distributed along the hepatic parenchyma. This increase indicates the high proliferative activity of dysplastic cells (Figure 4b). In the quantitative PCNA analysis we found an approximately 6-fold increase in the PCNA labeling index when compared to control group ($p < 0.05$). However, animals with preneoplasia and treated with gallic acid showed a significant decrease (61%) in the PCNA labeling index when compared with the preneoplasia group ($p < 0.05$).

Discussion

The identification of the appearance of foci of altered hepatocytes and preneoplastic lesions is essential to the identification of molecular and immunohistochemical markers, establishing the appearance of hepatocarcinogenesis, and implementing timely and adequate therapy [18].

High EGFR expression of is related to several types of carcinoma [19]. EGFR overexpression occurs in 40-70% of patients with CHC, which has been correlated with aggressive tumors,

metastases, chemotherapy resistance, poor clinical outcomes and low patient survival [20-22]. Given the importance of EGFR in hepatocarcinogenesis, this is now considered an attractive molecular target for therapeutic agents against cancer [23,24].

Because treatment options for HCC are still very limited, there has been a search in recent years for natural bioactive compounds with anticancer properties, including the use of polyphenols [25] given observed studies where their intake reduces the risk of developing cancer [26]. There has been an increase in their consumption as anticancer agents by the general public, with accompanying scientific interest in identifying its molecular targets in cancer. One of the most studied polyphenols associated with cancer prevention is gallic acid, and several anticancer properties have been ascribed to it [27]. Countless reports indicate gallic acid has multiple molecular mechanisms of action in cancer and other pathologies. Some even indicate that the combined use of gallic acid with antineoplastic agents increases the antitumor effects [28].

The effect of gallic acid on hepatocellular carcinoma has been previously demonstrated in an animal model. The authors concluded that the main effects were due to its antioxidant and anti-inflammatory properties, and also by the induction of apoptosis [29]. It has been reported, for example, that gallic acid increases Bax protein expression and decreases Bcl-2 protein expression [30], suppresses Src-Stat3-dependent signaling [31], reduces expression levels of RNAm of genes related to cell migration [32] and induces ATM-Chk2 activation [33].

In the present study we found a high expression of EGFR not only in foci of dysplastic hepatocytes, but also in large areas of the parenchyma without apparent lesions. In addition, areas with overexpression of phosphorylated EGFR in tyrosine 1068 (EGFR pY1068) were observed, although their expression was lower. EGFR is a tyrosine kinase-like receptor that has

autophosphorylation sites associated with the activation of signaling pathways associated with migration, proliferation, cell death, angiogenesis and ubiquitinylation [34]. This work researched the changes in the EGFR phosphorylation tyrosine 1068 because this phosphosite participates in the activation of the downstream signal pathways (PI3K/AKT and MAP/ERK) associated with proliferative signals. It was observed that gallic acid significantly inhibited EGFR expression and pY1068 EGFR phosphorylation in animals with preneoplastic lesions, and the effect of gallic acid on the EGFR signaling pathway has already been documented. For example, it has been shown that it inhibits the activation of EGFR-induced EGF mediated by AKT and ERK in breast cancer cells [35]. In addition to that, it has been reported gallic acid modifies EGFR turnover in EGFR mutant NSCLC cells [36]. There are no reports indicating it also modifies phosphorylation of EGFR. However, there are reports on how it can inhibit phosphorylation (activation) of key proteins in proliferation pathways such as Akt, Erk/c-Jun, Rb, STAT1 and STAT3 [37].

As already mentioned, one of the characteristics of dysplastic hepatocyte foci is their increase in proliferative capacity. For this reason, we sought to evaluate the effect of gallic acid on the levels of cell proliferation marker PCNA as they appeared in liver tissue with induced preneoplastic lesions in rats. We observed a marked increase in the number of positive nuclei for the PCNA marker in the preneoplasia group, indicating the high proliferative activity of the dysplastic cells distributed in the hepatic parenchyma. However, animals with preneoplastic lesions treated with gallic acid showed a significant reduction in PCNA expression, indicating a strong antiproliferative effect. Our results were similar to those reported by other authors in the sense that gallic acid is able to reduce the positive nuclei for PCNA (77.5%) in a model of hepatocarcinogenesis induced by DEN [38] as well as in transgenic

adenocarcinoma of the mouse prostate (TRAMP) [39].

It has been reported that gallic acid suppresses proliferation and survival signals associated with MAPK/ERK and PI/AKT in malignant mesothelioma cells [40]. It has also been shown gallic acid inhibits other molecular targets and cell signaling pathways associated with cell proliferation and apoptosis. For example, it inhibits the proliferation associated with pAKT and pSTAT3 in PC3 cells [41]. Our results show it inhibits the expression and phosphorylation of the specific tyrosine site that leads to cell proliferation. Histopathological and immunohistochemical analysis demonstrated an improvement in liver morphology, and a reduction of preneoplastic liver foci and cell proliferation, as well as improvement in liver functionality. According to current results, gallic acid appears to exert its hepatoprotective effects by inhibiting the expression and phosphorylation of EGFR in foci of altered hepatocytes and preneoplastic lesions.

Gallic acid produces hepatoprotection by modulating the expression and phosphorylation of EGFR, thereby inhibiting proliferation as well as possible signs of cell survival in induced preneoplastic liver foci in rats.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare

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