RESEARCH ARTICLE

The Protective Effects of Adenosine Deaminase Inhibitor and Quercetin Against Hepatocellular Carcinoma Induced by Thioacetamide in Male Rats via Downregulation of iNOS, Ki67 and Pan-CK

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Abstract:

The liver is the largest and most vulnerable organ in the body, several factors can cause liver cirrhosis and the onset of hepatocellular carcinoma. This study aims to investigate the possibility for erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) as adenosine deaminase inhibitor which reduces liver injury, neutrophil infiltration and the levels of proinflammatory cytokines and also it is considering as a target of liver cirrhosis and beginning of hepatocellular carcinoma protective, quercetin which is one of the most common flavonoids has an antioxidant, antitumor and chemopreventive effect on the liver-induced preneoplastic lesions and their combination against thioacetamide as a hepatotoxic and a carcinogenic compound. Biochemical, histopathological and immunohistochemical studies were carried out on male albino rats model to evaluate this possibility. Thioacetamide-treated rats showed a significant increase in liver functions tests (P = 8.6 X 10-7 for alanine transaminase, P = 1.89 X 10-8 for γ-glutamic transpeptidase and P = 8.8 X 10-13 for total bilirubin), α-fetoprotein (P = 7.95 X 10-13) and the number of Ki67+ cells (P = 3.0 X 10-6), and caused an elevation in the expression of inducible nitric oxide synthase (iNOS) and Pan-cytokeratin (Pan-CK) in hepatic tissue of rats.

In conclusion results of the present study showed that treatment with quercetin, EHNA or their combination protect liver from development of HCC by attenuated changes in liver functions tests, expression of iNOS, Ki67 and Pan-CK, and histopathological changes induced by thioacetamide

Introduction

Cirrhosis is the most common form of liver pathology that leads to the development of hepatocellular carcinoma (HCC), a type of primary liver cancer that is considered the most lethal cancer in the world(1). Most cases of cirrhosis and the beginning of HCC are attributable to infections caused by hepatitis B and C viruses (2), obesity, iron overload, environmental pollutants and several dietary carcinogens, such as aflatoxins, thioacetamide and nitrosamines (3). These insults are initiated by liver damage that leads to the release of inflammatory cells, cytokines, growth factors and reactive oxygen species (ROS) (4, 5) which leads to cytotoxicity, mutagenicity and carcinogenicity.

Thioacetamide (TAA) is an organic compound with the formula CH_3CSNH_2 which is used in rubber, chemicals, pesticides (6), and in making metal salt nanoparticles (7). It is a potent hepatotoxin that usually causes acute or chronic liver diseases, cirrhosis, liver cell adenomas, and HCC subsequently in experimental animal models (8). TAA undergoes an extensive metabolism that leads to the formation of sulfoxide and sulfone which circulate through vital organs like liver, kidney and bone marrow (9). The main site of TAA metabolism is the liver and the production of ROS considers the main reason for TAA toxicity in the liver (10).

Adenosine deaminase (ADA), is an important enzyme, found in all mammalian cells (11). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), an ADA inhibitor, is a crystalline compound soluble in organic solvents such as DMSO and is less toxic than other ADA inhibitors because it keeps the cellular capacity for the deamination of purines nucleotides like (adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP)) (12). EHNA prevents adenosine breakdown so it can raise adenosine concentration which has anti-inflammatory activities and induces apoptosis in a variety of cancer cells mediated by intrinsic and/or extrinsic pathways (13). For the intrinsic pathway, extracellular adenosine is taken into cells by adenosine transporters and converted to adenosine monophosphate (AMP) by adenosine kinase to activate AMP-activated protein kinase (AMPK), responsible for apoptosis in lung, breast cancer cells and hepatoma cells (14). For the extrinsic pathway, adenosine release from cells to the plasma membranes of neighboring cells, where it binds to specific adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) and causes apoptosis in a variety of cancer cells according to the concentration of adenosine and the type of receptors (13).

Quercetin (QU) is one of the most common flavonoids which is found in several fruits and vegetables, has various pharmacological actions including, hepato-protective, reno-protective and neuroprotective effects (15). It has a beneficial effect on liver diseases and tumors in animal models and induces apoptosis of cancer cells by disruption of the mitochondria and release of cytochrome c and the activation of caspases (16, 17). Moreover, QU can inhibit metabolic activity and induce apoptosis in liver cancer cell lines like HepG2, HuH7, and Hep3B2 (18). It has potential chemopreventive or therapeutic effects in the treatment of liver cancer (19).

In our study, we investigate the potential role of QU, EHNA or their combination on TAA-induced liver toxicity in male rats using different histopathological, biochemical and molecular techniques.

Materials and methods

Chemicals

Thioacetamide (TAA), Quercetin (QU) and adenosine deaminase inhibitor erythro- 9-(2-hydroxy-3-nonyl) adenine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Enzyme immunoassay kit used for measurement of α-fetoprotein (AFP) was purchased from Bioscience, South San Francisco. kits for the determination of alanine transaminase (ALT), γ- glutamic transpeptidase (GGT) were purchased from Agappe diagnostics Switzerland, GMBH. Total bilirubin (TB) measurement kit was purchased from Diamond diagnostic, Egypt. All other chemicals were of the highest purity commercially available.

Animals

Fifty adult male albino rats (120-150 g) were obtained from the animal house of the Faculty of Medicine, Assuit University, Assuit, Egypt. Rats were housed in cages and were kept at room temperature (30 ± 3^0C) with a normal 12/12 h light/dark cycle and supplemented with standard commercial pellets for feeding water and labium. All experiments followed protocols approved by the Institutional Animal Care and Life Committee, Assiut University.

Experimental groups

Animals were divided into 5 groups of 10 animals each. Group I: served as a control was intraperitoneally (IP) injected with dimethyl sulfoxide (DMSO). Group II: rats were IP injected with TAA (200 mg/kg) twice a week for 3 months (20). Group III: rats were IP injected with QU (100 mg/kg) 30 min before TAA injection (21). Group IV: rats were IP injected with EHNA (150 µM/kg) 30 min before TAA injection (22). Group V: rats were IP injected with QU (100 mg/kg) and EHNA (150 µM/kg) 30 min before TAA injection.

Collection and preparation of the samples for biochemical analysis

At the end time of the experiment, rats were sacrificed under anesthesia and were used for the collection of blood and liver. Blood was collected in tubes

containing EDTA, centrifuged at 3000 rpm for 10 minutes for separation of plasma for biochemical analysis.

Estimation of AFP as a liver tumor marker

Plasma AFP level as marker of liver tumor was estimated by enzyme immunoassay test kit for quantitative determination of AFP from Bioscience, South San Francisco. (Catalog Number: 10101). In brief, coated wells in the holder were labeled. 20 µl of samples, standard and control were added to appropriate wells followed by addition of 100 µl of zero buffer into each well. Samples in wells were mixed for 20 sec and incubated at room temperature (RT) for 30 min. Incubation mixture was removed by flicking plate content into a waste container. Wells were washed 5 times by washing buffer (1X). Wells were stroked sharply onto paper towel to remove all residual water droplets. 150 µl of enzyme conjugated reagent were added into each well, gently mixed for 5 seconds and incubated at RT for 30 min. The incubation mixture was removed by flicking plate content into a waste container. Each well was washed 5 times by washing buffer (1X). The wells were stroked sharply onto paper towel to remove all residual water droplets. 100 µl of TMP substrate were added into each well, gently mixed for 5 seconds and incubated at RT for 20 min. 100 µl of stop solution were added to each well to stop reaction. Wells were gently mixed for 30 second to make sure blue color change to yellow color. Optical density was readied at 450 nm within 15 min. and the concentration was calculated by internal calibration standard using VIDAS microliter reader.

Estimation of ALT

Plasma ALT activity was determined calorimetrically using commercial kit from Agappe Diagnostics, Switzerland, GMBH. In brief, working reagent was prepared by mixing R1 and R2 in 4:1 ratio. Large glass tubes were labeled for each sample. 1000 µl of working reagent and 100 µl of samples were added in each tube. All tubes were mixed and incubated for 1 min at 37°C. Change in absorbance per min for 3 min (ΔOD/min) was detected. ALT activity (U/L) was calculated with the following formula: ALT activity (U/L) = (ΔOD /min) X 1745.

Estimation of GGT

Plasma GGT activity was determined calorimetrically using commercial kit from Agappe Diagnostics Switzerland, GMBH. In brief, working reagent was prepared by mixing R1 and R2 in 4:1 ratio. Large glass tubes were labeled for each sample. 1.0 ml of working reagent and 100 µl of samples were added in each tube. All tubes were mixed and incubated for 1 min at 37°C. Change in absorbance per min for 3 min (ΔOD/min) was detected. GGT activity (U/L) was calculated with the following formula: GGT activity (U/L) = (ΔOD /min) X 1158.

Estimation of TB

Total bilirubin concentration in plasma was determined using commercial kit from Diamond Diagnostic, Egypt. In brief, large glass tubes were labeled for each sample, sample blank and blank. 50 µl sample and 1.0 ml normal saline were added to each sample blank tubes. 50 µl sample and 1.0 ml reagent (R) were added in

sample tubes and 1.0 ml of reagent (R) was added in blank tube, respectively. All tubes were mixed and incubated for 3 min at room temperature. Absorbance of specimen (A sample) and sample blank (A sample blank) against reagent blank was measured at 546 nm. Total bilirubin concentration was calculated with the following formulas: Total bilirubin concentration (mg/dl) = ΔA sample X 28 where ΔA Sample = A sample – A sample blank.

Histopathological examination of liver tissue and quantification of liver cirrhosis

Liver was washed in phosphate buffer saline (PBS) pH 7.4 and morphologically examined. The other part of liver was preserved in 10 % neutral formalin solution for fixation. The fixed parts were trimmed, washed and dehydrated in ascending grades of alcohol. Tissue specimens were then cleared in xylene, embedded in paraffin and sectioned at 4-6 µm thickness. Slides were deparaffinized in xyelen, rehydrated and placed in hematoxylin stain for 1 min then washed for 5 min in running tap water. Slides were then stained with eosin, quickly rinsed in distilled water, dehydrated in ascending grades of alcohol and mounted. Slides were imaged using Olympus BX60 microscope and digital camera system (DP70, Olympus, Tokyo, Japan) under X200 magnification. For the quantification of liver cirrhosis, slides were stained in Picro-Sirius Red as described previously in (23). Brifly, slides were deparaffinized in xyelen, rehydrated and placed in Picro Sirius Red solution for 1 hr at RT. Slides were washed in two changes of acidified water for 5 min each at RT. Slides were dehydrated in three changes of 100% ethanol, cleared in xylene and mounted. Images of liver sections in each group were acquired by using the Olympus BX60 microscope and digital camera system (DP70, Olympus, Tokyo, Japan). All images were analyzed using the ImageJ software (version 1.45b; National Institutes of Health, Bethesda, MD, USA). The whole area and the fibrosis area stained with Picro Sirius Red were measured. The percentage (%) of intensity was calculated with the following formula: (fibrosis area / whole area) X 100.

Immunohistochemical studies of Ki67, Pan-CK and iNOS

Liver tissue sections were deparaffinized by placing them in aseries of xylene, rehydrated, and washed in (PBS) pH 7.4. For antigen retrieval, slides were incubated for 45 min in 10 mM citrate buffer at pH 6.0 and heated at 98 °C. To block endogenous peroxidase activity, slides were incubated in PBS containing 3% H_2O_2 for 45 min. Slides were then incubated for 1 h at room temperature or overnight at 4^0C with rat polyclonal antibodies specific for Ki67 at a dilution of (1:300), Pan-CK at a dilution of (1:100) and iNOS at a dilution of (1:20. Slides were washed with distilled water, followed by PBS then incubated with horseradish peroxidase-conjugated secondary antibodies for 15 min. Slides were subsequently washed twice with PBS. Then, the peroxidase reaction was developed with DAB-chromogen, and the slides were washed for 5 min in tap water and counterstained with Mayer hematoxylin from (Dako) for 5 min and then dehydrated, mounted and examined under the microscope (24).

Statistical analysis

All data were statistically analyzed using one-way analysis of variance (ANOVA), the Tukey-HSD test was used to find the significant difference between groups at $P < 0.05$. The results were analyzed as mean $\pm$ SEM (standard error of the mean). These analyses were carried out using Statistical Package for Social Sciences (SPSS) version 16.

Results

Treatment of EHNA and QU reduced the high level of AFP and liver function tests induced by TAA

The AFP level is the most common predictor for HCC. As compared to control rats the level of AFP was significantly elevated in TAA-treated group. Groups of rats co-treated with QU, EHNA and their combination showed improvement in the AFP level in comparison to TAA-treated group, it also showed that no significant difference between the treatment with QU, EHNA and control group (Fig. 1A). As compared to control rats the activity of ALT was markedly elevated in HCC-induced group. Groups of rats co-treated with QU, EHNA and their combination showed improvement in the activity of ALT in comparison to TAA-treated group but still higher than control (Fig. 1B). As compared to control rats the activity of GGT and the level of TB were markedly elevated in HCC-induced group. Groups of rats co-treated with QU, EHNA and their combination showed improvement in the activity of GGT and the level of TB in comparison to TAA-treated group but still higher than control but it also showed that treatment with EHNA gave a better result than treatment with QU and their combination (Fig. 1C and D).

Table 1. Plasma level of tumor marker AFP and liver functions (ALT, GGT and TB) of control and different treated groups.

G\P	Control	TAA	TAA +QU	TAA+ EHNA	TAA+QU+EHNA
AFP (ng/ml)	0.25±0.10[a]	3.18±0.27[c]	0.38±0.02[a]	0.42±0.11[a]	1.45±0.07[b]
ALT (U/L)	37.5±3.24[a]	191.43±10.43[c]	115.71±10.00[b]	143.71±9.50[b]	117.14±6.88[b]
GGT (U/L)	2.00±0.18[a]	7.33±0.43[d]	4.83±0.46[bc]	3.50±0.33[ab]	5.67±0.70[cd]
TB (mg/dl)	0.12±0.01[a]	0.26±0.01[d]	0.19±0.003[c]	0.14±0.01[ab]	0.16±0.01[b]

Results presented as mean $\pm$ SEM, the different letters (a, b, c and d) indicate significant difference at $P < 0.05$ between different groups, where n=6.
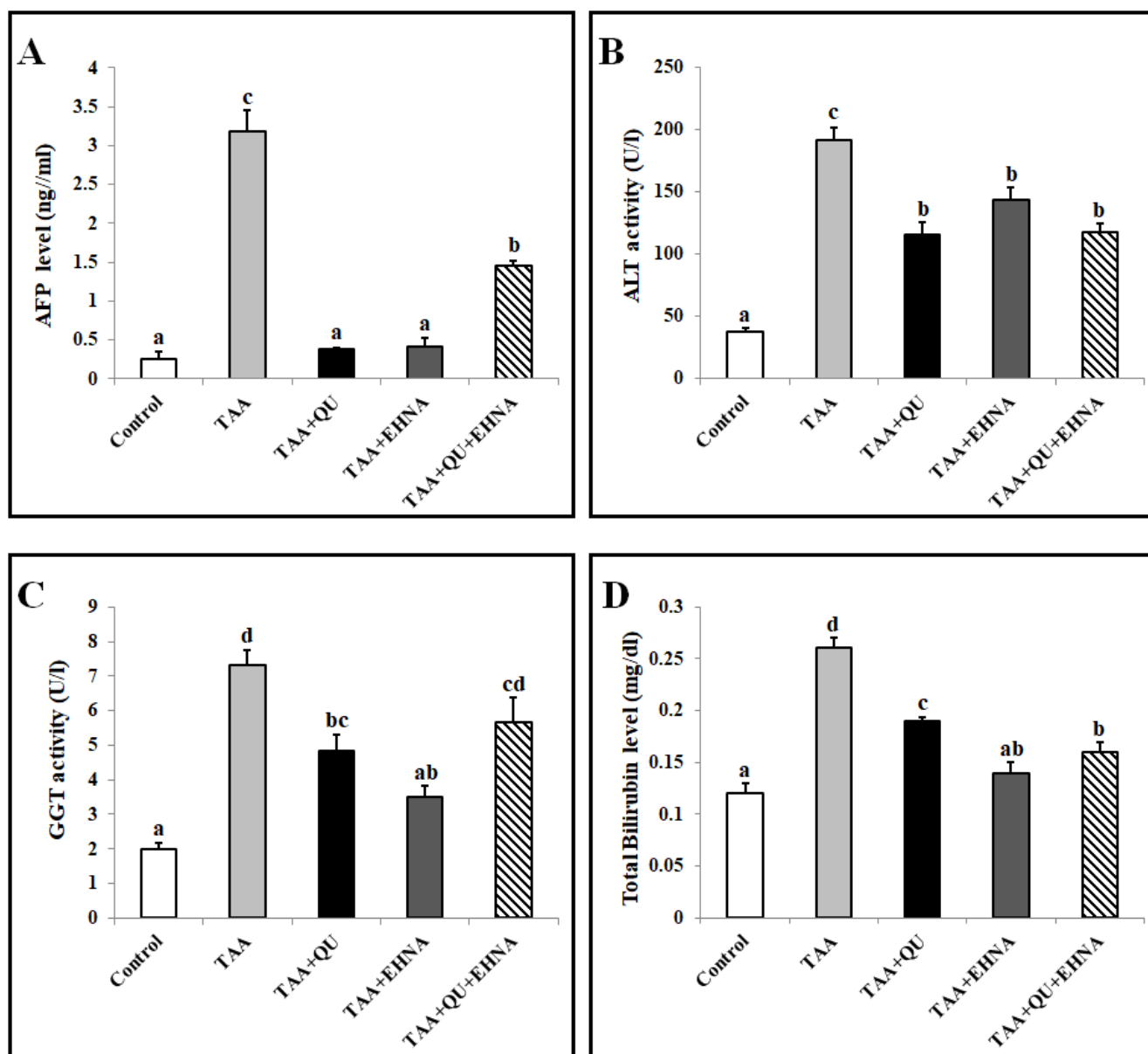
Figure. 1. Level of AFP (A), activities of ALT and GGT (B and C) and the level of TB (D) in plasma of control and different treated groups. Results presented as mean ± SEM, the different letters (a, b, c and d) indicate significant difference at p < 0.05 between different groups, where n=6.

Treatment with EHNA or QU recovered the histopathological alterations in hepatic tissues induced by TAA

Liver of control rats showed normal lobular architecture with distinct hepatic cells, sinusoidal space and a central vein (Fig. 2A, arrow). Liver of TAA-treated group showed hepatocytes necrosis (Fig. 2B), loss of normal lobular architecture with the presence of regenerating nodules separated by fibrous septa (Fig. 2C, arrow), sinusoidal dilatation and adenoma (Fig. 2D, arrow), progressive increase in the number of cells having large nuclei and open chromatin pattern with eosinophilic staining of their cytoplasm were observed (clear cells) (Fig. 2E), giant hepatocytes with enlargement and darkening of nuclei with clumping of chromatin

(Fig. 2F, arrow), the proliferation of bile duct and increased mitotic figures in portal areas (Fig. 2G, arrow). Liver of rats co-treated with QU, EHNA and their combination (Fig. 2H-J) showed marked recovery in hepatic morphology as the large fibrous septa or collagen deposition was reduced, the proliferation of bile duct, clear cells, pyknotic nuclei and lymphocytes infiltration were decreased compared with TAA-treated group.
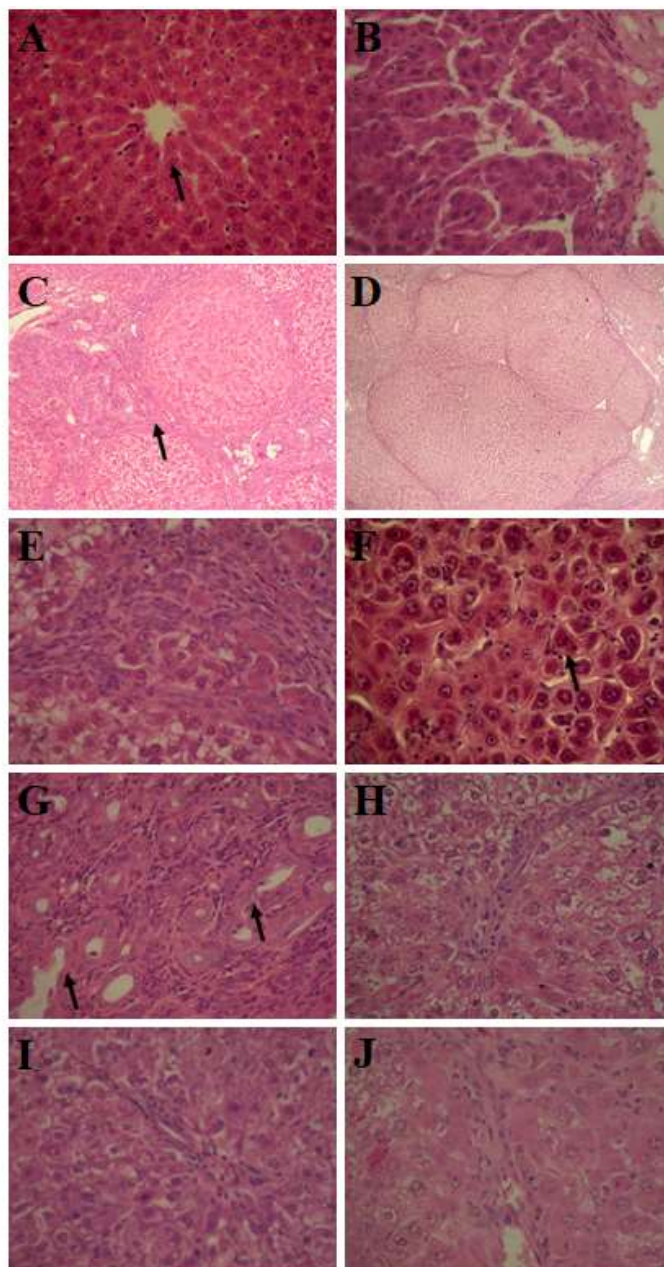

Figure 2. Hepatic tissues stained with H&E images (X200), control group (A), TAA-treated group (B-G), TAA+QU group (H), TAA+EHNA group (I) and TAA+QU+EHNA group (J).

EHNA and QU administration decreased liver cirrhosis induced by TAA

Liver of the control group showed normal hepatic structure and no collagen deposition (Fig. 3a). In rats treated with TAA showed extended collagen deposition and large septa forming hepatic pseudo lobules compared with the control group (Fig. 3b, arrows). Liver of rats co-treated with QU, EHNA and their combination showed marked reduction in collagen deposition and decrease in hepatic fibrosis compared with TAA-treated group (Fig. 3c-e, arrow).
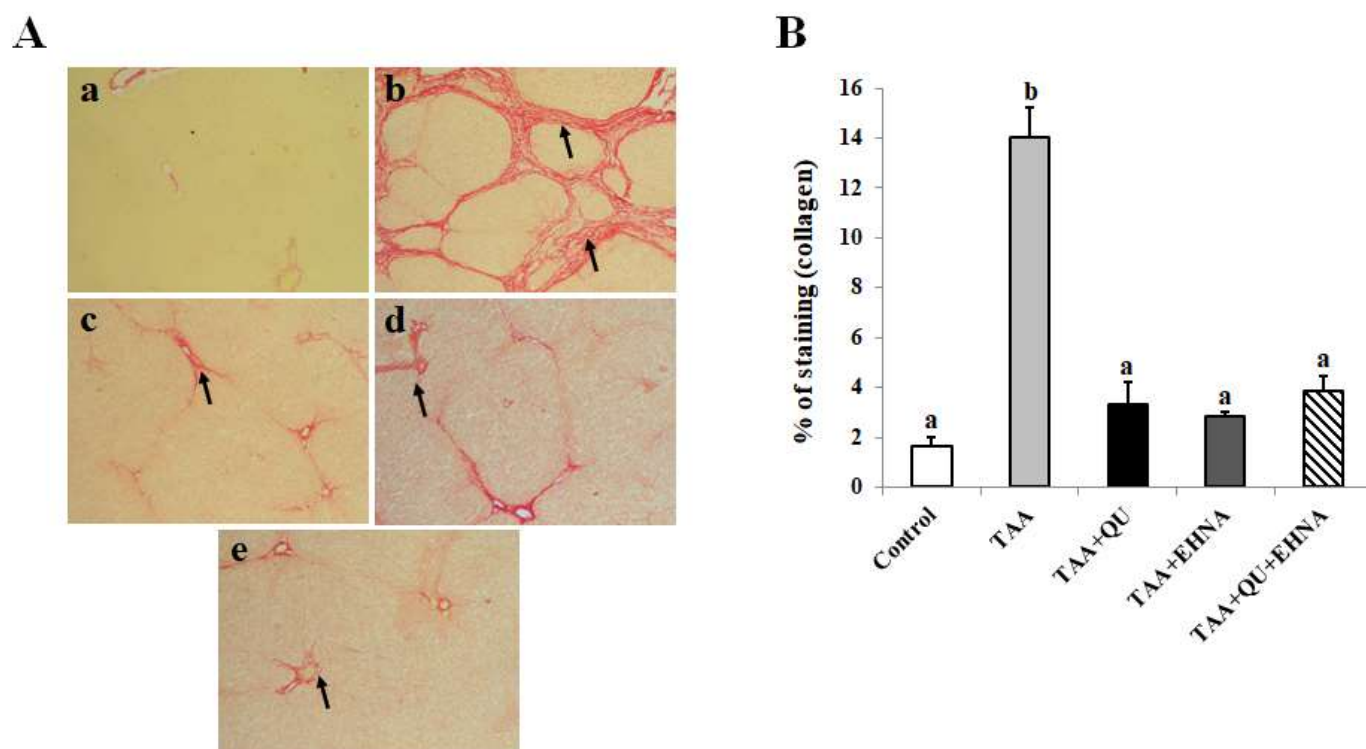

Figure. 3. (A) Collagen deposition in liver fibrosis induced by TAA in rats stained with Picro-Sirius Red (X100). Control group (a), TAA-treated group (b), TAA+QU group (c), TAA+EHNA group (d) and TAA+QU+EHNA group (e). (B) Quantitative analysis of Picro Sirius Red staining as mean ± SEM in liver tissue of control and different treated groups, the different letters (a and b) indicate significant difference at $p < 0.05$ between different groups, where n =6.

EHNA and QU reduced the expression of iNOS

The expression response of iNOS in liver of all groups was analyzed using immunohistochemistry. In the control group the expression area of iNOS was very low (Fig. 4A), in the TAA-treated group there was elevation of iNOS protein expression compared with the control group (Fig. 4B). On the other hand, co-treatment with QU, EHNA and their combination decreased the expression of iNOS compared with TAA-treated group but still higher than control but it also showed that treatment with EHNA gave a better result than the treatment with QU and their combination (Fig. 4C-E).
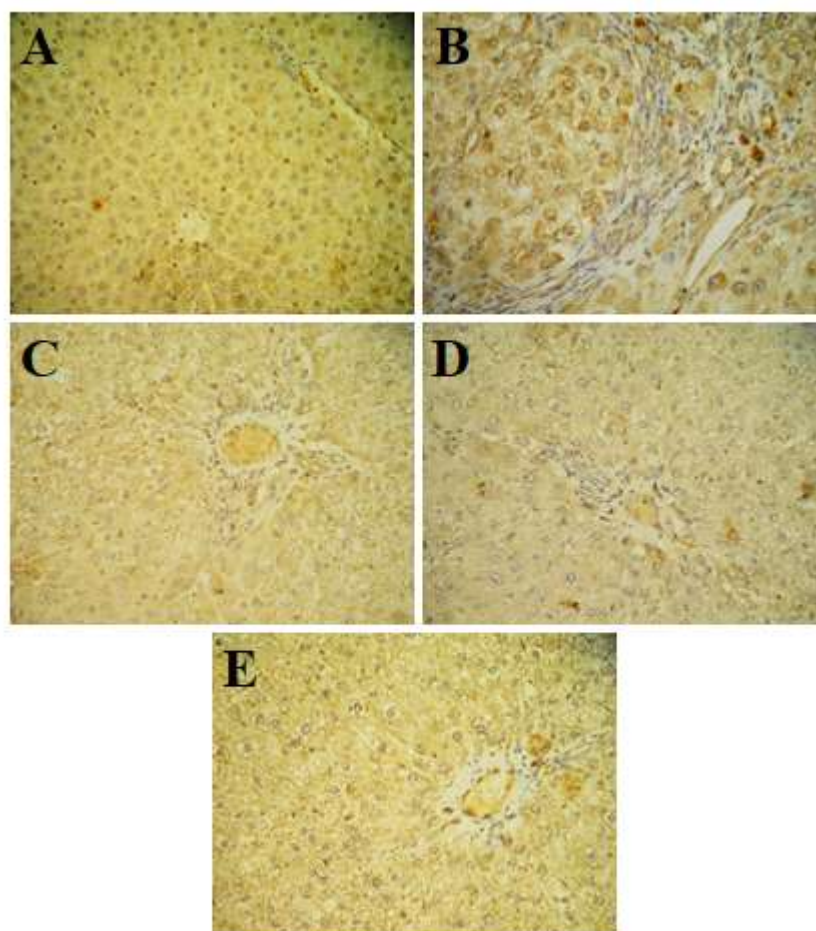
Figure 4. Immunohistochemical staining showed the expression of iNOS labeling (X200) in normal rat tissue (A) TAA-treated group (B), TAA+QU group (C), TAA+EHNA group (D) and TAA+QU+EHNA group (E).

EHNA and QU reduced the proliferation in hepatic tissue

Immunohistochemistry with anti-Ki67 antibody was used to assess cell division in the liver of all groups. Ki67 immunoreactivity was localized in the nucleolus and nucleus mainly in the nuclear membrane. In the control group the expression area of Ki67 was very low in the nucleus of hepatocytes and cholangiocytes (Fig. 5a, arrow), in the TAA-treated group there was a massive increase in Ki67 labeled cells compared with the control group (Fig. 5b, arrows). These dividing cells are mainly found in the nucleus of hyperplastic cholangiocytes (Fig. 5b, upper arrow) and in the nucleus of hepatocytes (Fig. 5b, lower arrow). On the other hand, co-treatment with QU, EHNA and their combination caused a significant reduction in the expression area of Ki67 in the nucleus of hepatocytes and cholangiocytes compared with TAA-treated group but still higher than control (Fig. 5c-e) but it also showed that treatment with EHNA gave a better result than treatment with QU and their combination.
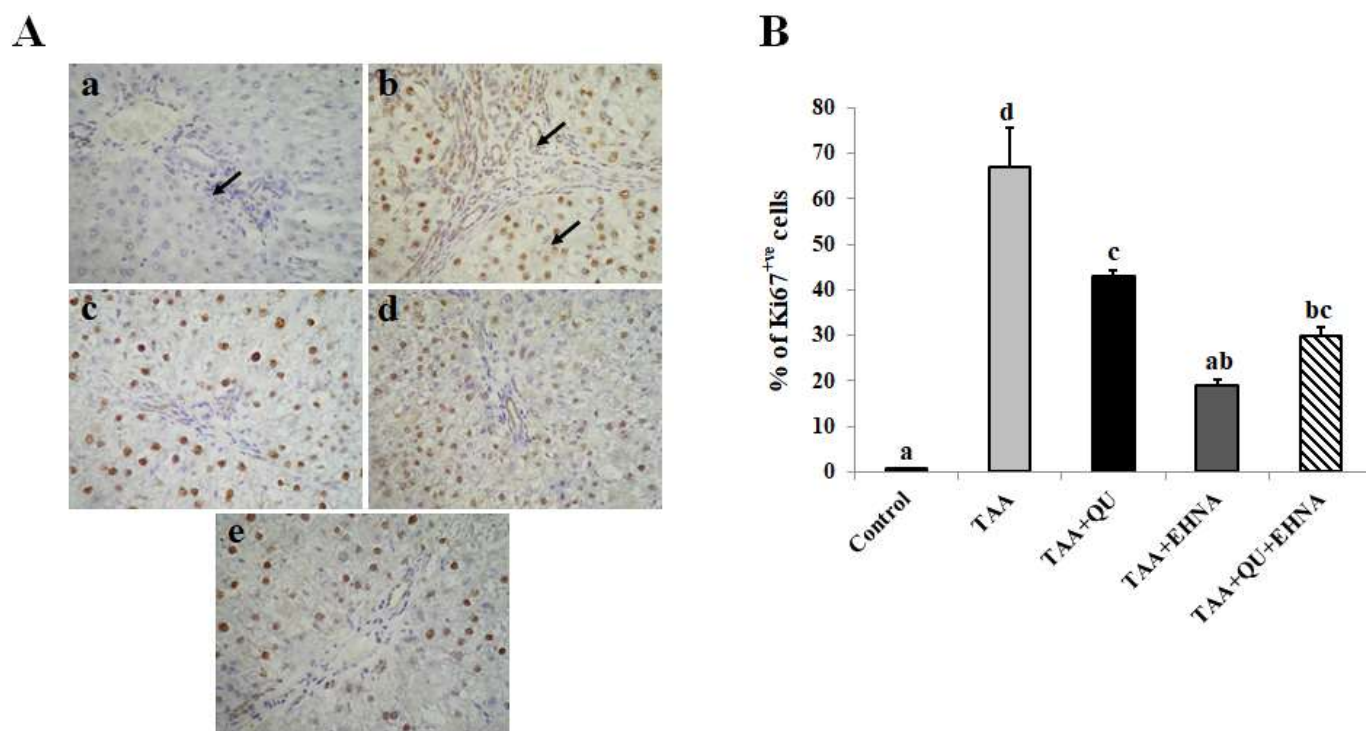
Figure 5. (A) Immunohistochemical analysis of Ki67 showed the level of Ki67 (X200) labeling in the control group (a) TAA-treated group (b), TAA+QU group (c), TAA+EHNA group (d) and TAA+QU+EHNA group (e). (B) A graph demonstrates the quantifications of the percent of Ki67 labeled areas as mean ± SEM in liver tissue of control and different treated groups, the different letters (a, b, c and d) indicate significant difference at P < 0.05 between different groups, where n =6.

EHNA and QU decreased the expression of Pan-CK, hepatocellular carcinoma and cholangiocarcinoma marker

Immunohistochemistry with anti-Pan-CK antibody was used to investigate HCC and cholangiocarcinoma (CCA) in the liver of all groups. In the control group, no Pan-CK-labeled in hepatocytes and the epithelium of bile ducts (Fig. 6a and b, arrow), in the TAA-treated group there was a massive increase of Pan-CK-labeled in hepatocytes (Fig. 6c, arrow) and in the epithelium of dysplastic bile ducts (Fig. 6d, arrow), in TAA+QU group the Pan-CK-labeled in hepatocytes and in the epithelium of bile ducts were reduced (Fig. 6e and f, arrows), in TAA+EHNA group there were only a few Pan-CK-labeled in hepatocytes and in the epithelium of bile ducts (Fig. 6g and h, arrow), in TAA+QU+EHNA group there were also very few Pan-CK-labeled in hepatocytes and in the epithelium of bile ducts (Fig. 6i and j, arrow), but it also showed that treatment with EHNA and their combination gave a better result than treatment with QU.
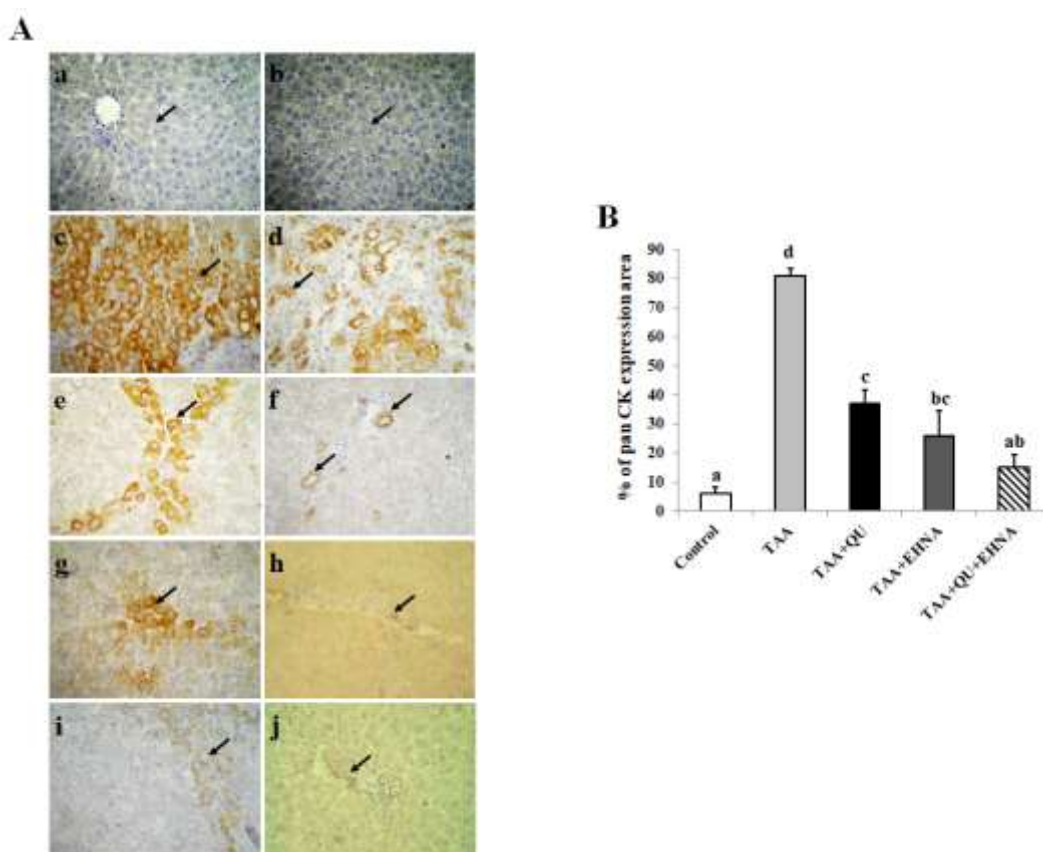
Figure 6. (A) Immunohistochemical analysis of Pan-CK (X200) labeling in control group (a and b), TAA-treated group (c and d), TAA+QU group (e and f), TAA+EHNA group (g and h) and TAA+QU+EHNA group (i and j). (B) A graph demonstrates the quantifications of the percent of Pan-CK labeled areas as mean ± SEM in liver tissue of control and different treated groups, the different letters (a, b, c and d) indicate significant difference at p < 0.05 between different groups, where n=6.

Discussion

In the present study, we found that adenonsine deaminas inhibitor and quercetin protect the liver from cirrhosis and the initiation of hepatocellular carcinoma caused by thioacetamide toxicity. The liver is more prone to toxic injuries than other organs, TAA was listed in the (Third Annual Report on Carcinogens) and an additional study has been identified (25), which found that TAA induced damage, DNA repair and uncontrol apoptosis in the liver, and also caused hepatic cell proliferation, neovascularization, activation of oncogenes, and inactivation of tumor suppressor genes are all involved in liver cirrhosis and the formation of HCC and CCA (26). AFP is a major mammalian embryo-specific and tumor-associated protein that is also produced during the embryonic period by the visceral endoderm of the gestational sac and, later by the liver (27, 28). AFP level measurement may be useful as a sensitive marker system for the early detection of recurring HCC, even before the clinical symptoms are evident (29). The current results showed that the AFP level was significantly elevated ($P = 7.95 \times 10^{-13}$) in TAA-treated group that is may be due to hepatic intoxication that caused alterations in the hepatocytes and increased transcription of AFP gene or posttranslational modification affecting AFP production (30). Also, our results

showed that the administration of TAA significantly elevated the activities of ALT ($P = 8.6 \times 10^{-7}$) and GGT ($P = 1.89 \times 10^{-8}$), and the level of TB ($P = 8.8 \times 10^{-13}$). This elevation due to marked hepatic membrane damage (31) or may be due to the possible effect of tumor initiation on hepatic tissues (32). Moreover, GGT enzyme is used as a tumor marker (33), it is detected in altered hepatic foci induced by carcinogens cause initial lesions until the tumor formation in most animal models (34). Elevated GGT activity was confirmed by the increases of plasma TB level due to blockage of bile ducts (35). Co-treatment with QU and EHNA decreased AFP, ALT, GGT activities and TB level due to their ability to inhibit the transcription of AFP gene (36), also due to they have antitumor and hepatotoxic preventing effects that preserved cell membrane stabilization (37). But their combination didn't give a better result due to QU may work as adenosine antagonist which has a high *in vitro* and *in vivo* affinity for the A_1 and A_{2A} adenosine receptors (38).

In TAA group the histopathological features and the Sirius Red staining showed severe liver cell damage, liver cirrhosis and preneoplastic lesions in rats supporting the observed changes in plasma analysis and these results are in agreement with (39). These features may be due to TAA has carcinogenic properties through different mechanisms: i) releasing of reactive oxygen species (ROS) which damages DNA, protein, and lipid molecules, ii) inducing mutations in proto-oncogenes leading to cell division, iii) inducing the proliferation of hepatocytes and hyperplasia of bile duct epithelium leading to the formation of microscopic precancerous lesions (40), also multiple injections of TAA were found to induce preneoplastic changes in rats in two stages: initially by enhancing apoptosis and then precancerous lesions are formed (41). After treatment with QU, EHNA and their combination, the recovery of histological features may be due to the potential chemopreventive of QU and EHNA (42). Also, EHNA may increase the adenosine concentration which has anti-inflammatory and antitumor activities by binding to its receptors (A1, A2A, A2B and A3) (13). In addition, QU may cause apoptosis in cancer cells by induction of stress proteins, disruption of mitochondrial, the release of cytochrome c, and activation of caspases (43), and also by cell cycle arrest in different cell types (17).

Nitric oxide is a potent biological mediator that influences physiological processes in every organ and tissue and it is a free radical produced by hepatocytes after exposure to cytokines (44). It is rapidly oxidized in blood and tissues to form nitrate and nitrite (45). Our results showed an elevation in the hepatic iNOS protein expression in TAA-treated rats due to the inflammatory reaction (46, 47). These results are in line with (39, 48). Administration of QU or EHNA decreased the elevation of iNOS protein expression in hepatic tissue because they have powerful anti-inflammatory effects by inhibiting NOS gene expression (47). EHNA elevates endogenous adenosine concentrations which could provide the binding to (A2A) adenosine receptor results in anti-inflammatory effects, including the decreased neutrophil release of (ROS) (49). But their combination didn't give a better result due to adenosine antagonistic effect of QU that has a high in vitro and in vivo affinity for the A1 and A2A adenosine receptors (38).

Ki67 proteins were expressed during cell proliferation, it was highest at the G2/M phase of the cell cycle except for the G0 phase (50). The present study showed a significant elevation of Ki67 expression ($P = 3 \times 10^{-6}$) in the nucleus of liver cells and cholangiocytes of rats treated with TAA due to the higher mitotic activity (51), and it also might be that is an early event in the pathogenesis of hepatic neoplasia which is one of the most common features of liver cirrhosis supporting the observed changes in histopathological studies (52). (53) indicated

that the number of Ki67 positive cells was increased significantly due to paralleled c-met proto-oncogene overexpression. Treatment with QU or EHNA significantly reduced (P = 0.011 and P = 4.7 X 10-5, respectively) the number of Ki67 positive cells compare with TAA group. This may be because QU can inhibit the cell proliferation and the growth of different cancer cell lines by causing the arrest of cell cycle phases such as G2/M or G1 (54), and EHNA cause apoptosis of cancer cells by increasing the adenosine concentration which increases the inhibition of cell proliferation, invasion and metastasis of the tumor by interacting with a variety of cell signaling proteins (55). But their combination didn't give a better result due to QU is adenosine antagonist which has ahigh *in vitro* and *in vivo* affinity for the A_1 and A_{2A} adenosine receptors (38).

Pan-CK is the epithelial-specific tumor marker in liver cells and in the epithelium of bile ducts which is used for the identification of circulating tumor cells (56, 57). Moreover, the tumor cells can undergo the epithelial to mesenchymal transition, which can result in downregulation of epithelial cell-specific molecules including cytokeratins (25), and this transition may grant tumor cells to stem cell properties enabling self-renewal (58). Our study showed distinct staining for Pan-CK in liver cells and in the epithelium of bile ducts of rats treated with TAA due to DNA damage caused genetic changes and up-regulation of the proto and oncogenes leading to uncontrolled cell division supporting the observed changes in Ki67 immunohistochemical analysis (41). (59) reported that TAA could induce liver `severe' bile duct proliferation and eventually CCA. The present results showed that the co-treatment with QU, EHNA and their combination significantly decreased the expression of Pan-CK (P = 7 X 10^{-5} and P = 7.12 X 10^{-7}, respectively) due to QU has antitumor effects which inhibit the progression of hepatic carcinogenesis (60). Moreover, EHNA increases adenosine concentration by binding to A_{3A} receptor-induced cell cycle arrest in the G0/G1 phase (13).

Conclusion

From this study it was found that treatment with EHNA alone and QU alone produced better results than treatment with both against TAA induced liver cirrhosis, initiation of HCC and CCA through decreased of iNOS, Ki67 and Pan-CK expression. So that our results suggest that EHNA or QU can be recommended to treat liver damage.

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