Therapeutic Role of Quercetin against Experimentally Induced Hepatocellular Carcinoma in Female Albino Rats and their offspring

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ABSTRACT

Aim: To evaluate the potential role of quercetin against N-methyl nitrosourea (MNU)-induced hepatocellular carcinoma (HCC) in pregnant rats and their offspring. **Material & Methods:** Twenty-four female rats were used in this study. Six rats were preserved without treatment and the other eighteen female rats were induced by a single dose of MNU (50 mg /kg B wt). After confirmation of the positive tumor marker test, female rats were placed with the males for mating. The pregnant rats were divided into four groups (n=6). Group1: control rats, group2: Quercetin supplemented rats (20 mg/kg B.Wt, group3: MNU-induced rats, and group4: MNU-treated rats followed by supplementation with quercetin. At the end of the weaning period, the mothers and their offspring (at 21 days old postnatal) of all groups were sacrificed, the liver was removed immediately for histological and immunohistochemical investigations. Also, blood samples were collected, centrifuged, and processed for the estimation of antioxidants. **Results**: In the control and quercetin groups, the histological investigation of the liver of mother rats and their offspring appeared with normal architecture. In Group3 (MNU-induced group) the liver sections of mother's rats revealed degenerated hepatic lobules with pronounced cellular hyperplasia (HCC) especially around the central vein and portal area as well as numerous Kupffer cells and fat droplets. However, the liver sections of offspring displayed little cellular hyperplasia but the central and portal veins appeared damaged and congested with blood. Immunohistochemically, the liver sections of MNU-induced mother's rats and their offspring appeared strongly stained with *α-FP* antibody and negatively stained with caspase-3 antibody. Furthermore, the levels of serum antioxidants; SOD, CAT&GSH were significantly decreased however the levels of MDA and NO were significantly increased in MNU-induced mother rats and their offspring if compared with control. In group 4, quercetin was able to restore the histological and immunohistochemical changes in the liver induced by MNU. Also, the levels of antioxidants, as well as MDA and NO, were markedly restored near to the normal level as in control. **Conclusion:** Quercetin has a powerful therapeutic role against MNU-induced HCC in pregnant rats and their offspring.

Keywords: HCC, Quercetin, Antioxidants, *α1FP*, Caspase-3.

INTRODUCTION

Cancer is one of the most commonly diagnosed diseases, and it's associated with morbidity and mortality. It is a major health problem worldwide (**Siegel et al., 2017**). It is categorized as the second major cause of death after cardiovascular diseases (**Jain et al., 2016).** Cancer is expected to be the leading cause of death and the single most important barrier to increased life expectancy in the world (**Bray et al., 2018**). Globally, liver cancer remains a health challenge and its range is growing all over the world (**Vilanuevla, 2019).** It is reported that, by 2025, >1 million individuals will be affected by liver cancer annually. Hepatocellular carcinoma (HCC) is the most common type of liver cancer and represents ~90% of cases(**Llovet et al.,2021**).

Hepatocellular carcinoma (HCC) is a worldwide neoplasm for which early diagnosis is difficult and the prognosis is usually poor (**Mohamed et al., 2014). Dimitrios et al. (2017)** reported that HCC is the most frequent primary liver malignancy and the third cause of cancer-related death in Western Countries. The well-established causes of HCC are chronic liver infections such as hepatitis B virus or chronic hepatitis C virus, nonalcoholic fatty liver disease, consumption of aflatoxins, heavy alcohol abuse, and tobacco smoking (**Naghavi et al., 2015; Ružić et al., 2018; Goto et al., 2020).** Interleukin-1b (IL-1b) is a central component of the cytokine milieu that accompanies both acute and chronic inflammation and viral disease (**Allen, 2009).** Moreover, hepatic chronic damage often leads to progression to liver cirrhosis and, in most cases, to HCC (**Kawabata et al., 2015; Bae et al., 2018**).

HCC remains a challenge to early diagnose, and treat effectively; treating management is focused on hepatic resection, orthotopic liver transplantation, ablative therapies, chemoembolization, and systemic therapies with cytotoxic drugs, and targeted agents (**Siegel et al., 2016; Tarini et al., 2018).** These methods utilized in mainstream medicine are almost associated with significant unforeseen effects which pose a challenge in its management. It was thought that chemotherapy drugs specifically kill the cancer cells only but progressive studies revealed that they may also damage the normal cells resulting in the chemotherapy dose-dependent side effects (**Lee& Longo., 2011**). Chemotherapy can cause various side effects including asthenia (**Schmitz et al., 2010**), alopecia (**Lundstedt et al., 2012),** nausea, vomiting, diarrhea (**Baena-Canada et al., 2011**), leucopenia, and neutropenia as well as infection, and developing neurological toxicity (**Schmitz et al., 2010**; **Urquhart, 2011).** In addition, pain, heart problems, pulmonary problems, cognitive dysfunction with difficulties in memory, concentration, and language (**Schmitz et al., 2010**), anxiety, and finally depression that adversely affect the performance status of patients (**Baena-Canada et al., 2011; Urquhart, 2011**).

It has been reported that some natural products can inhibit tumor cell growth and metastasis and induce apoptosis suggesting a growing application of these natural compounds as an alternative medicine for the treatment of human tumors (**Wen et al., 2011**; **Mishra et al., 2013**).).

Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the six subclasses of flavonoid compounds **(Kelly, 2011).** It is widely distributed throughout the plant kingdom and is one of the most important antioxidants of plant origin (**Fernández-Palanca et al., 2019).** Quercetin is found in plant foods, such as leafy greens, tomatoes, berries, broccoli, onions, and apples. It plays an important role in fighting free radical damage, the effects of aging, and inflammation (**Khan et al., 2016**). Quercetin has been recognized and employed as an alternative drug in treating different cancers alone or in combination with other chemotherapeutic drugs **(Kelly, 2011; Rauf et al., 2018).** It had been reported that fatty acid esters of quercetin-3-O-glucoside were found to exhibit significant inhibition of HepG2 cell proliferation. Furthermore, quercetin-3-O-glucoside esters showed significantly low toxicity to normal liver

cells than sorafenib, a chemotherapy drug used in the treatment of HCC (**Sudan & Rupasinghe, 2015**). **Oršolić & Car (2014)** added that quercetin can protect the blood, liver, and kidney cells of mice against hyperthermic intraperitoneal chemotherapy (HIPEC) - induced injury.

Anti-inflammatory, antioxidant, and anticancer activities are some of the mainly described quercetin mechanisms of action (**Boots et al., 2008; Kawabata et al., 2015**; **Khan et al., 2016).** Besides, the therapeutic potential of this flavonoid

has been evaluated in a broad variety of human disorders, including diabetes, cardiovascular, neurodegenerative (**Oboh et al., 2016**), Alzheimer's diseases (**Babaei et al., 2018),** hypertension, intestinal microbiota, and kidney dysfunction (**Elumalai et al., 2016; Babaei et al., 2018).** Other studies have also proved the beneficial activities of quercetin against liver cirrhosis development and pulmonary associated complications (**Tieppo et al., 2009; Casas et al., 2017)**.

Accordingly, the current work is mainly designed to evaluate the therapeutic role of quercetin against HCC experimentally induced in pregnant rats and consequent complications on the offspring.

MATERIALS and METHODS

1. Chemicals

N-methyl nitrosourea (MNU) ($C_2H_5N_3O_2$) with molecular weight 103.08 gram (Catalog no. N1517-1G) was purchased from Sigma, (St. Louis, MO).

2. Quercetin

Quercetin is a plant flavonoid with chemical formula ($C_{15}H_{10}O_7$). It is obtained from international laboratory USA and orally administered every other day at doses of 20mg/kg body weight dissolved in saline solution from the fourth day of pregnancy till the end of weaning.

3. Experimental Animals

For this study, thirty-two (24 females and 8 males) Wister albino rats (*Rattus norvegicus*) with age (8-10weeks) weighing 180 $\pm$ g were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). The animals were kept in wire-bottomed cages in a room under a standard condition of illumination with a 12-hours light-dark cycle at $25 \pm 1°C$ and 50% relative humidity. They were provided with tap water and a balanced diet of *libitum*.

4. Induction of HCC

After an acclimatization period of two weeks; a total of 12 females were treated with a single dose of MNU intra-peritoneally (50 mg in 6.67 ml phosphate-citrate buffered saline (PCBS)/kg body wt) (**Steinetz, et al., 2006**). After two months of MNU injection, blood samples were collected from the tail vein using capillary tubes. The blood samples were centrifuged at 5000 rpm for 10 min then kept frozen at -80 ^{0}C. Cancer antigen (CA 15.3) was measured in the serum by ELISA technique for confirmation of tumor induction. After confirmation of the positive tumor marker test, all females were placed with the males. After 3-4 days and ensuring of pregnancy via observation of vaginal plug and using vaginal smear method, pregnant females were separated from males and the day 0 of pregnancy (E0) was determined.

5. Experimental groups

The pregnant rats were divided into four groups as follows, six for each group (n=6).

Group 1: (control) they have received a daily oral dose of 1 ml saline solution.

Group 2(Quercetin): Pregnant rats' orally administered with 20mg/kg body weight of quercetin every other day that dissolved in saline solution from the fourth day of pregnancy till the end of weaning (**Vásquez-Garzón et al ., 2013).**

Group 3(HCC): Pregnant rats with HCC, induced with a single dose of MNU intra-peritoneally (50 mg in

6.67 ml phosphate-citrate buffered saline (PCBS)/kg body wt) **(Steinetz, et al., 2006)**

Group 4: Pregnant rats with HCC that treated with 20 mg/kg body weight of quercetin every other day from the 4th day of pregnancy till weaning.

6.   Sample collection and tissue preparation

At the end of the experimental period (21st days postnatal), the mother rats and their offspring (at 21 days old) were sacrificed by decapitation. Blood samples were collected; serum was separated by centrifugation at 860 Xg and kept frozen at ⁻ 20ºC for estimation of physiological parameters. The animals were dissected and the whole liver of mothers and their offspring were removed immediately, washed in normal saline, and cut into two halves. One half was fixed in 10% neutral buffered formalin for histological and immunohistochemical studies and the other half was kept frozen for estimation of biochemical parameters.

7.   Investigated parameters

7.1 Histological technique for hematoxylin and eosin stain.

The formalin-fixed liver was dehydrated with an ascending ethanol series, cleared with xylene, and embedded in paraffin. A 5-6 μm thick sections of liver were obtained, stained with hematoxylin and eosin (Bancroft & Gamble, 2008). The obtained sections were investigated under a bright field light microscope and photographed.

7.2.   *Immunohistochemical labeling of alpha1 fetoprotein (α1FP) and caspase3*

i.Alpha1 fetoprotein (α1FP)

Immunohistochemical staining for anti-alpha1 fetoprotein (Rabbit, EPR9309, Abcam, 1:50) was performed according to manufacturer instructions. Briefly, the liver slides were initially left in an oven at 60°C for 12 h. The sections were then de-paraffinized in xylol and rehydrated in successive alcohol baths. After, epitope retrieval was performed in a citrate buffer at pH 6.0 in a vegetable steamer for 30 minutes.

The endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min and proteins for 10 minutes. Next, overnight incubation with the primary antibody was conducted. After removing the antibody, the complement was placed and the HRP conjugate (Advance HRP Polymer) was applied for 30 minutes. Staining was viewed using 3,3′-diaminobenzidine substrate-chromogen (DAB) solution followed by counterstaining with hematoxylin. The sections were examined and photographed by phase-contrast light microscopy. Incidences of cellular accumulations of *α1FP* were determined for each group.

ii.Caspase3.

Five μm thick paraffin-embedded liver sections from mother's rats and their offspring were cut, mounted onto positively charged slides, de-paraffinized, rehydrated in descending grades of alcohol, and washed in PBS. Endogenous peroxidase activity was inhibited using 3% H_2O_2 in methanol for 40 min at room temp. The tissue sections were retained at normal room temperature and processed for antigen retrieval by digestion in 0.05 % trypsin. After thorough washing in TRIS buffered saline (TBS), pH 7.6, the sections were incubated for 45 min with diluted 1:10 monoclonal primary antibody (Anti-caspase3; clone DO-7 Dako). Slides were then rinsed in PBS and subsequently incubated in the presence of the secondary antibody for 20 min. For all sections, the complex sites were shown brown using 3, 3 diaminobenzidine tetrahydrochloride with fresh hydrogen peroxide substrate (**Rohan, et al, 1998**). The sections were counterstained with Mayer's hematoxylin, mounted, and photographed by phase-contrast light microscopy. Incidences of cellular accumulations of caspase3 protein were determined for each group

7.3 Serum analysis

7.3.1 Measurement of serum antioxidants (catalase, superoxide dismutase &reduced glutathione).

Catalase activity was determined spectrophotometrically by the method of **Koroliuk et**

al. (1988). Briefly, 10 µL of the sample was incubated with 100 µmol/mL of H_2O_2 in 0.05 mmol/L Tris-HCl buffer pH = 7 for 10 min. The reaction was terminated by rapidly adding 50 µL of 4% ammonium molybdate. The yellow complex of ammonium molybdate and H_2O_2 was measured at 410 nm. One unit of catalase activity was defined as the amount of enzyme required to decompose 1 µmol H_2O_2 per min.

The determination of the Superoxide Dismutase (SOD) activity was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. Briefly, 300 µL of the mixed substrate was added to 200 µL of diluted hemolysates. The samples were mixed well and 75µL xanthine oxidase was added to reactions. The absorbance was measured at 505 nm and the SOD activity was then calculated according to the manufacturer's instruction (Ransod®-Randox Lab, Antrim, UK) and expressed as U/mL (**Bahrami et al., 2016**).

Reduced glutathione (GSH) activity was determined using a GSH assay kit (Randox Lab., Ltd., UK) according to the manufacturer's protocol of **Ellman (1959)**. To evaluate GSH levels in samples, 15 µL of hemolysates was mixed with 260 µL assay buffer (0.1 M sodium phosphate and 1 mM EDTA, pH: 8) and 5 µL Ellman reagents. Samples were incubated for 15 min at room temperature and the $TNB2^-$ formation was quantified in a spectrophotometer by measuring the absorbance of visible light at 412 nm. Absorbance values were compared with a standard curve generated from a standard curve from known GSH.

7.3.2 Estimation of serum Malondialdehyde (MDA)

MDA levels in samples were measured using the thiobarbituric acid reaction method of **Placer et al. (1966).** Quantification of the thiobarbituric acid reactive substances was determined at 532 nm by comparing the absorption to the standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of 1, 1, 3, 3-tetra methoxy propane. To measure the MDA level, a working solution containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25N hydrochloric acid was prepared. For each sample, 250 µL serum and 500 µL working solution were mixed and placed in boiling water for 10 min. After cooling the samples were centrifuged at 3000 rpm for 10 min. Finally, 200 µL of each supernatant was transferred to microplates and the optical density of samples was measured at 535 nm. The values of MDA were expressed as (nmol/ml).

7.3.3 Determinations of Nitric oxide serum levels

Nitrite ($NO2^-$) and nitrate ($NO3^-$) are stable final products of NO metabolism and may be used as indirect markers of NO presence. Total NO concentration is commonly determined as a sum of nitrite and nitrate concentrations. NO concentration was determined in serum using an indirect method based on measurement of nitrite concentration in serum according to Griess's reaction (**Jablonska et al., 2007**). In the samples analyzed, nitrate was reduced to nitrite in the presence of cadmium (Sigma-Aldrich, Steinheim, Germany), and then converted to nitric acid that gave a color reaction with Griess's reagent (Sigma-Aldrich, Steinheim, Germany). Nitrite concentrations were determined by spectrophotometric analysis at 540 nm (UVN-340 ASYS Hitech GmbH microplate reader; Biogenet, Eugendorf, Austria) regarding a standard curve. NO products were expressed as (**nmol/ml**)

7.3.4 Determination of serum Interleukin-1β (IL-1β).

IL-1β concentration was measured in serum (stored at −80 °C) by an enzyme-linked immunosorbent assay (ELISA) using commercially available rat-sensitive assay kits (Bender Medsystems, Vienna, Austria, [BMS622MST, BMS625MST, and BMS623MST]). The assay was carried out according to the manufacturer's instructions. The IL-1β assay was performed in duplicate and reported in Pg/mL.

7.3.5 Determination of serum levels of carcinoembryonic antigen (CEA)

Serum carcinoembryonic antigen (CEA) was quantified by ELISA using a kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer's instructions provided with the CEA assay kit.

7.3.6 Flow cytometric detection of Bax in liver tissues.

A 100 µl of cell suspension (1×10^6) cell/ml was prepared by isolation of mononuclear cells from the liver tissues by processing with Tris Edita buffer. The cells were washed with PBS/BSA (Bovine serum albumin) with 2 ml and then centrifuge at 2000 rpm for 5 min. The supernatant was discarded and re-suspend the pellet in 100 µl of PBS. A 7 µl of Bax marker was mixed well then incubated the tube for 30 min at room temperature in dark. The cells were washed with 2 ml PBS/BSA, and centrifuged at 2000 rpm for 5 min, and discarded the supernatant. Finally, cells were re-suspended in 200 µl of 4% par formaldehyde in PBS and then fixed until acquired by flow cytometry.

This analysis was performed in the Mansoura University Hospital using FACS (flow activated cell sorter) Calibur Flow Cytometer (Becton Dickin-son, Sunnyvale, CA, USA) equipped with a compact air-cooled low power 15 mW Argon ion laser beam (488 nm).

8. Statistical Analysis:

Statistical analysis and correlations were performed using SPSS program version 20. Data are presented as Mean ± standard error (SE). Analysis of variance (ANOVA) followed by Tukey analysis was used for comparisons between groups. The level of statistical significance was set at probability $P < 0.05$.

RESULTS

1. **Histological observations**

In control and quercetin supplemented mother's rats **(Figure1 A-D)** and their offspring **(Figure2 A-D)**, the liver sections displayed normal histological structure. The hepatic lobules appeared regular and separated from each other by connective tissues. Each hepatic lobule consisted of hepatic strands radiating from a central vein and separated by well-organized blood sinusoids. The hepatic strands consist of polyhedral hepatocytes with rounded centrally located nuclei. The blood sinusoids are lined with intact endothelial cells. The hepatic lobules are interconnected by the portal area which is represented by a branch of the hepatic vein, a branch of the hepatic artery, and bile ductule.

In comparison to control mothers rats, the liver sections of MNU-induced carcinogenesis in mother's rats displayed severe deleterious histological alterations in their architectures. Such alterations included pronounced cellular hyperplasia (HCC) especially in the central veins and portal area but appeared little in the hepatic lobules. Also, the hepatic lobules appeared degenerated with scattered pyknotic cells, lipid droplets, and Kupffer cells (Figure1 E-H).

In MNU induced mothers rats post-supplemented with quercetin, the liver sections showed evident amelioration in the structure of hepatic lobules, central vein, and portal area. On the other mean, quercetin successfully restored the histological changes induced by MNU that become appeared more or less similar to the control liver (Figure1 I&J).

The liver sections from 21 days old offspring maternally induced with MNU displayed mild deleterious histological changes if compared with their mothers. These changes included damaged and dilated central, congested portal vein, dilated blood sinusoids, pyknotic hepatocytes, and scattered Kupffer cells. Moreover, the liver sections displayed little hyperplasia (HCC) that restricted only around the portal area (Figure2 E&F). On the other hand, the liver sections of offspring maternally induced with MNU and treated with quercetin displayed remarkable amelioration in their histological architecture (Figure2 G&H).
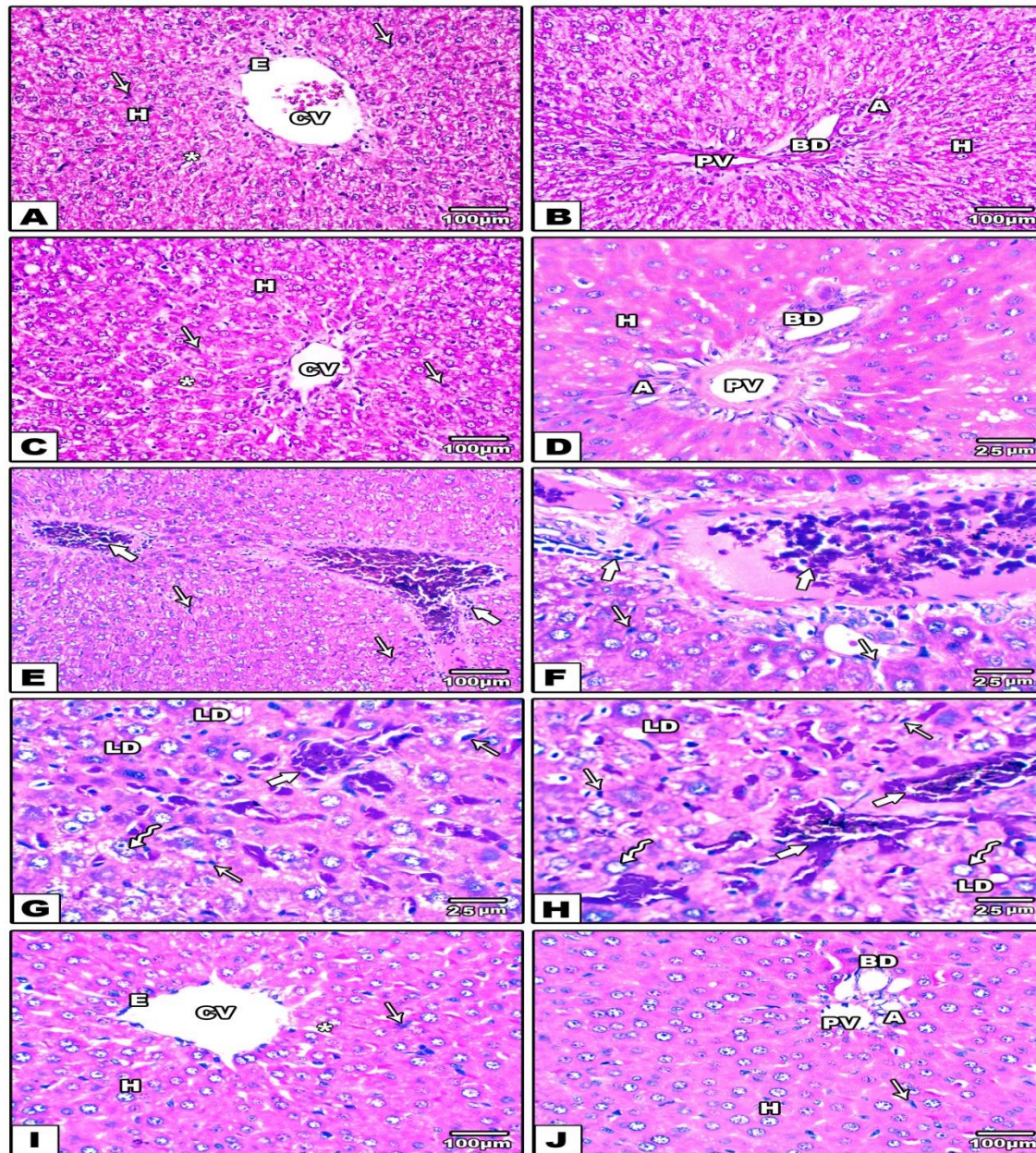
Figure1: Photomicrograph of histological sections through the liver of mother rats among the different studied groups. A & B: Control; C & D: Quercetin; E-G: MNU and I & J: MNU& Quercetin. In images A-D**,** the liver sections appear with the normal histological architecture with complete differentiation of hepatic lobules, blood sinusoids (*), central vein, and portal area (portal vein, hepatic arteriole, and bile ductile). In images E-H, the liver sections display pronounced cellular hyperplasia (HCC) (thick arrows), multiple Kupffer cells (thin arrows), pyknotic hepatocytes (zigzag arrows), and scattered lipid droplets. In images I&J, the liver sections appear ameliorated to be more or less similar to control sections with the exception in figures C&D still showing multiple Kupffer cells (fine arrow).

Abbreviations: Central vein (CV), Portal vein (PV), Hepatocyte (H), Lipid droplets (LD), Arteriole (A), Bile ductule (BD), and Endothelium (E).
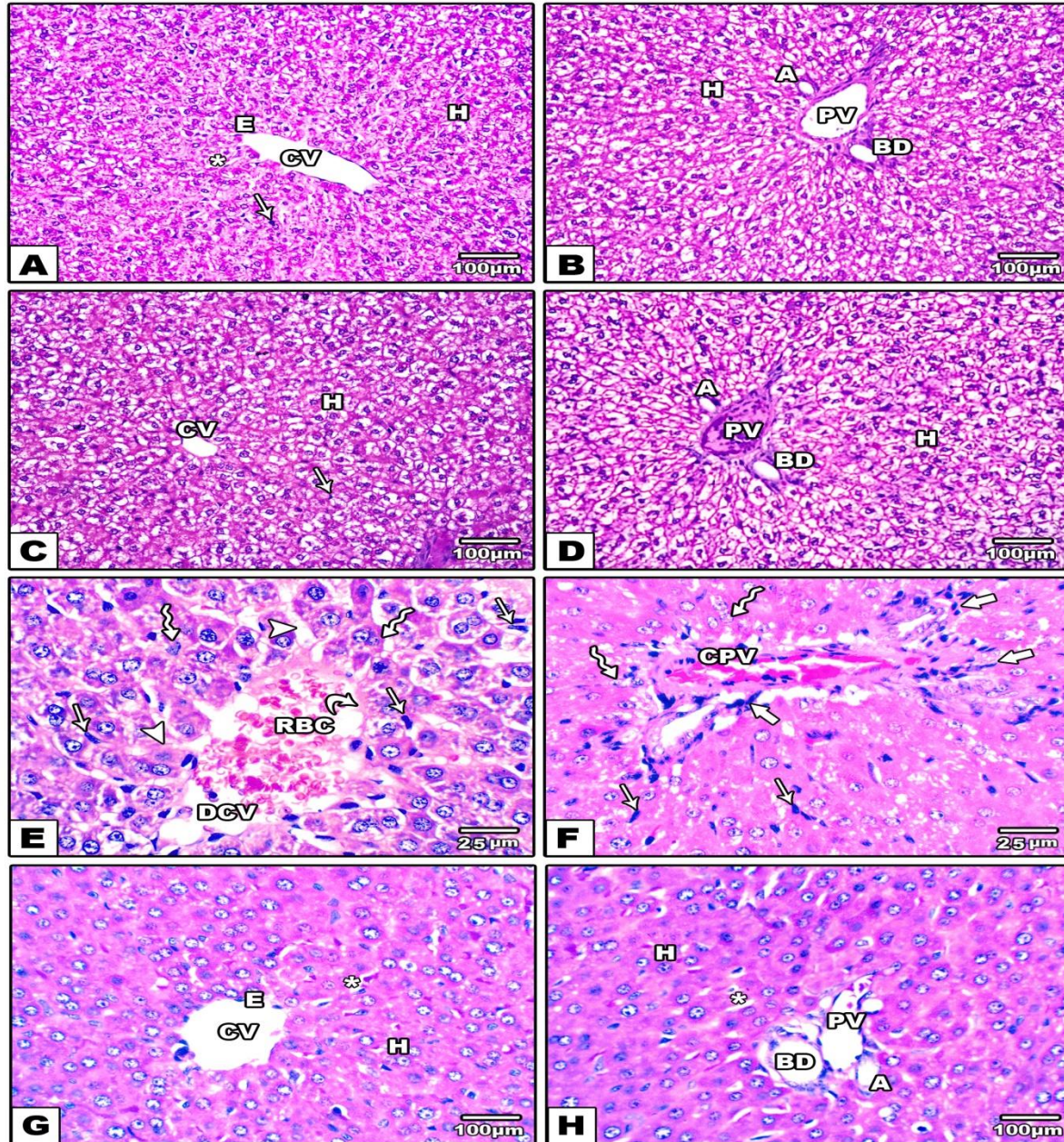
Figure 2: Photomicrograph of histological sections through the liver of 21 days old offspring among the different studied groups. A & B: Control; C &D: Quercetin; E & F: MNU and G&H: MNU& Quercetin .Note: In images A-D, the liver sections appear with the normal histological architecture with complete differentiation of hepatic lobules, blood sinusoids (*), central vein, and portal area (portal vein, hepatic arteriole, and bile ductile). In images E&F, the liver sections show degenerated dilated central vein with damaged endothelial lining (curved arrow), dilated blood sinusoid (arrowheads) and multiple kupffer cells (thin arrow), pyknotic hepatocytes (zigzag arrows). Also, the central vein appears congested with little HCC (thick arrows) around the portal area. Images G&H: display remarkable amelioration in the histological architecture of liver sections.
Abbreviations: Central vein (CV), Portal vein (PV), Hepatocyte (H), Arteriole (A), Bile ductule (BD), Endothelium (E), Red blood cell (RBC), Dilated central vein (DCV), Congested portal vein (CPV).

2. Immunohistochemical observations *of α1FP and caspase3 in liver tissues*

The liver sections from either control or quercetin-treated mother' rats (Figure 3 A&B) and their offspring (Figure 3 A1&B1) respectively displayed negative reactions for anti-α1FP antibodies. In contrast, a strong positive immune expression for α1FP was recorded in the liver tissues of MNU-induced carcinogenesis in mother rats (Figure 3C) and their offspring (Figure 3C1). This expression was more confined to the cytoplasm of hepatocytes. In MNU-induced mother rats post-supplemented with quercetin the liver section displayed a very weak immune reaction for α1FP that restricted only to the hepatocytes around the central and portal vein (Figure3 D) while their offspring revealed negative reaction except for a few cells around the central vein and portal area displayed very weak immune-reaction (Figure3D1).

The liver sections from either control or quercetin supplemented mother' rats (Figure4 A&B) and their offspring (Figure A1&B1) showed weak to moderate immune reactions for anti-caspase3 antibodies. In comparison with control, the hepatocytes appeared negatively stained with anti-caspase 3 antibody in MNU-induced HCC in mothers rats (Figure4C) and their offspring (Figure4 C1). On the other hand, a very weak immune expression for anti-caspase3 antibody was recorded in the hepatic cells of MNU-induced in mother rats post-treated with quercetin and their offspring (Figure4D&D1).

3. Serum analysis

3.1 changes in Serum antioxidants SOD, CAT, and GSH

i. In mothers

In quercetin supplemented mother rats, the mean level of SOD appeared significantly higher than control however in MNU-treated mother rats this mean appeared significantly lowered (P< 0.001) than control. On the other side, the mean level of SOD appeared significantly higher in MNU treated rats post supplemented with quercetin if compared with MNU induced rats (table 1&figure5A).

In quercetin supplemented mothers rats, the mean level of CAT appeared with non-significant change (P >0.05) with control. In comparison with control, the mean level of CAT in MNU-induced mother rats appeared significantly decreased (P< 0.001). On the treatment of MNU treated mothers rats with quercetin the mean level of CAT appeared significantly elevated if compared with MNU induced rats but this level still significantly decreased if compared with control (table 1&figure5B).
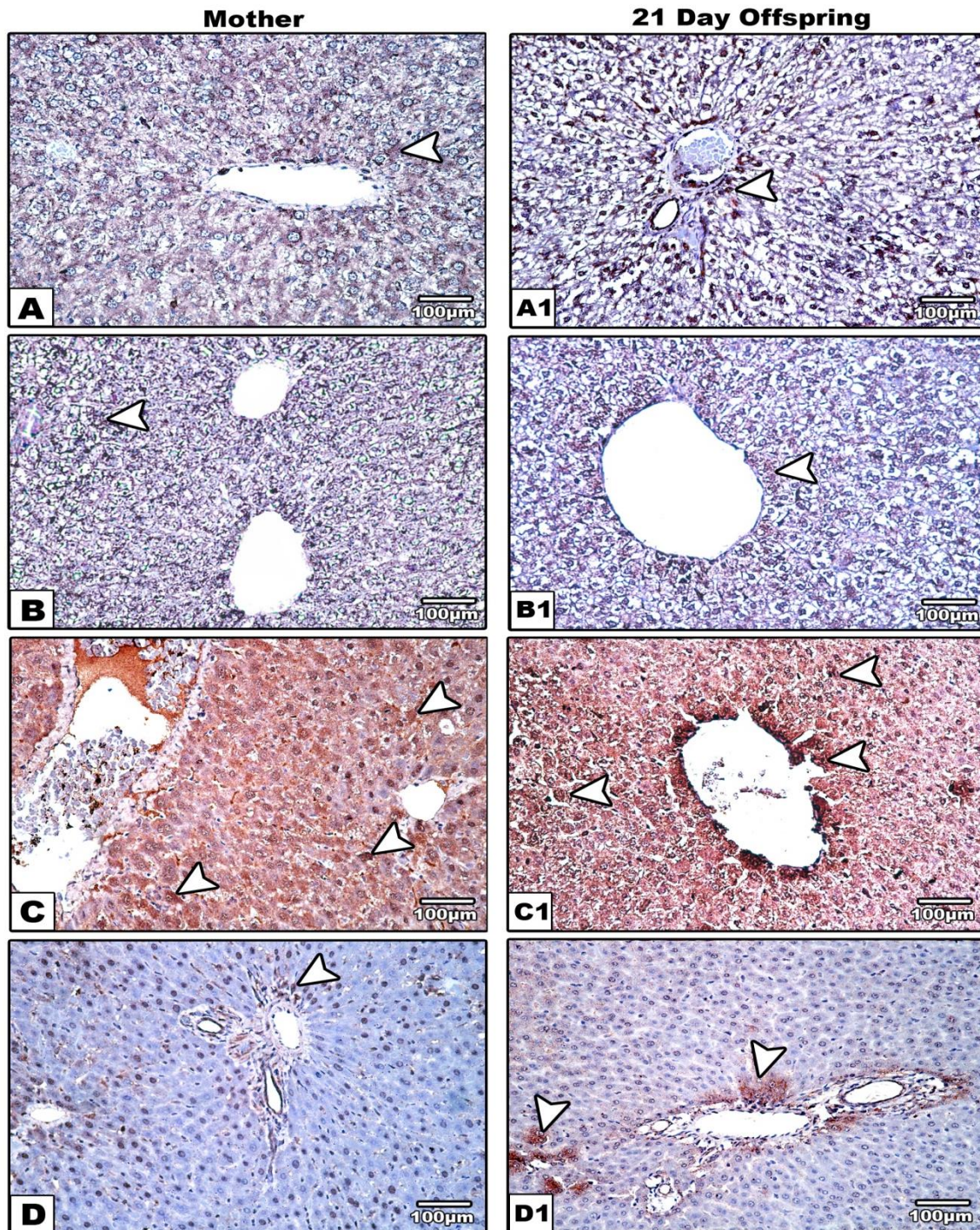
Figure3: Photomicrograph of paraffin-embedded sections through the liver of mother rats (A-D) and their offspring (A1-D1) stained with anti-alpha-fetoprotein antibody. A&A1 control, B&B1: Quercetin, C&C1: MNU, D&D1: MNU& Quercetin. Note a negative immunohistochemical reaction in A-B1, a strong positive immune expression appears in the hepatocytes of MNU-induced carcinogenesis in mother rats (C) and their offspring (C1), a weak reaction appears in image D and negative to very weak reaction in image D1.

Arrows heads refer to the localization of alph1-fetoprotein immuno-reactivity
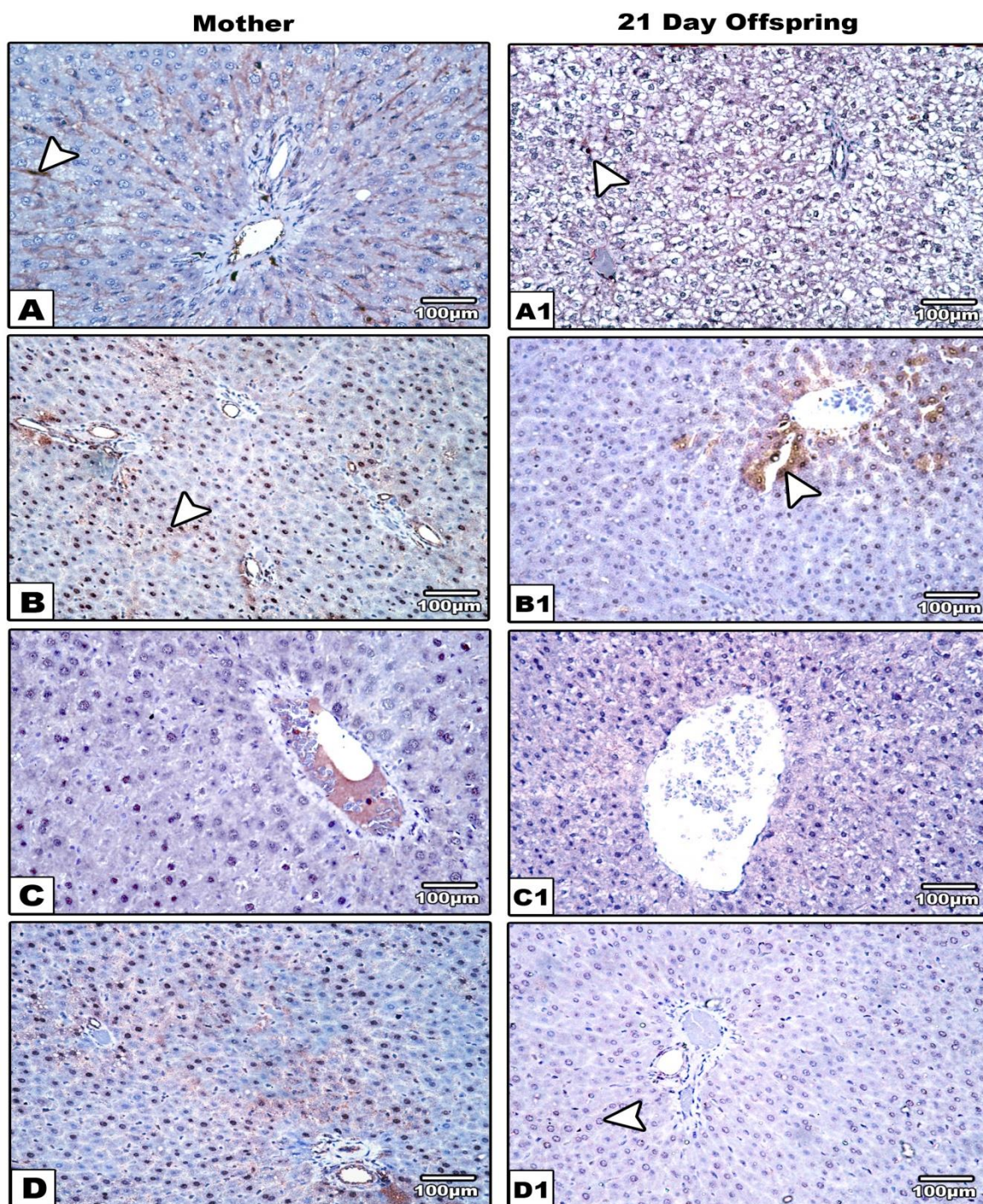
Figure4: Photomicrograph of paraffin-embedded sections through the liver of mother rats (A-D) and their offspring (A1-D1) stained with anti-caspase3 antibody. A&A1 control, B&B1: Quercetin, C&C1: MNU, D&D1: MNU& Quercetin. Note a weak to moderate caspase3 reaction in A-B1, a negative immune expression appears in the hepatocytes of MNU-induced carcinogenesis in mother rats (C) and their offspring (C1), and a very weak reaction appears in image D&D1.
Arrows heads refer to the localization of caspase3 immuno-reactivity

The mean level of GSH in quercetin supplemented mothers rats appeared significantly higher than control; however, this level appeared significantly lowered in the MNU-treated group. In the treatment of the MNU-induced group with quercetin, the mean level of GSH appeared significantly higher than the MNU-induced group but did not reach the normal value as control (table 1&figure5C).

ii. At 21 days old offspring

The mean level of SOD in maternally quercitin supplemented offspring showed non-significant with control (P >0.05). In comparing with control offspring, the level of SOD appeared significantly lowered (P< 0.001) in maternally MNU-induced offspring. On the other side, the mean level of SOD appeared significantly higher in maternally MNU induced offspring post supplemented with quercetin appeared with non-significant change if compared with control (table1&figure 5A1).

In maternally quercetin supplemented offspring, the mean level of CAT appeared significantly higher if compared with control. In comparison with control, the mean level of CAT in maternally MNU-induced offspring appeared significantly decreased (P< 0.001). On the other hand, the mean level of CAT in maternally MNU induced offspring post supplemented with quercetin appeared with non-significant change if compared with control (table1&figure5B1).

The mean level of maternally supplemented quercetin offspring appeared significantly higher than control; however, this level appeared significantly lowered in the maternally MNU-induced group. On the treatment of MNU-induced offspring with quercetin, the mean levels of GSH appeared significantly higher than the MNU-induced group but did not reach the normal value as control, however (table1&figure5C1).

3.2 Changes in Serum NO and MDA

i.In mother's rats

In quercetin supplemented mothers rats, the mean levels of NO and MDA appeared with non-significant change with control, however, in MNU-induced mother's rats these levels appeared significantly higher (P< 0.001) if compared with control. On the other side, the mean level of NO and MDA appeared significantly lowered in MNU induced rats to post supplemented with quercetin if compared with MNU

induced rats but still significantly higher than control (table1 &figure5D&E).

ii.At 21 days old offspring

In maternally quercitin supplemented offspring, the mean level of NO appeared significantly decline however the level of MDA showed non-significant change if compared with control. In maternally MNU-induced offspring the level of serum NO and MDA appeared significantly higher (P< 0.001) if compared with control. On the treatment of MNU-induced offspring with quercetin, the mean level of NO and MDA appeared significantly lowered if compared with MNU-group but still significantly higher than control (table1 &figure5D1&E1).

3.3 Changes in Serum CEA and IL-1β

i.   In mother's rats

In quercetin supplemented mothers rats, the mean levels of CEA and IL-1β appeared with non-significant change with control, however, in MNU-treated mother rats these levels appeared significantly higher (P< 0.001) if compared with control. On the other side, the mean level of CEA and IL-1β appeared significantly lowered in MNU treated rats post-supplemented with quercetin if compared with MNU treated rats but the level of CEA was still significantly higher than control (Table1 and figures6A&B).

ii.   At 21 days old offspring

In maternally quercitin-supplemented offspring, the mean levels of CEA and IL-1β appeared significantly unchanged if compared with control, however, in maternally MNU-induced offspring these levels appeared significantly higher (P< 0.001) if compared with control. On the other side, the mean level of CEA in maternally MNU treated offspring post supplemented with quercetin appeared with non-significant change with control (Table1 and figures6A1&B1).

4. Flow cytometric analysis Bax in the liver cells

The obtained flow cytometric data revealed that the mean percentage value of positively expressed liver cells for Bax antibody was markedly lowered in MNU-induced mothers rats (15.3%) and their offspring (18.3%) if compared with their control (23.7%and 20.1%) respectively. Post-treatment of MNU-induced mother's rats and their offspring with quercetin the percentage of positively expressed Bax cells was markedly elevated (20.5% and 19.9%) respectively (Figure 7).

Table1. The levels of serum SOD (U/ml), CAT, GSH, NO, MDA, CEA, and *IL-1β* among the different studied groups of mother rats and their offspring.

Parameter / Group (n=6)		Control (n=6)	Quercetin (n=6)	MNU (n=6)	MNU& Quercetin (n=6)	p
SOD (U/ml)	Mother	0.1033 ±0.0010	0.1123^a ±0.0015	0.0872^{ab} ±0.0017	0.1002^{abc} ±0.0017	<0.001*
	Offspring	0.1260 ±0.0018	0.1280 ±0.0018	0.1080^{ab} ±0.0018	0.1190^{abc} ±0.0018	<0.001*
CAT (ng/ml)	Mother	0.1908 ±0.0015	0.1910 ±0.0018	0.1160^{ab} ±0.0018	0.1860^{abc} ±0.0018	<0.001*
	Offspring	0.1888 ±0.0015	0.1950^a ±0.0018	0.1368^{ab} ±0.0017	0.1840^{abc} ±0.0018	<0.001*
GSH (ng/ml)	Mother	0.1950 ±0.0018	0.2020^a ±0.0018	0.1280^{ab} ±0.0018	0.1850^{abc} ±0.0018	<0.001*
	Offspring	0.2282 ±0.0017	0.2350^a ±0.0018	0.1600^{ab} ±0.0018	0.2017^{abc} ±0.0016	<0.001*
NO (nmol/ml	Mother	0.1197 ±0.0008	0.1190 ±0.0018	0.1900^{ab} ±0.0018	0.1368^{abc} ±0.0017	<0.001*
	Offspring	0.1115 ±0.0018	0.1080^a ±0.0018	0.1230^{ab} ±0.0018	0.1150^{abc} ±0.0018	<0.001*
MDA (nmol/ml	Mother	0.1548 ±0.0015	0.1550 ±0.0018	0.2100^{ab} ±0.0018	0.1660^{abc} ±0.0018	<0.001*
	Offspring	0.1288 ±0.0015	0.1260 ±0.0018	0.1442^{ab} ±0.0017	0.1410^{abc} ±0.0018	<0.001*
CEA (ng/ml)	Mother	0.1120 ±0.0014	0.1100 ±0.0021	0.2080^{ab} ±0.0018	0.1260^{abc} ±0.0018	0.1120 ±0.0014
	Offspring	0.1143 ±0.0010	0.1120 ±0.0018	0.1260^{ab} ±0.0018	0.1140^c ±0.0018	0.1143 ±0.0010
IL-1β (Pg/ml)	Mother	0.1282 ±0.0017	0.1260 ±0.0018	0.1460^{ab} ±0.0018	0.1298^{bc} ±0.0017	0.1282 ±0.0017
	Offspring	0.1128 ±0.0015	0.1110 ±0.0018	0.1368^{ab} ±0.0017	0.1090^{ac} ±0.0018	0.1128 ±0.0015

*Data were expressed in **mean ± SD.F: F for ANOVA test**, Pairwise comparison between every 2 groups was done using **Post Hoc Test, (Tukey)** .p: p-value for comparing between the studied groups. *: Statistically significant at p ≤ 0.05.**a:** Significant with **Control**, **b:** Significant with **Quercetin**, **c:** Significant with **MNU**, **d**: Significant with **MNU & Quercetin**.*
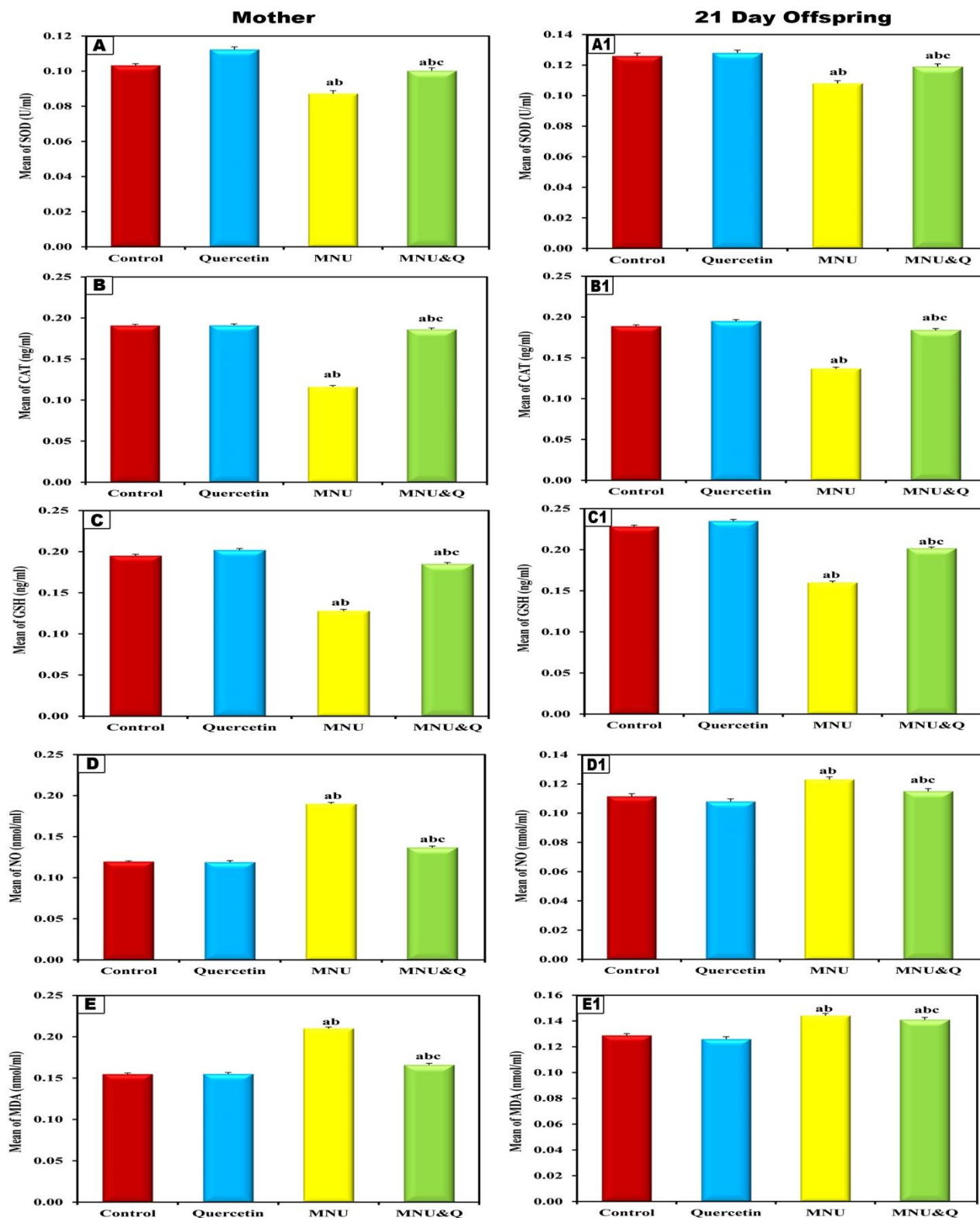
Figure 5: The levels of serum SOD, CAT, GSH, NO, MDA among the different studied groups of mother rats and their offspring.
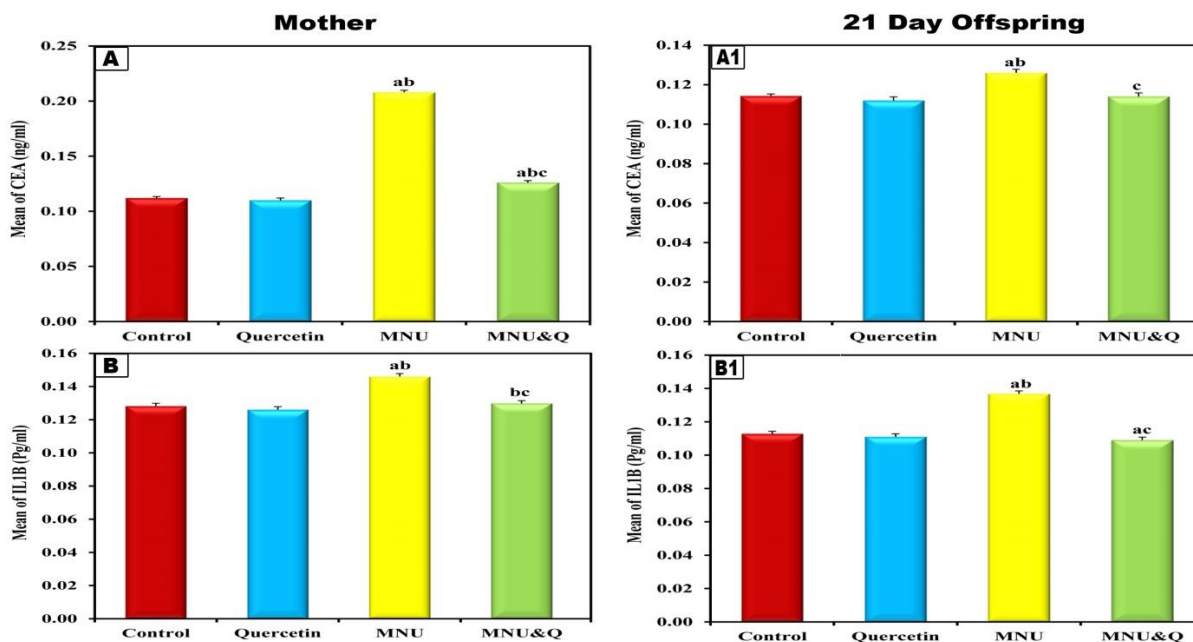
Figure 6: The levels of serum CEA (ng/ml) and IL-1β (pg/ml) among the different studied groups of mother rats and their offspring
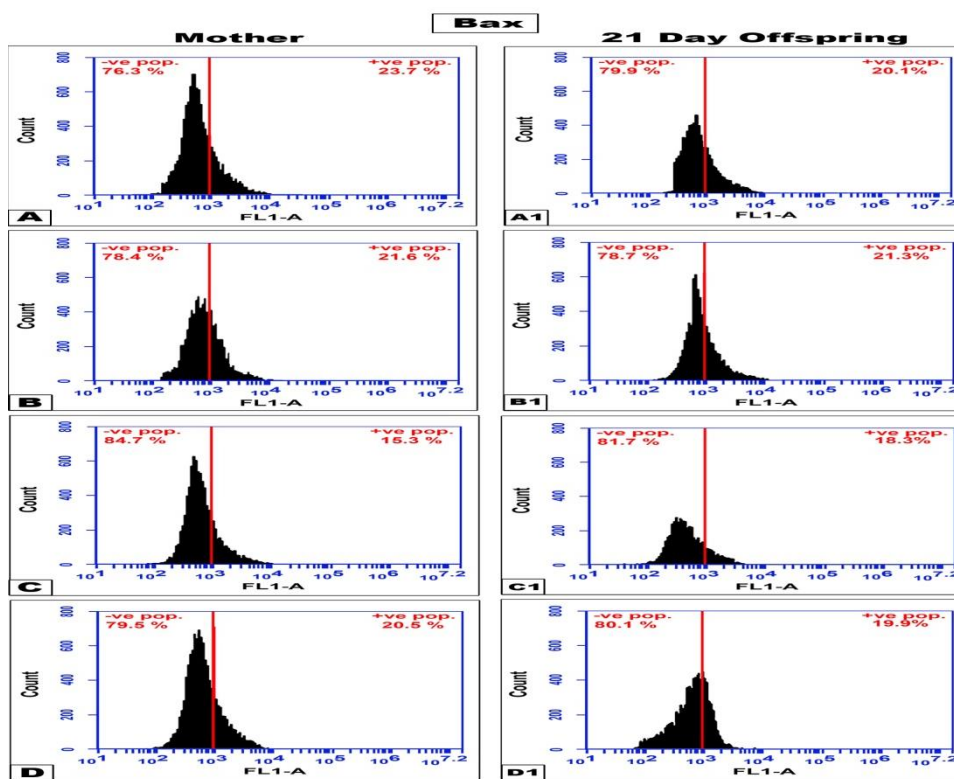

Figure 7: A flow cytometric chart showing the mean % value of positively expressed liver cells for Bax marker among the different studied groups of mother rats and their offspring.

DISCUSSION

Hepatocellular carcinoma (HCC) is a common malignant tumor with the highest incidence of the clinic, and it is also one of the top causes of cancer-induced deaths worldwide. Additionally, HCC is a polygenic chronic disease, which involved complex genes and signaling pathways; as a result, there are many obstacles on the way to achieving a successful therapeutic goal. Therefore, searching for more efficient alternative antitumor drugs to complement or to replace surgery, chemotherapy, and radiotherapy remains a highly important research area (**Yajid et al., 2018; Wu et al., 2019).**

Traditional medicines have been studied in various cancers for their preventive and therapeutic properties (**Dai et al., 2016)**. Flavonoids, polyphenolic plant secondary metabolites, have been confirmed to have effective antioxidant, anti-inflammatory, and antiproliferative activities, which are beneficial for human health (**Gibellini et al., 2011**). Quercetin is a typical flavonoid that has significant effects on inhibiting the development of various types of cancer like breast cancer (**Rivera et a.,2016**), ovarian cancer (**Liu et al.,2017**), carcinoma of the colon and rectum (**Yang et al.,2016**) and gastric cancer(**Zhang et al.,2015**)

It was found that quercetin affects pregnancy in an animal model (**Karampou et al., 2014; Khaksary-Mahabady et al., 2018**). According to **Vanhees et al. (2011)** when female mice were exposed to quercetin from 3 days before conception until day 14.5 of gestation, no negative effects on the placenta or fetal development were found (**Vanhees et al., 2011**). A previous study has shown that quercetin administrated to mice did not have any teratogenic effects on the fetus (**Prate et al., 2008).** Moreover, exposure to quercetin during pregnancy resulted in the up-regulation of genes involved in the enzymatic antioxidant defense system in the liver of fetuses at day 14.5 of gestation (**Vanhees et al.,**

2011). Accordingly, the current work is mainly designed to evaluate the therapeutic role of quercetin against MNU-induced HCC in pregnant rats and their offspring.

The histological results of the present work displayed that MNU has a powerful role in the induction of pronounced cellular hyperplasia (HCC) in addition to severe deleterious histological changes in liver tissues of pregnant mother rats however the liver tissues of their offspring appeared without HCC but with remarkable histopathological features. Supplementation of quercetin can attenuate the histopathological changes as well as inhibition of cellular hyperplasia induced by MNU in the liver tissues of mother rats.

Previous studies reported that MNU can experimentally induce deleterious histological alterations with subsequent pronounced cellular hyperplasia in the liver tissues in mice (**Somanah et al., 2016).** Several reports discussed that the histopathological criteria and carcinogenic action of MNU are mainly attributed to the methylation of DNA in the cells (**Kour et al., 2017).** Another study emphasized that MNU is a harmful industrial and environmental pollutant, potentially activates inflammatory cytokines (IL-1β, IL-6) in hepatic cells with increased expression of nuclear factor *kappa* B (NFκB) which might be responsible for the development of HCC (**Verma et al.,2012). Ragab et al.(2013)** declared that MNU can accelerate oxidative stress in the tissues resulting in increased free radicals production and elevation of lipid peroxidation which is a major cause for histopathological signs.

Quercetin antitumor effects have been described in different cancer types, including HCC (**Brito et al., 2016; Khan et al., 2016**). It has been described that quercetin-derived inhibition of liver cancer cell growth could be mediated by the disruption of different pathways, including protein kinase B

(Akt)/mammalian target of rapamycin (mTOR) **(Ji et al.,2019; Wu et al.,2019**), mitogen-activated protein kinase 1 (MEK1)/extracellular signal-regulated kinase 1/2 (ERK1/2) **(Ding et al., 2018)** and Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling routes **(Wu et al.,2019)**. Furthermore, it had been reported that quercetin has a potential role in the elevation of antioxidants levels, especially CAT, SOD, and GSH, and inhibition of ROS in the alcohol-induced liver cell damage **(Lee et al., 2017)**. Furthermore, it had been found that quercetin is converted to circulating forms, 3-O-methyl quercetin (3MQ) and quercetin-3-O-glucuronide (Q3GA), during absorption in the liver. These metabolites play an essential role in the elevation of antioxidant enzymes **(Duenas et al., 2011)**. This explains the ameliorative role of quercetin against the MNU- induced histopathological signs in liver tissues in this study.

In this study, the recorded histopathological features without HCC in the liver tissues of maternally MNU-induced offspring may be attributed to little leakage of MNU-during gestation and breastfeeding. The ameliorative role of quercetin against MNU-induced liver damage in offspring is fundamentally attributed to the circulating quercetin metabolites (3MQ and Q3GA) that can cross the placenta and comes out with milk during lactation **(Khaksary-Mahabady et al., 2018).**

The obtained results in this work revealed that the serum antioxidants; CAT, SOD, and GSH were significantly decreased in MNU-induced carcinogenesis in mothers rats and their offspring, this is followed by a significant elevation of NO and MDA. On the other hand treatment with quercetin was successfully ameliorated the disrupted levels of antioxidants as well as NO and MDA caused by MNU near to the normal values. The ameliorative effects of quercetin against MNU-induced oxidative

damage agree with findings of previous studies of **Liu et al. (2012)** who found that quercetin metabolites can protect the primary hepatocyte of rats against ethanol-induced oxidative cell damage. **Lee et a.(2017)** added that the anti-oxidative effects of quercetin and its metabolites are mediated by diminished ROS generation, decreased lipid peroxidation (MDA) and NO as well as up-regulation of activity on antioxidant enzymes such as superoxide dismutase SOD, CAT, and GSH. Furthermore, during oxidative stress, GSH, an important cellular component for preventing oxidative stress, is consumed, resulting in rapid depletion **(Armstrong et al., 2002).** Our obtained results showed that quercetin help hepatocytes to attenuate lipid peroxidation and maintain the reduced form of GSH. Also, the obtained result declared that quercetin-treatment increased SOD and CAT activity in HCC cells. Such results are in line with the finding of **Lee et al. (2017)** who found that the cytoprotective effects of quercetin and its metabolites are mediated by diminishing lipid peroxidation, increasing the reduced form of GSH, and increasing the activity of antioxidant enzymes.

Previous researches emphasized that the levels of serum α1FP, CEA, and IL-1β (tumor markers) are proportional to the development of HCC **(Wu et al., 2012; Hiroshima et al., 2016)**.). α1FP is a tumor-associated fetal protein, has long been employed as a serum fetal tumor marker to monitor disease progression. In this study, there was strong positive α1FP immune expression in the liver tissues of MNU-induced HCC in mothers rats. Such observation goes parallel with the finding of **Wu et al.(2012)**. The decrease in α1FP tumor markers after quercetin supplementation might be due to a decrease in the production rate of tumors **(Srigopalram et al., 2012)**.

Apoptosis has been established as one of the mechanisms of quercetin-induced cell death in HCC **(Wu et al., 2019)**, as it was demonstrated by the

increase in pro-apoptotic proteins expressions, such as Bax and cleaved caspases-3 **(Ji et al., 2019)**. It has been reported that supplementation of high doses (over 100 mg/body weight) to mice can induce apoptosis in the liver cells and brain of mice however if the dose is lowered than this value leads to regulation of apoptosis **(Choi and Kim, 2010).** In the current work, the applied dose of quercetin was 20mg/body weight which successfully maintained the apoptotic pathway in the liver tissues that was confirmed by observation of weak expression of csapse3. Moreover, the flow cytometric analysis applied in this study confirmed that the percentage values of positively Bax expressed liver cells were markedly lowered in MNU-induced HCC in the mother's rats and their offspring if compared with the control. However, post-treatment with quercetin this percentage was markedly elevated. The obtained results go parallel with the finding of **Wu et al. (2019).** Several studies reported that quercetin metabolites can promote autophagy in several cell lines of liver cancer, being related to apoptosis induction and suppression of tumor progression **(Ji et al., 2019; Wu et al., 2019).**

Conclusion

In conclusion, quercetin has a clear anti-proliferative and pro-apoptotic effect against HCC and likely a modulating role on tumor cell cycle progression which needs to be investigated further.

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