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Efficacy of zinc oxide nanoparticles on hepatocellular carcinoma-induced biochemical and trace element alterations in rats

Samir A. E. Bashandy[1*], Omar A. H. Ahmed-Farid[2], Sherif Abdelmottaleb Moussa[3], Enayat A. Omara[4], Gehad A. Abdel Jaleel[1], Fatma A. A. Ibrahim[3]

[1]Pharmacology Department, National Research Centre, Cairo, Egypt.
[2]Physiology Department, National Organization for Drug Control and Research, Cairo, Egypt.
[3]Biophysics Group, Department of Biochemistry, Genetic Engineering and Biotechnology Division, National Research Centre, Cairo, Egypt.
[4]Pathology Department, National Research Centre, Cairo, Egypt.

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ABSTRACT

The purpose of this study is to evaluate the probable roles of zinc oxide nanoparticles (ZnO-NPs) at a dose level of 5 mg or 10 mg/kg as antitumor in hepatocellular carcinoma (HCC) animal model. HCC was induced in rats by using diethylnitrosamine and carbon tetrachloride. The treatment of HCC rats with ZnO-NPs alleviated the significant increase in cancer markers, alpha-fetoprotein (AFP), glypican-3 (GPC3), and Vascular Endothelial Growth Factor (VEGF). The treatment of HCC rats with ZnO-NPs relieved the increase in liver enzymes and histopathological changes. Also, ZnO-NPs lessened the increase in the inflammatory markers. Moreover, the treatment of HCC rats with ZnO-NPs led to a significant decline in hepatic malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine and a significant increase in reduced form of glutathione and DNA content of hepatic cells as compared to the HCC group. Additionally, ZnO-NPs prevented the significant increase in hepatic copper and manganese levels or the decrease in zinc level in rats with HCC. Furthermore, ZnO-NPs can modulate plasma glucose level and lipid profile associated with improved hepatic mucopolysacchrides and ATP that altered in the HCC group. In conclusion, the treatment of HCC rats with ZnO-NPs offered an anticancer remedy that may be considered as a new trend for control HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent disease worldwide (Torre *et al*., 2012). Many risk factors such as chronic alcohol consumption, viral hepatitis and fatty liver disease have been participated in HCC associated fibrosis and cirrhosis (Parikh and Hyman, 2007). Oxidative stress and cytokine/adipocytokine pathways may involve nonalcoholic fatty liver disease-related HCC pathogenesis which likely participates in the development of HCC (Starley *et al*., 2010). Cancer cells directed metabolism in favor of their demand. Metabolic modifications allow tumor growth, proliferation, and survival as they supply cancer cells with energy and macromolecule biosynthesis. Enhanced consumption of glucose, a common feature of cancers, reinforces the formation of mediate compounds for the induction of lipids, proteins, and nucleic acids synthesis. Otherwise, increased glutamine uptake and glutaminolysis permit cancer cells to accomplish intermediates in the Krebs cycle that are directed toward biosynthetic reactions (Schulze and Harris, 2012). It was reported that hypoglycemia in HCC was due to a rise in glucose consumption by a rapid-growing tumor (Sharma *et al*., 2014). Various cancer cells have been shown to promote the activity of mevalonate pathway enzymes, a precursor for cholesterol synthesis (Mullen *et al*., 2016). The metabolic reprogramming in HCC includes mitochondrial malfunction. The elevated free radicals production and the decrease in adenosine triphosphate generation may participate in the HCC development (Hsu *et al*., 2013).

Nanomedicine may reflect a recent direction in the universe. It is interested in the biomedical accomplishment of nanotechnology in the rapid identification and remedy of diseases.

*Corresponding Author
Samir A. E. Bashandy, Pharmacology Department, National Research Centre, Cairo, Egypt. E-mail: Bashandysamir @ gmail.com

In newly years, many scientists attempt to use the distinctive characters of nanoparticles, particularly zinc oxide nanoparticles (ZnO-NPs) in the management of some diseases (McNeil, 2009). It was reported that ZnO-NPs resemble biomolecules in their activity; therefore, they can maintain cellular homeostasis (Vizirianakis, 2011). Traditional treatment of cancer as chemotherapy exhibited unfavorable effects and affects most cells. The most unfavorable effects of cancer chemotherapy are nausea, hair loss, mouth sores, memory impairment, diarrhea, headache, dry mouth, fatigue, abdominal cramps, and vomiting (Aslam *et al*., 2014). Therefore, searching for other replacement medication for cancer has become urgent (Gowda *et al*., 2013). ZnO-NPs showed the cytotoxic effect on different cancer cell lines (Elsayed *et al*., 2020). ZnO-NPs represented a promising preclinical anticancer efficacy in HCC and could be considered as a novel strategy for the treatment of HCC in clinical practices (Hassan *et al*., 2017). No previous studies on the effect of zinc nanoparticles on trace elements in HCC have been notified. Therefore, the target of the present study is the evaluation of the zinc nanoparticles *in vivo* as medication for HCC via investigation cancer or inflammatory markers, oxidative stress parameters, and hepatic trace elements. Zinc nanoparticles were used as they have new biomedical applications and display potential toxicity against some cancerous cells.

MATERIALS AND METHODS

Chemicals

Methanol (HPLC grade) was secured from Loba Co., India. Perchloric acid was bought from Loba Co., India. Sulphosalsilic acid and P-amino benzyl glutamate and pyrogallol were purchased from TMMEDIA Co., India. The 1,1,3,3-tetraethoxypropane, reduced form of glutathione (GSH), and oxidized form of glutathione (GSSG) were gotten from Sigma Aldrich (USA). Other used chemicals were of purity grade and were bought from known supplies.

Synthesis of ZnO-NPs

For the synthesis of the samples, we dissolved 2 g of zinc acetate dehydrate in 14 ml of methanol, under magnetic stirring for 2 hours. Then, the resulting solution underwent rapid drying to obtain powder aerogels. Furthermore, we conducted drying in supercritical conditions of ethanol at 250ºC, with a heating rate of 45ºC/hours. The secured powder was placed in an oven at 500°C for 2 hours to get ZnO-NPs (About 50 nm). Nanopowder was recognized by x-ray diffraction. Moreover, the nanopowder was characterized according to morphology and structure properties (Omri *et al*., 2016)

Preparation of ZnO-NPs solution

The nanoparticles of zinc oxide were added to 1% sodium carboxymethyl cellulose as a stabilizer, moved by a magnetic stirrer for 5 minutes, and followed by exposure to ultrasonic vibration for 15 minutes (Wang *et al*., 2008).

Induction of HCC

The rats were injected IP with a single dose of DENA (200 mg/kg body weight). After 2 weeks, animals received CCl_4 (3 ml/kg) dissolved in corn oil (1 : 1 volume) injected subcutaneously once a week for 6 weeks (Singh *et al*., 2009).

Ethics statement

Animal handling was executed following the guidance of Laboratory Animals of the National Institutes of Health (NIH publication No. 85–23, revised 1996) and National Research Centre (NRC) in Egypt with ethical approval No. 19218.

Animals and treatment

Male Wistar rats (weighing 150–170 g and 3 months old) were brought from National Research Centre (NRC) of Egypt. They were given freely standard pellet diet and water. The animals remained at adjusted temperature (22°C ± 2°C) with equal light and dark cycle. Thirty-two rats were sorted into the following groups:

Group 1. Normal rats served as control injected with the same volume of vehicle (0.2 ml 1% sodium carboxymethyl cellulose) IP.

Group 2. Rats with HCC injected with the same volume of vehicle IP.

Group 3 and 4. Rats with HCC injected IP with 5 and 10 mg/kg ZnO-NPs daily for 8 weeks (Bashandy *et al*., 2018).

Samples collection

The blood was gathered after 2 months from rats killed under ether anesthesia and plasma was separated and kept at −30°C. The liver samples were handled for histological study and for evaluation of malondialdehyde (MDA), reduced glutathione (GSH), oxidized glutathione (GSSG), superoxide dismutase (SOD), catalase (CAT), nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and coenzyme Q10 (CoQ10). Homogenized liver samples were obtained and the supernatant was stored at −80°C until assay.

Determination of cancer markers in plasma

Alpha-fetoprotein (AFP) was determined by ELISA technique using kits get from Sunlong Biotech Co. Ltd., China, while VEGF and glypican-3 (GPC3) were assayed by the same technique using kits purchased from Lifespan Biosciences, Inc., USA.

Hepatic oxidative stress parameters

Hepatic MDA, GSH, GSSG, NO, and CoQ10 (Karatas *et al*., 2002; Papadoyannis *et al*., 1999; Yoshida, 1996; Yubero *et al*., 2014) levels were evaluated by the HPLC system of Agilent HP 1200 series (USA). SOD activity was assayed in the homogenate by the method of Marklund and Marklund (1974).

Determination of hepatic ATP and 8-hydroxy-2′-deoxyguanosine (8-OHdG)

Moreover, the detection of ATP and 8-OHdG was carried out by HPLC (Lodovici *et al*., 1997; Teerlink *et al*., 1993;,).

Anti-inflammatory markers

Plasma C-reactive protein (CRP) and interleukin 6 (IL-6) were assayed by immunoassay (ELISA) using kits get from Sunlong Biotech Co. Kit, China. Tumor necrosis factor-alpha

(TNF-α) was assayed using an enzyme immunoassay kit produced by R&D Systems (USA).

Liver function and lipid profile tests

Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, total protein, glucose, and lipid profile were evaluated calorimetrically using kits of Salucea Company, Netherlands, while gamma-glutamyl transferase (GGT) was determined kinetically using kits produced by Egyptian company for biotechnology.

Determination of hepatic trace elements

Hepatic zinc, copper, and manganese concentrations (µg/g dry tissue) were evaluated by the use of atomic absorption spectrophotometry (PyeUnicam, Cambridge, UK). Samples of tissues were put in an oven and dried. Certain weight of dried samples was digested with nitric acid and perchloric acid (2 : 1, *v/v*) in a sand bath and wet residues were watered to 5 ml and centrifuged.

Histological analysis

After the fixation of liver samples, they were stained with hematoxylin and eosin for investigation of the histopathological changes. Moreover, some liver sections were stained with Masson trichrome stain which was used for demonstrating the collagen fibers.

Histochemical examination

Sections of 5 µm thickness produced were stained with periodic acid Schiff (PAS) to demonstrate muco polysaccharides (MPS) or with Feulgen stain for DNA. Both Feulgen stain (DNA) and PAS sections are subjected to measurement of optical density using an image analyzer (Leica Qwin 500).

Statistical analysis

The rough data were analyzed statistically at $p < 0.01$ using analysis of variance and LSD comparison test by using SPSS software, version21.

RESULTS

Cancer markers

Table 1 showed that the treatment to hepatocarcinoma rats with of ZnO-NPs significantly ($p < 0.01$) lowered the increase in plasma levels of cancer markers (α-fetoprotein, GPC3, and VEGF) when compared with the HCC group. The effect of ZnO-NPs on all parameters tested or histopathological study is dose dependent; the effect of high dose is more pronounced than low dose.

Hepatic oxidative stress parameters

The oxidative stress parameters are affected by HCC as manifested by a significant ($p < 0.01$) rising of MDA, GSSG, SOD, and NO levels (Table 2) in comparison with the control group. Otherwise, hepatic GSH and CoQ10 lowered significantly ($p < 0.01$) relative to the control. In the HCC rats treated with ZnO-NPs, GSH and CoQ10 were elevated significantly ($p < 0.01$), while MDA and NO were diminished significantly ($p < 0.01$) as compared to the HCC group.

Table 1. Effect of zinc oxide nanoparticles on plasma AFP, GPC3, and VEGF in HCC rat model.

Treatment parameter	Control	DENA	DENA + ZnO-NPs L	DENA + ZnO-NPs H
AFP (ng/ml)	8.45 ± 0.37	33.64 ± 1.26[a]	25.08 ± 0.75[ab]	18.49 ± 0.58[abc]
GPC3 (pg/ml)	11.04 ± 0.26	40.52 ± 2.31[a]	32.07 ± 0.88[ab]	24.11 ± 0.63[abc]
VEGF (ng/l)	53.64 ± 2.94	191.34 ± 7.83[a]	140.11 ± 4.68[ab]	116.73 ± 5.29[abc]

Each value is the mean ± SE; $n = 8$.
L = 5 mg/Kg; H = 10 mg/Kg; AFP = alpha-fetoprotein; GPC3 = glypican-3; VEGF = vascular endothelial growth factor.
[a]Significant difference compared to the control group at $p < 0.01$.
[b]Significant difference compared to the HCC group at $p < 0.01$.
[c]Significant difference compared to the HCC + ZnO-NPs L group at $p < 0.01$.

Table 2. Effect of zinc oxide nanoparticles on hepatic oxidative stress parameters in HCC rat model.

Treatment Parameter	Control	HCC	HCC + ZnO-NPs L	HCC + ZnO-NPs H
MDA (nmol/g)	36.63 ± 1.00	56.15 ± 1.96[a]	45.75 ± 1.19[ab]	40.31 ± 2.06[b]
GSH (µmol/g)	4.25 ± 0.07	2.25 ± 0.15[a]	2.60 ± 0.12[ab]	3.47 ± 0.17[abc]
GSSG(µmol/g)	0.32 ± 0.04	0.41 ± 0.01[a]	0.39 ± 0.02	0.34 ± 0.01[b]
SOD (U/G)	44.38 ± 0.86	52.82 ± 1.20[a]	45.81 ± 1.70	46.53 ± 1.75
NO (µmol/g)	0.59 ± 0.03	1.00 ± 0.032[a]	0.86 ± 0.02[ab]	0.65 ± 0.02[bc]
CoQ10 (nmol/g)	1.18 ± 0.04	0.62 ± 0.03[a]	0.97 ± 0.06[ab]	1.23 ± 0.04[bc]

Each value is the mean ± SE; $n = 8$.
L = 5 mg/Kg; H = 10 mg/Kg; MDA = malondialdehyde; GSH = reduced form of glutathione; GSSG = oxidized form of glutathione; SOD = superoxide dismutase; NO = nitric oxide; CoQ10 = coenzyme Q10.
[a]Significant difference compared to the control group at $p < 0.01$.
[b]Significant difference compared to the HCC group at $p < 0.01$.
[c]Significant difference compared to the HCC + ZnO-NPs L group at $p < 0.01$.

Anti-inflammatory markers

As displayed from Figure 1, the treatment of HCC rats with ZnO-NPs significantly ($p < 0.01$) inhibited the increase in plasma CRP, IL-6, and TNF-α levels when compared with the HCC group.

Glucose, mucopolysacchrides (MPS), and ATP

The injection of HCC rats with ZnO-NPs mitigated significantly ($p < 0.01$) the decrease in plasma glucose or hepatic MPS, and ATP levels (Fig. 2) as compared with the HCC group.

Liver function and lipid profile tests

The values of liver enzymes and bilirubin in HCC + ZnO-NPs were significantly ($p < 0.01$) lower than those of the HCC group (Table 3). Moreover, plasma cholesterol, triglycerides, and low-density lipoprotein (LDL) levels in the HCC group (Table 4) were significantly increased ($p < 0.01$) while high-density lipoprotein (HDL) was lowered significantly ($p < 0.01$) as compared to control. The lipid profile parameters were improved by ZnO-NPs treatments. The protein level in HCC + ZnO-NPs did not change significantly as compared to control.

Hepatic trace elements

The hepatic levels of zinc, copper, and manganese in rats with HCC treated with ZnO-NPs were presented in Figure. 3. The levels of zinc were significantly ($p < 0.01$) lower in rats with HCC in comparison to controls (125 *versus.* 153 µg/g). The hepatic copper content was significantly higher ($p < 0.01$) in rats with HCC than controls (47.69 *versus.* 17.65 µg/g) as well as

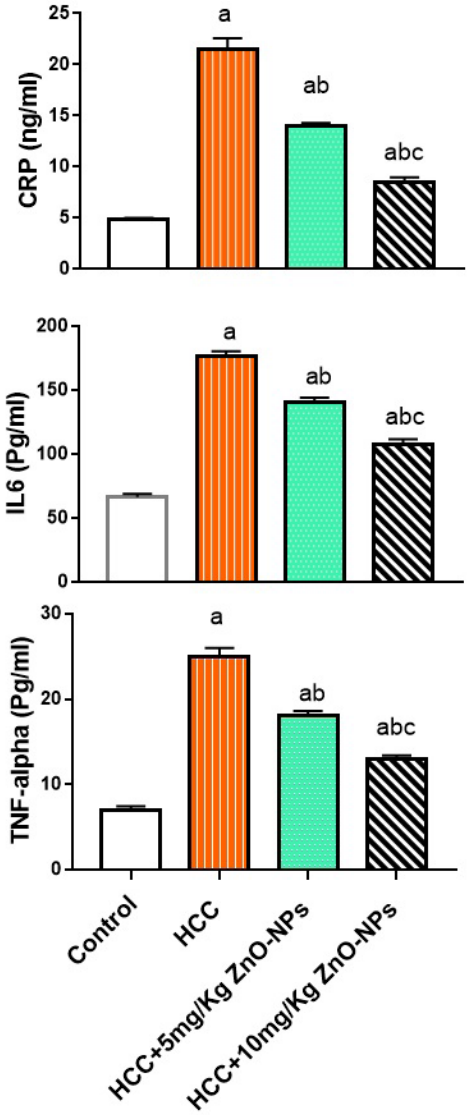

Figure 1. Plasma inflammatory markers in various groups; CRP = C-reactive protein; IL-6 = interleukin 6; TNF-alpha = tumor necrosis factor-alpha. (a): significant difference from the control group, (b): significant difference from the HCC group, and (c): significant difference from the HCC + 5 mg/Kg ZnO-NPs (zinc oxide nanoparticles) group at $p \leq 0.01$. Each value is the mean ± SE; $n = 8$.

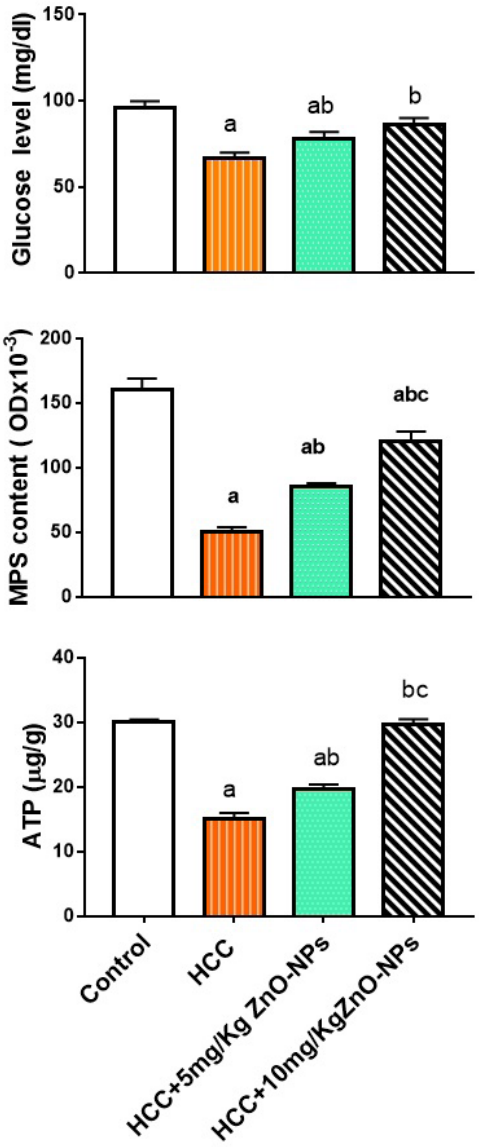

Figure 2. Plasma glucose, hepatic MPS (mucopolysaccharides), and ATP (adenosine triphosphate) levels in various groups. (a): significant difference from the control group, (b): significant difference from the HCC group, and (c): significant difference from the HCC + 5 mg/Kg ZnO-NPs (zinc oxide nanoparticles) group at $p \leq 0.01$. Each value is the mean ± SE; $n = 8$.

Table 3. Effect of zinc oxide nanoparticles on liver function in HCC rats.

Treatment Parameter	Control	HCC	HCC + ZnO-NPs L	HCC + ZnO-NPs H
AST (U/ml)	139.7 ± 5.10	195.5 ± 1.80[a]	148.2 ± 2.10[b]	146.7 ± 6.9[b]
ALT (U/ml)	42.2 ± 3.50	113.5 ± 2.70[a]	76.1 ± 3.5[ab]	70.6 ± 6.9[ab]
GGT (U/L)	6.73 ± 0.22	37.95 ± 1.24[a]	25.00 ± 0.61[ab]	17.39 ± 0.58[abc]
T Bil (mg/dl)	1.8 ± 0.09	3.7 ± 0.31[a]	1.90 ± 0.06[b]	1.85 ± 0.07[b]
Protein (g/dl)	7.80 ± 0.15	8.90 ± 0.37[a]	7.90 ± 0.50	7.50 ± 0.5

Each value is the mean ± SE; $n = 8$.
L = 5 mg/Kg; H = 10 mg/Kg; AST = aspartate aminotransferase; ALT = alanine aminotransferase; GGT = gamma-glutamyl transferase; T Bil: total bilirubin.
[a]Significant difference compared to the control group at $p < 0.01$.
[b]Significant difference compared to the HCC group at $p < 0.01$.
[c]Significant difference compared to the HCC + ZnO-NPs L group at $p < 0.01$.

Table 4. Effect of zinc oxide nanoparticles on plasma lipid profile in HCC rat model.

Treatment Parameter	Control	HCC	HCC + ZnO-NPs L	HCC + ZnO-NPs H
Cholesterol (mg/dl)	90.50 ± 0.85	224.6 ± 8.60[a]	193.8 ± 60.4[ab]	143.6 ± 5.00[abc]
Triglyceride (mg/dl)	67.00 ± 1.50	192.1 ± 4.7[a]	128.3 ± 6.6[b]	119.7 ± 7.50[ab]
HDL (mg/dl)	73.7 ± 3.3	46.20 ± 1.13[a]	66.5 ± 2.80[b]	68.9 ± 2.15[b]
LDL (mg/dl)	16.94 ± 0.8	98.20 ± 1.50[a]	56.00 ± 2.08 [ab]	40.56 ± 1.60[ab]

Each value is the mean ± SE; $n = 8$.
L = 5 mg/Kg; H = 10 mg/Kg; HDL = high-density lipoprotein; LDL = low-density lipoprotein.
[a]Significant difference compared to the control group at $p < 0.01$.
[b]Significant difference compared to the HCC group at $p < 0.01$.
[c]Significant difference compared to the HCC + ZnO-NPs L group at $p < 0.01$. high- and low-.

manganese concentration (17.33 µg/g *versus.* 5.34 µg/g). Injection of ZnO-NPs to HCC rats caused a notable increase ($p < 0.01$) in zinc and a remarkable decrease ($p < 0.01$) in copper or manganese when compared with the HCC group.

Hepatic DNA content and 8-OHdG

The DNA content in liver cells (Fig. 4) of all groups showed a grand decrease ($p < 0.01$) relative to control, but it increased significantly in HCC rats injected with any doses of ZnO-NPs as compared to the HCC group. As demonstrated in Figure 4, the 8-OHdG level in HCC + ZnO-NPs groups lowered significantly ($p < 0.01$) when compared to the HCC group.

Histopathological results

The hepatic cells of rats with HCC showed loss of hepatic lobular construction, ballooning appearance, malformed cord arrangement, defective sinusoids, pyknotic nuclei, and pronounced steatosis. Also, portal tracts were extended with massive necrosis, inflammatory cell infiltration, and fibrous tissue with periportal and pericellular fibrosis (Fig. 5B) as compared to liver control sections (Fig. 5A).

Treatments of HCC rats with 5 mg/kg ZnO-NPs preserved partly the hepatic normal architecture. The hepatocytes were still with a moderate degree of steatosis. Inflammation cell infiltration and portal tracts to pericellular were also noticed as shown in Figure 5C.

Inflammation cell infiltration, swollen hepatocytes, and dilated central vein were also present in the livers of the HCC + high dose ZnO-NPs (10 mg/kg) group but they were notably reduced in extent fibrous tissue and were less frequent compared to the HCC group (Fig. 5D).

Masson's trichrome staining

The microscopic investigation of liver tissue of the HCC group revealed multiple fibrotic extensive fibrosis (Fig. 6B) as compared to the control group (Fig. 6A). Fibrotic lesions were mild in the livers of the groups treated with low and high doses of ZnO-NPs compared to the HCC group (Figs. 6C and D).

DISCUSSION

HCC resembles the main health global dilemma. It is the most existing cancer in the world (Ferlay *et al*., 2015). In Egypt, HCC incidence is more in males than in females and hepatitis C participates in HCC development (Zekri *et al*., 2008). Many researchers try to find a new plan for control HCC. In the present work, our goal is the appreciation of the effectiveness of ZnO-NPs on the treatment of HCC in male rats. Our results demonstrated the anticancer activity for ZnO-NPs as evidenced by a reduction in the elevation of AFP, GPC3, and VEGF (cancer markers). It has appeared that ZnO-NPs have an antiangiogenesis effect as the VEGF level (an angiogenic marker) is decreased by ZnO-NPs treatment to rats with HCC. Furthermore, ZnO-NPs were confirmed by the Food and Drug Administration as a recent and efficient therapy for cancer (Shen *et al*., 2013). It has been reported that ZnO-NPs significantly decreased the elevated serum levels of HCC-related tumor marker AFP, alpha-l- fucosidase, and the apoptotic marker caspase-3 and also significantly reduced oxidative stress markers which are in agreement with previous results (Hassan *et al*., 2017). ZnO-NPs can yield toxicity toward cancer cells via the production of free radicals and oxidative stress, in addition to the motivation of apoptosis (Vinardell and Mitjans, 2015). ZnO produced much higher cytotoxicity than nonmetal nanoparticles. This was significantly approved by glutathione

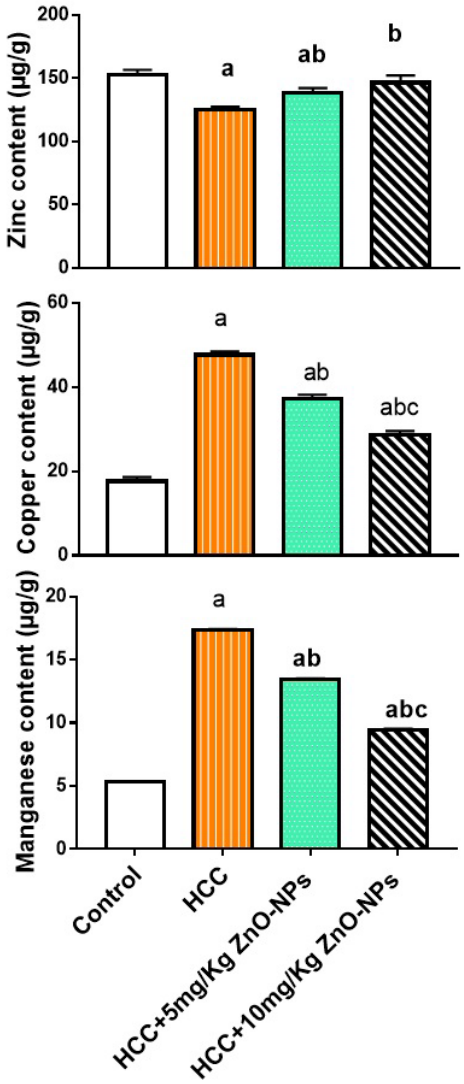

Figure 3. Hepatic trace element levels in various groups. (a): significant difference from the control group, (b): significant difference from the HCC group, and (c): significant difference from the HCC + 5 mg/Kg ZnO-NPs (zinc oxide nanoparticles) group at $p \leq 0.01$. Each value is the mean ± SE; $n = 8$.

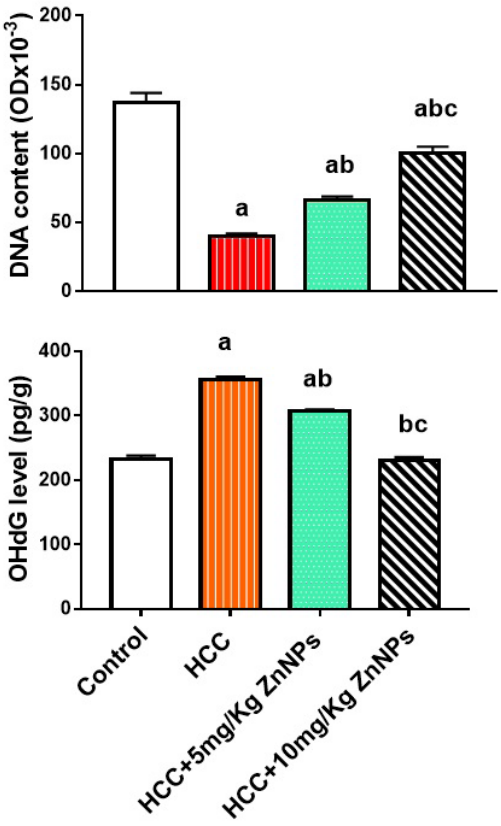

Figure 4. Hepatic DNA (deoxyribonucleic acid) and 8-OHdG (8-hydroxy-2′-deoxyguanosine) levels in various groups. (a): significant difference from the control group, (b): significant difference from the HCC group, and (c): significant difference from the HCC + 5 mg/Kg ZnO-NPs (zinc oxide nanoparticles) group at $p \leq 0.01$. Each value is the mean ± SE; $n = 8$.

depletion, MDA production, SOD inhibition, and reactive oxygen species (ROS) generation. Therefore, oxidative stress may be a key route in producing the cytotoxicity of nanoparticles (Yang *et al*., 2009). Also, the cytotoxic effects of zinc on cancer cells have been reported. Zinc can promote apoptosis or inhibit cell cycle activity and hence it can reduce the growth and metastasis of cancerous cells. Moreover, zinc showed that metabolic effects originate in part from the effects of zinc on inhibition of citrate oxidation which are essential for the synthesis of energetic requirements for the malignant process (Costello and Franklin, 2012). Angiogenesis plays an important role in the advancement of HCC (Poon *et al*., 2001). VEGF motivates endothelial cell proliferation and vascular permeability (Poon *et al*., 2001). GPC3 is frequently produced in HCC. Remarkably elevated expression of GPC3 was demonstrated in HCC tumor tissues. Cell surface GPC3 may combine with growth factors which are likely implicated in the rapid development of HCC (Wu, 2015). AFP is a tumor sign for HCC and helps in cancer diagnosis (Yi *et al*., 2013).

Oxidative stress known as an essential agent implicated in the pathogenesis of HCC and ROS is proved to be the main reason for liver cancer (Fu and Chung, 2018). Carcinogenesis may proceed if a lack of harmony between oxidative stress and the efficiency of antioxidant defense happens. It appears that the DNA damage is predominantly linked with the initiation process of cancer. It is now well established that ROS can damage DNA and that GSH can protect against this type of damage (Valko *et al*., 2007). DNA mutation is a decisive step in carcinogenesis and raised levels of oxidative DNA lesions (8-OHdG) have been recognized in many tumors (Guo *et al*., 2016) Here, rats with HCC exhibited oxidative stress as noticed from an increment of hepatic MDA (a signal of lipid peroxidation) and a reduction of GSH or CoQ10 levels. The significant increase in lipid peroxidation may be the elucidation for the liver damage noticed in this study. The participation of lipid peroxidation in the cell membrane damage due to chain reaction is well affirmed (Ayala *et al*., 2014). Administration of ZnO-NPs to rats with HCC in this study opposed oxidative stress as shown by the conservation of antioxidant levels of hepatic GSH, CoQ10, SOD, and lowering of MDA (Oxidized lipids). Moreover, ZnO-NPs treatment to HCC

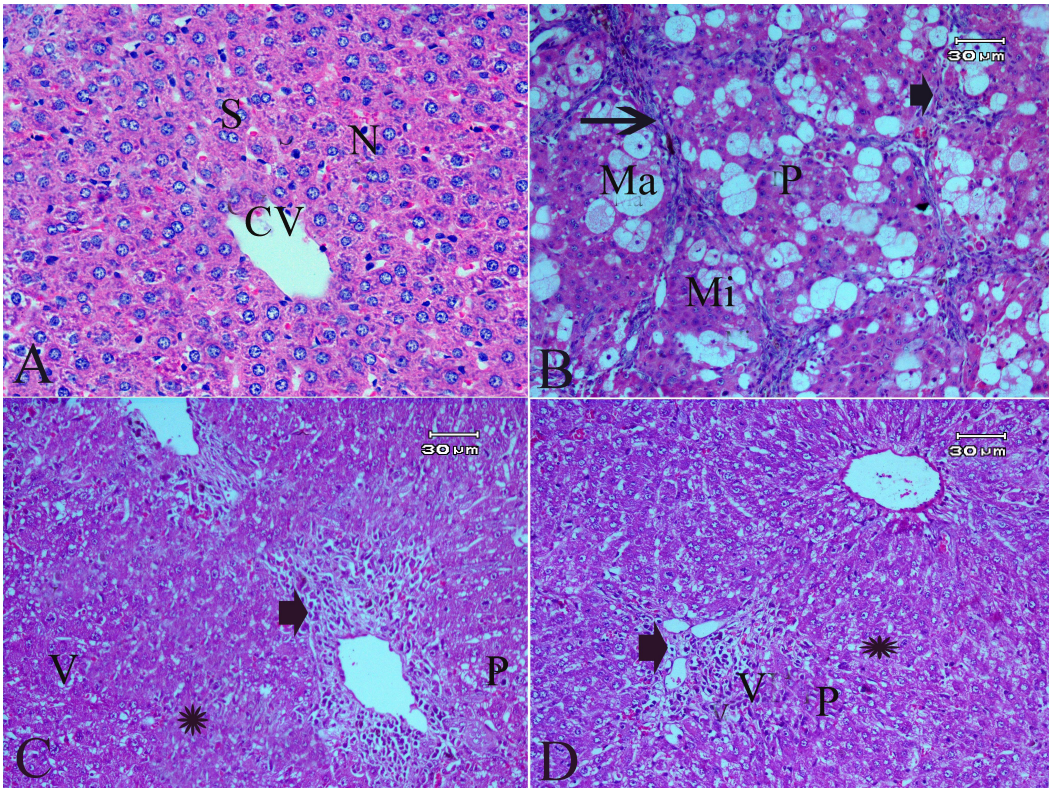

Figure 5. Effect of zinc nanoparticles on liver histopathology of rats with HCC (400×; H&E stain). The control group showing normal central vein (CV), sinusoids (S), and nucleus (N). (B) The liver of the HCC group showing loss of hepatic lobular architecture, ballooning changes of hepatocytes, pyknotic nuclei (P), macrovesicular (Ma), microvesicular steatosis (Mi), portal tracts extended with massive necrosis (arrowhead), inflammation cell infiltration or fibrous tissue, and periportal and pericellular fibrosis (arrow). (C) The liver of the HCC + 5 mg/Kg Zn-ONPs group showing partly preserved hepatic normal architecture, moderate degrees of steatosis (Star), the hepatocytes were still, vacuolated (V), swollen with narrow sinusoids. Inflammation cell infiltration (arrowhead), and portal tracts to pericellular fibrosis were extended with moderate fibrous tissue was observed. (D) The liver of the HCC + 10 mg/Kg ZnO-NPs group showing inflammation cell infiltration (arrowhead) and hepatocytes that were still swollen, but they were notably reduced in extent and were less frequent compared to the HCC group whereas microvesicular steatosis (star) was still present in the surrounding liver parenchyma with pyknotic nuclei (P).

rats decreased liver function markers as AST, ALT, and GGT suggesting a decrease in liver pathology. It was reported that ZnO-NPs showed antioxidant activity via scavenging of free radicals (Tettey and Shin, 2019) that may participate in the restoration of the antioxidant system. GSH also inhibited free radical damage, and its decrease is connected with the progression of cancer (Vieira *et al*., 2011). Additionally, CoQ10 is a vigorous antioxidant that conserves cells from free radical-stimulated oxidative damage (Singh *et al*., 2007). Since increased levels of ROS have been proposed to enhance the expansion of HCC by motivating DNA and genes changes (Machida *et al*., 2004), the anticancer activity of ZnO-NPs can be attributed to the decrease in 8-OHDG level, a marker for oxidative DNA damage and carcinogenesis (Valavanidis *et al*., 2009). The decrease in the 8-OHDG level due to treatment of HCC rats with ZnO-NPs can be referred to as the decrease in TNF-α level that enhanced the formation of 8-OHDG through an increase in oxidative stress (Wheelhouse *et al*., 2003). Another interpretation for the anticancer of ZnO-NPs is related to the reduction of NO level in the liver that motivated tumor angiogenesis by elevation of the blood flow in the tumor cells and so induces tumor development and growth (Oktem *et al*., 2004). Furthermore, zinc is implicated in some vital cellular processes related to cancer progress through the stimulation of DNA repair and hindering apoptosis (Ho, 2004).

Our results demonstrated an anti-inflammatory activity of ZnO-NPs as proved by a reduction in CRP, IL-6, and TNF-α levels in the HCC rat model. The antioxidant and anti-inflammatory characteristics of ZnO-NPs were mentioned (Nagajyothi *et al*., 2015) in the lipopolysaccharide animal model. It was suggested that ZnO-NPs lowered hepatic damage induced by thioacetamide by reducing IL-6 and lipid peroxidation levels (Bashandy *et al*., 2018). IL-6 could be believed a convenient tumor marker for HCC particularly with AFP (Porta *et al*., 2008). Inflammatory cells are tumor developers that make a suitable environment for tumor growth, DNA damage, angiogenesis, and metastasis (Allin *et al*., 2009). Our results illustrated fibrosis in the liver of rats with HCC. The development of HCC involves several stages and includes inflammation-associated fibrosis and cirrhosis via activation of hepatic stellate cells by cytokines and ROS, produced in the injured liver (Huang *et al*., 2014). Serum CRP- (an inflammatory marker) is upregulated in HCC (Carr *et al*., 2018).

ZnO-NPs can modulate alteration of lipid metabolism and carbohydrate metabolism observed in HCC rats and reduce the elevation of cholesterol, triglycerides, and LDL observed in HCC rats. It has been reported that zinc given to diabetic patients led to the decrease in lipid parameters (cholesterol, LDL, and triglycerides) and the increase in HDL-cholesterol (El-Ashmony *et al*., 2012). The present results suggested that ZnO-NPs affect

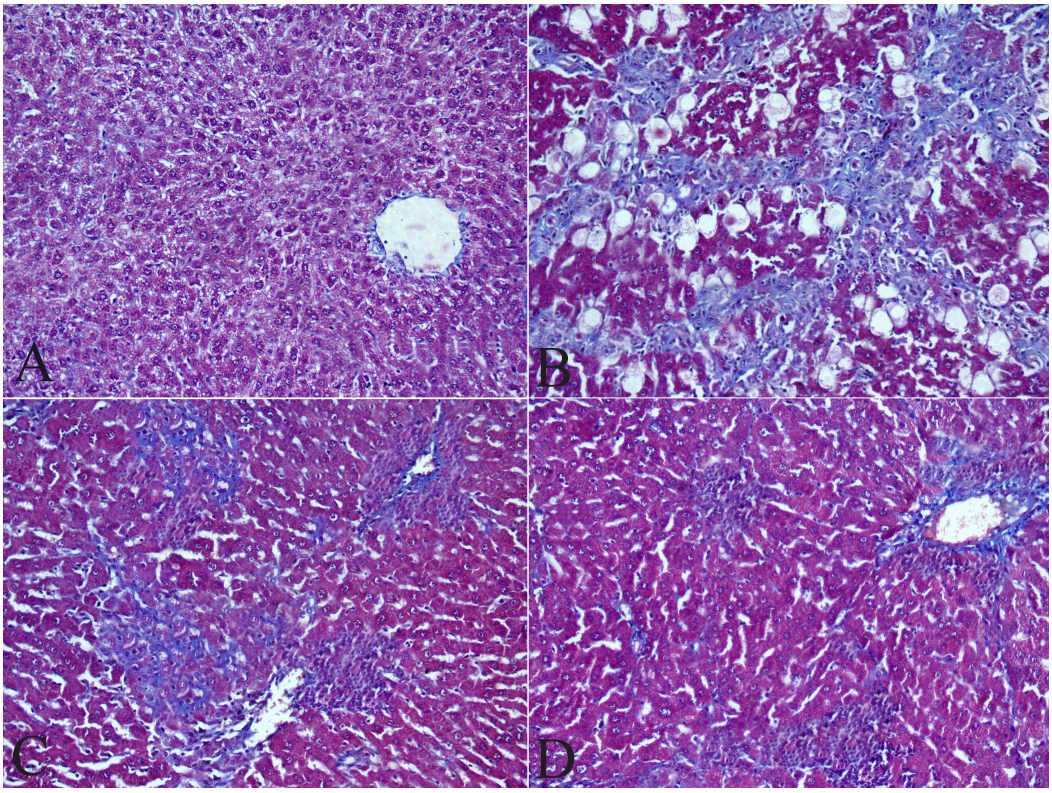

Figure 6. Effect of zinc nanoparticles on liver fibrosis (400×; Masson's trichrome stain). (A) Liver section of control rat showing no collagen fibers. (B) Liver section of the HCC group showing marked increased fibrosis, multiple fibrotic nodules, and blue collagen fibers which are seen among periportal and pericellular areas. (C) Liver section of the HCC + 5 mg/kg ZnO-NPs group showing fewer strand fibers of collagen among periportal and pericellular areas. (D) Liver section of the HCC + 10 mg/kg ZnO-NPs group showing fewer collagen fibers among periportal and pericellular areas.

lipid metabolism indirectly by hindering the elevation of TNF-α level in HCC rats. TNF-α motivates atherogenic pathways through the lessening of HDL production, stimulation of the process of cholesterogenic gene expression, and inhibition of cholesterol elimination (Tacer *et al*., 2007). Moreover, ZnO-NPs antagonized the decrease in the glucose level, hepatic ATP, and mucopolysaccharides levels. It was reported that zinc can widely modify energetic metabolism and is major in reconditioning the declined energetic metabolism of cellular physiology (Yang *et al*., 2017).

Trace elements may be engaged in the development of HCC. There is an association between copper, magnesium, and zinc and the severity of cirrhosis or HCC (Arirudran *et al*., 2014). Here, the hepatic zinc level decreased significantly in the HCC group, while copper and manganese levels increased significantly. Copper was concluded to be elevated in serum patients with liver cirrhosis (Nangliya *et al*., 2015). The increase in hepatic copper level in HCC rats may be due to the elevation of Ceruloplasmin, a major copper-carrying protein (Pousset *et al*., 2001). The histopathological changes observed in the HCC group can be attributed to copper accumulation that results in oxidative liver injuries (Hatano *et al*., 2000) or to a decrease in zinc content that has antioxidant properties (Jarosz *et al*., 2017). It was observed that the manganese absorption in patients with hepatic cirrhosis is higher than that in normal adults (Takikawa, 1990). The increased manganese level in the liver of HCC may be due to enhancement of its absorption through the intestine. It has appeared that the improved liver structure of HCC rats treated with ZnO-NPs can be explained by the increase in hepatic zinc level and the decrease in hepatic copper or manganese concentrations.

In conclusion, the treatment of HCC rats with ZnO-NPs showed an anticancer effect which may be due to modulation of oxidative stress, metabolic disorders, oxidative damage of DNA, inflammatory markers, and trace elements. Considering the highly specific characteristics of ZnO-NPs and their selectivity and toxicity toward cancer cells may make them a new therapeutic for next-medication cancer treatment. Although ZnO-NPs improve many parameters involved in the development of HCC, some pathological changes were notified in the liver of the HCC + ZnO-NPs groups. These may mean that ZnO-NPs need more modifications to become more effective.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

Study protocol was approved by National Research Centre (NRC), Egypt with ethical approval No. 19218.

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