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RESEARCH ARTICLE

Preventive Effect of ZnO-Metformin Nanocomposite Against Carbon Tetrachloride-Induced Hepatotoxicity

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ABSTRACT

Objective(s): The protective effect of metformin on liver disorders has been reported. Studies have documented the hepatoprotective benefits of metformin against liver disorders, but due to its low bioavailability and untoward side effects, metformin has limited therapeutic utility. Nanotechnology has increased efficiency and reduced the side effects of several drugs.

Methods: A ZnO-metformin nanocomposite was synthesized. The protective effects of metformin (150 mg/kg), ZnO (10 mg/kg), and the ZnO-metformin nanocomposite (50, 100, 150 mg/kg) were compared in a CCl4-induced hepatocellular damaged mouse model. Serum liver enzymes, oxidative stress biomarkers, and histological changes in hepatocytes were evaluated.

Results: The levels of AST, ALT and ALP in the blood were prevented by the metformin and ZnO-metformin nanocomposite against the action of CCl4, as well as liver damage associated with it. Furthermore, metformin or/and ZnO-metformin nanocomposite reversed the SOD, GPx and GR activity that was found to be lowered due to CCl4, as well as increased MDA levels under the same condition. The extent of hepatoprotection with respect to the two agents in question also differed significantly in terms of dose. The ZnO-metformin nanocomposite hepatoprotective effects occurred at lower doses than metformin. ZnO alone did not alter the CCl4-induced hepatic injury.

Conclusions: The ZnO-metformin nanocomposite was a more efficient hepatoprotective agent at lower doses than metformin. The ZnO-metformin nanocomposite has the potential to be a replacement for metformin as a hepatoprotective drug candidate.

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INTRODUCTION

The liver can be adversely affected by toxins, drugs, and industrial and agricultural chemicals [1, 2]. Hepatocytes are highly sensitive to the effects of such chemicals [3]. Carbon tetrachloride (CCl_4) is a hepatotoxic agent used to induce hepatocellular damage in experimental animals [4-6]. A primary mechanism of CCl_4 and related hepatotoxic agents is activating free radicals and inhibiting antioxidants [7]. Numerous studies have evaluated chemicals with hepato-protective properties [8-10], including medications in repositioning studies [8-10]. Metformin effectively manages

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hepatocellular damage caused by toxins, drugs, and abused substances by inhibiting oxidative stress, inflammation, and cell death [1, 2, 11-13]. The role of metformin in inhibiting free radicals and activation of antioxidant enzymes in hepatocytes has also been reported [14, 15].

Nanotechnology can increase the efficiency of existing and new drugs, reduce their side effects, increase their potencies, improve drug delivery, and potentiate the drug mechanism by activating the target receptors and improving the pharmacokinetics and pharmacodynamics of the drug [16-18]. However, the effect of a metformin nanoformulation in managing hepatotoxicity in nonclinical and clinical studies is unclear [16-18].

A ZnO-metformin nanocomposite was synthesized, and its hepatoprotective effects were investigated by examining several liver enzymes, oxidative stress changes, and the histology of hepatocytes in an experimental rodent model of CCl_4-induced hepatotoxicity.

MATERIALS AND METHODS

Drugs, Substances, and Equipment

Metformin hydrochloride (1,1-dimethylbiguanide hydrochloride) and zinc nitrate tetrahydrate (Zn (NO3)2•4H2O) were obtained from Sigma-Aldrich (USA) and were dissolved in regular saline solution. Both substances were prepared freshly before usage, and the volume was adjusted to 0.3 mL per mouse. All other materials and solvents used were of the highest available purity (DNA Biotech Co, Tehran, Iran). A Shimadzu 8400S infrared spectrometer (Shimadzu 8400S, Japan) with pressed KBr disks was used for infrared spectroscopic analysis. X-ray diffraction patterns were obtained using a Shimadzu 7000S instrument (Shimadzu 7000S, Japan). The ZnO-metformin nanocomposite was dissolved in regular saline solution for administration to the mice.

Synthesis of ZnO-Metformin Nanocomposite

To encourage the formation of hydrogen bonds, metformin hydrochloride (0.0166 g, 0.1 millimole) was dissolved in 10 mL of methanol and subjected to heating at 50-60°C. The solution's pH ranged from 6 to 7. Zinc nitrate tetrahydrate (1.3 g, 5 millimole) and KOH (0.056 g, 1 millimole) were added gradually to the methanol solution (pH between 7 and 8). In the next stage of the process, the mixture was refluxed and stirred at a steady rate (1400 rpm) for 2 hours in an oil bath at ~ 150°C. After cooling, the unreacted white solid ZnO was removed by ethanol filtration. The ZnO-metformin nanocomposite solution was allowed to precipitate over several days (four days) at 20 to 25 °C. The resulting black solid was washed with diethyl ether to remove impurities and air dried [19-22]. The melting point of the ZnO-metformin nanocomposite was 223°C [19-21].

Characterization of ZnO-Metformin Nanocomposite

The synthesized ZnO-metformin nanocomposite was compared to metformin using FTIR and XRD to assess particle size, morphology, and chemical composition [19-21]. The X-ray diffraction pattern was obtained within the temperature range of normal conditions, utilizing Cu Kα radiation (1.54 Å) with a current of 40 mA, while scanning the angle of 2θ from 10° to 80°. The X-ray diffraction spectrometer (Shimadzu 7000S) was then set to a voltage of 40 kV at room temperature. The structural analysis was carried out using FTIR spectroscopy. Functional groups were identified based on their characteristic vibration modes, which were captured using FTIR (Shimadzu 8400S) in the spectral range of 400 cm-1 to 4000 cm-1. This range encompassed both the functional and fingerprint regions, serving as the primary method for identifying the complete molecule [19-22].

Animals

Fifty-six male adult mice, weighing approximately 25-30 g on average, were acquired from the Iran University of Medical Sciences in Tehran, Iran. Subsequently, they were transferred to Masih Danshvari Hospital at the Shahid Beheshti University of Medical Sciences. The animal rooms maintained a controlled environment at a temperature of 22 ± 0.5°C, with a relative humidity between 40 and 60%, and a 12-hour light/dark cycle. The mice were provided with unrestricted access to feed (Pars Animal Feed Co, Tehran, Iran) and local water. The experimental protocol was conducted in compliance with the Guidelines of Animal Ethics and Welfare, following the standardized ARRIVE procedures [23]. This protocol received approval from the Institutional Animal Care and Use Committee, with the research protocol and ethical code number IR.SBMU.NRITLD.REC.1402.213.)

Experimental Procedures

The mice were split into seven sets, each with eight mice, using a random method. The amounts of metformin and CCl4 were chosen based on earlier studies. In Group 1 (sham), mice got normal saline (0.2 mL/mouse, inside the belly) every day for four weeks. Group 2 (no treatment) mice were given CCl4 (1.5 mg/kg, inside the belly) twice a week for two weeks, then no treatment for two weeks. Group 3 (with treatment) animals received CCl4 (1.5 mg/kg, inside the belly) twice a week for two weeks, then got metformin (150 mg/kg, inside the belly) every day for the following two weeks. Groups 4-6 got CCl4 treatment (1.5 mg/kg, injected) twice a week for first two weeks, then daily ZnO-metformin nanocomposite at doses of 50 mg/kg, 100 mg/kg, or 150 mg/kg, injected, for next two weeks. However, Group 7 mice received CCl4 (1.5 mg/kg, injected) twice a week for first two weeks, and then treated with 10 mg/kg of ZnO, injected daily for next two weeks.

On Day 28, the mice were put to sleep using thiopental (50 mg/kg, injected)[24, 25], and their livers were surgically removed. One part of each liver was kept in 10% buffered formalin for histopathological analysis. The rest of the liver was frozen at -20 ºC to check oxidative stress [25, 26]. Blood samples were taken from the heart and kept to clot for 50 minutes at room temperature before measuring serum levels of AST, ALT, and ALP [27].

AST, ALT, and ALP Levels and Oxidative Stress Parameters

Serum AST, ALT, and ALP levels were assessed using commercially available kits (Stanbio kits, EKF Co, Boerne USA) with a UV-rate auto-analyzer (Hitachi 736-60, Japan)[28-30].By measuring the absorbance of NADH at 340 nm, serum AST, ALT, and ALP were quantified and reported as Iu/mL. An International Unit (IU) per liter represents the quantity of enzyme needed to oxidize one micromole of NADH per minute)[28-30].

Oxidative Stress Parameters

Lipid peroxidation, which is the process of oxidative damage to lipids, was evaluated through the measurement of malondialdehyde (MDA) levels. The activity of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR), was determined using established methods as described before [25, 31].

Histology

The tissues underwent standard processing procedures [32, 33], and thin sections measuring five micrometers were stained with hematoxylin and eosin. The histopathology assessment of hepatocytes was performed by examining an area of 1.30 mm within the liver subfield across all sections [32, 33].

Statistical Analysis

All data were processed using GraphPad PRISM v.11 software (California, USA) to calculate the means ± SEM. Significant differences among all groups were assessed using ANOVA and Tukey's post-test, with a significance level of $p < 0.001$. An F-test with (6, 49) degrees of freedom was conducted for all parameters, with the F ratio and p-value reported in parentheses after each experimental parameter

RESULT

ZnO-Metformin Nanocomposite

The diffraction patterns of ZnO, identified as the hexagonal wurtzite structure, were compared to the JCPDS database (card no. 036-1451), which provides standard XRD reference patterns for different materials. This comparison, depicted in Figure-1 A, confirmed the high crystallinity of the ZnO-NPs. At the following orientations (31.65°, 34.28°, 36.30°, 47.57°, 56.57°, 62.87°, 67.92°, 69.04°), the diffraction peaks observed were indexed to hexagonal wurtzite phase of ZnO as shown in Table-1. Analyzing the FTIR spectra of metformin (Figure-1 C) and ZnO-metformin nanocomposite (Figure-1 B) was noticed that the peaks of C = N bonds at 1629 and 1587 cm-1 moved to the right hand side (increased frequency), this indicated that there is a metformin and zinc cation interaction. Additionally, the absorption band at 459 cm-1 confirmed the presence of a Zn-N bond. These results further supported the successful synthesis of the ZnO-metformin nanocomposite.

Effect of ZnO-Metformin Nanocomposite on CCL_4-Induced Oxidative Stress

In comparison with the sham group (Figure-2), administration of CC14 to the animals led to a significant growth in MDA hepatic levels (39.99; $p<0.001$) coupled with a simultaneous decrease in SOD (17.84; $p<0.001$), GPx (12.59; $p<0.001$) and GR (21.27; $p<0.001$). ZnO-metformin nanocomposite administration (at 50, 100, and

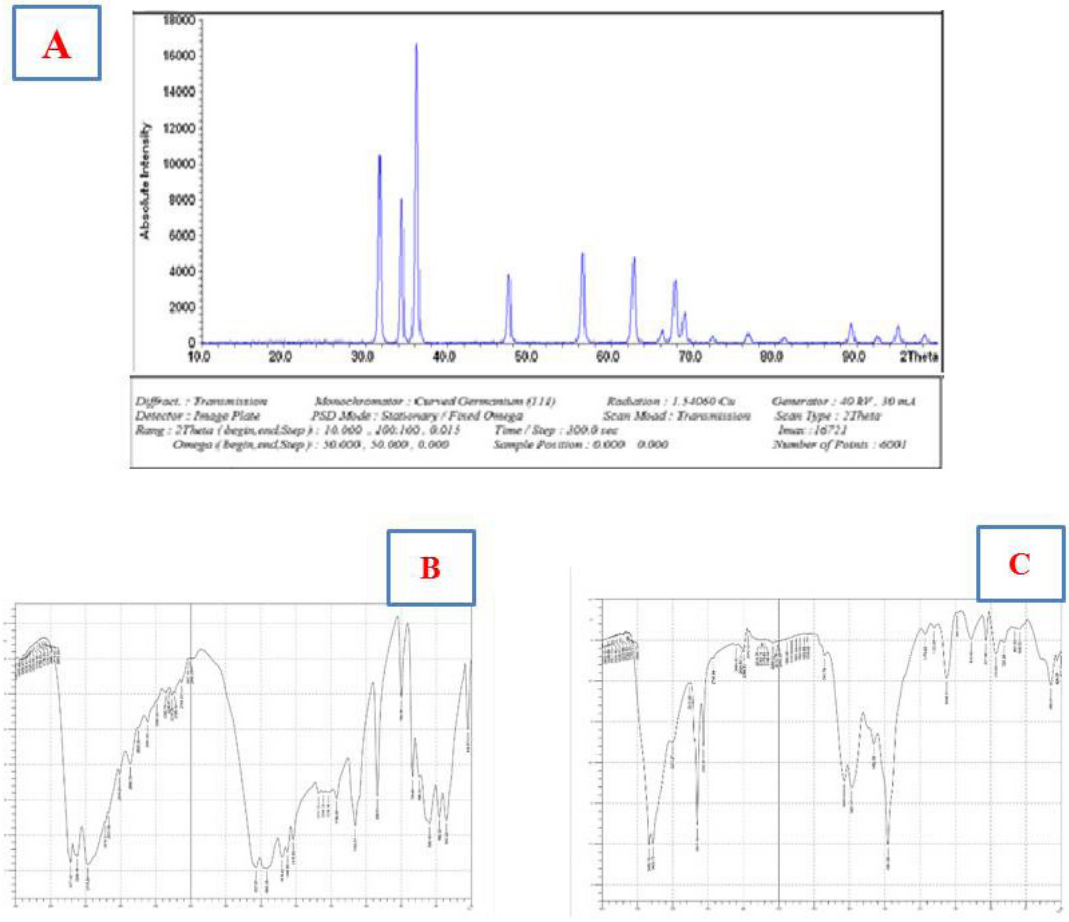

Fig.1. (A)XRD patterns of ZnO-metformin nanocomposite, (B) FT-IR of ZnO-metformin nanocomposite, and (C) FT-IR of Metformin.

150 mg/kg doses) however attenuated CCl4-induced oxidative stress as evidenced by a reversal of MDA (39.99; p<0.001), SOD (17.84; p<0.001), GPx (12.59; p<0.001) and GR (21.27; p<0.001) concentrations in comparison to the negative control group (Figure-2). Furthermore, the CCl4-induced elevation of MDA (39.99 p<0.001) and SOD (17.84 p<0.001), GPx (12.59 p<0.001) and GR (12.62 p<0.001) decrease were all reduced when metformin was administered at 150mg/kg compared to a negative control group (Figure-2). In general, the use of CCl4 and ZnO (10 mg/kg) together did not result in any significant difference from the effect of CCl4 alone (Figure-2).

Effect of ZnO-Metformin Nanocomposite on CCl_4-Induced Hepatic-related Function of Serum Enzymes

In comparison to the control group, there was a significant increase in AST (14.08; p<0.001), ALT (13.50; p<0.001) as well as ALP (12.62; p<0.001) levels after the circulation of CCl4 in the body (Figure-3). The administration of ZnO-metformin nanocomposite in combinations 50, 100 and 150 mg/kg significantly reduced levels of AST (14.08; p<0.001), ALT (13.50; p<0.001), and ALP (12.62; p<0.001), which was induced by CCl4 in comparison to 1.5 mg/kg of CCl4 alone treated group (Figure-3). The CCl4-induced increases in AST (14.08; p<0.001), ALT (13.50; p<0.001), and ALP (12.62; p<0.001) levels were also reduced by use of metformin alone at a dose of 150 mg/kg compared to the negative control group (Figure-2). The outcome observed in the CCl4 (1.5 mg/kg) treated negative control group was the same as that produced by ZnO at a dose of 10 mg/kg (Figure-3).

Table 1. Table of data of XRD of ZnO-metformin nanocomposite. Peak intensity, position, and width, full width at half-maximum (FWHM) data.

POS:(2Th)	FWHM Left (2Th)	d_spacing (A)	ReI_Int(%)
31.65	0.089	2.83	60.68
34.28	0.148	2.62	37.73
36.30	0.148	2.47	100.00
47.57	0.148	1.92	22.63
56.57	0.133	1.63	31.32
62.87	0.20	1.48	24.26
67.92	0.177	1.38	20.26
69.04	0.177	1.36	9.61

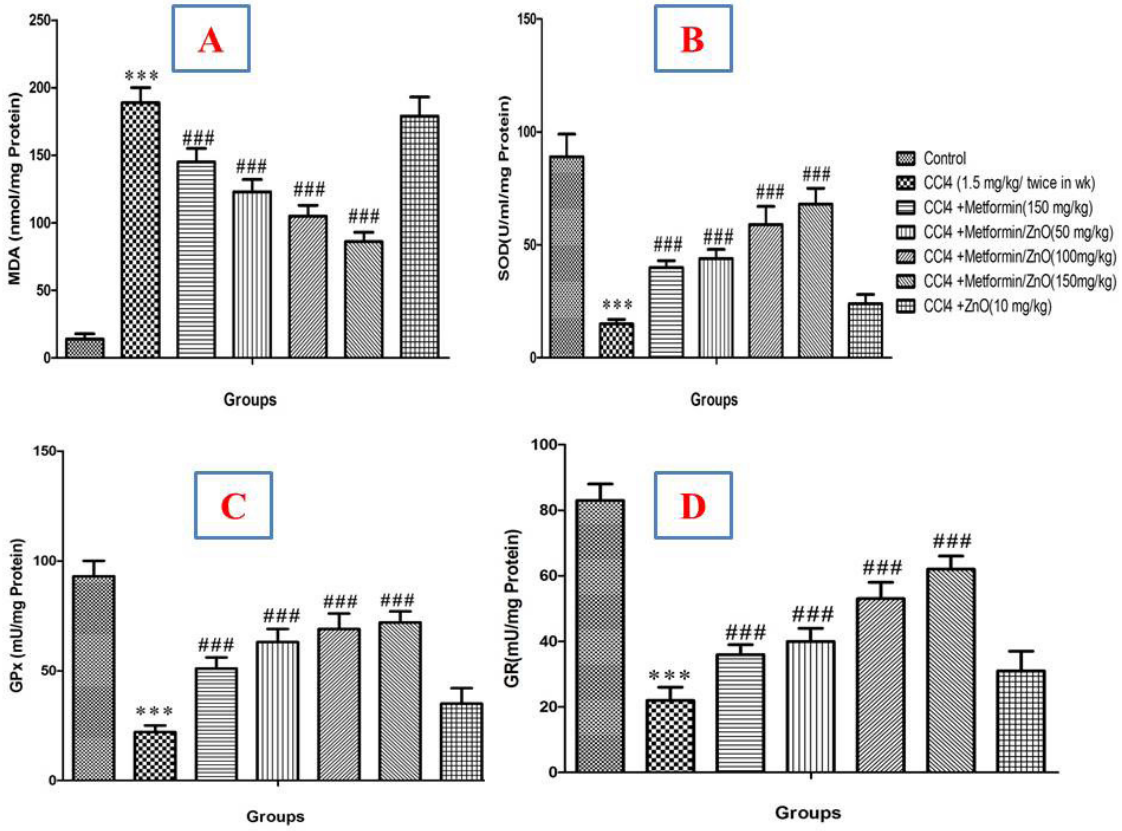

Fig. 2. Effect ZnO-metformin nanocomposite (50,100, and 150 mg/kg) and metformin (150 mg/kg) on CCL_4- induced MDA(A) level and also SOD(B),GPx(C) and GR(D) activity. All data are expressed as mean ± SEM (n=8).
*** p<0.001 vs. CCL (1.5 mg/kg twice in wk) group
p<0.001 vs. control group

Histological Studies

The hepatocytes of the groups treated with CCl4 also showed noticeable alterations compared to those of the sham group (Figure-4). Nevertheless, the administration of metformin in a dose of 150 mg/kg mitigated the degeneration of hepatocytes as compared with the negative control group (Figure-4). Similarly, the ZnO-metformin nanocomposite at the doses of 50, 100, and 150 mg/kg reduced the CCl4-induced hepatic cell degeneration of the negative control group. In contrast, CCl4-induced hepatocellular degeneration was noted in all the animals treated with a combination ZnO (10 mg/kg) and CCl4 in comparison to the negative control group (Figure-4).

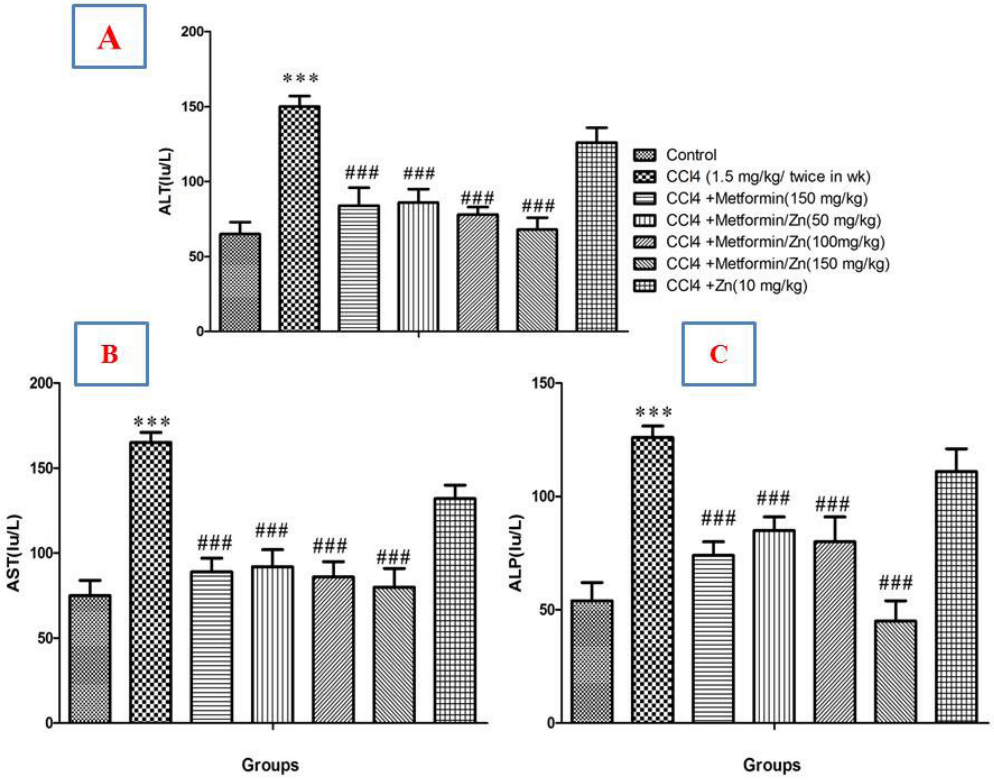

Fig. 3. Effect ZnO-metformin nanocomposite (50,100, and 150 mg/kg) and metformin (150 mg/kg) on CCL_4- induced ALT(A), AST(B) and ALP(C) activity. All data are expressed as mean ± SEM (n=8).
*** p<0.001 vs. CCL (1.5 mg/kg twice in wk) group
p<0.001 vs. control group

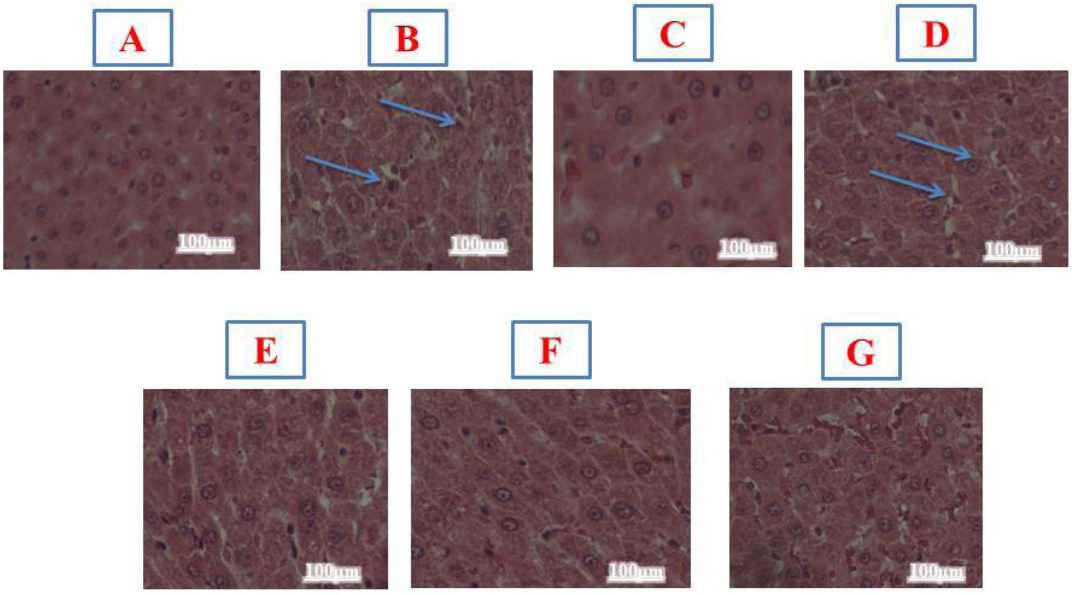

Fig. 4. Representative Images of Hematoxylin Eosin-stained Hepatocyte in control (A) CCL_4 (B), CCL_4+Metformin(C), CCL_4+Metformin/ZnO (50,100, and 150 mg/kg)(D,E and F), CCL_4+ ZnO(10mg/kg)(G) treatment groups. Arrows indicate the vacuolation and degeneration in Hepatocytes (Magnification x400. Scale bar 100 µm).

DISCUSSION

The liver cells or hepatocytes are typically affected by many dangerous substances for instance carbon tetrachloride (CCl4) [34]. This substance can lead to a dysfunction in the mitochondria of liver cells and an imbalance in respiratory enzymes leading to oxidative stress when it is administrated into young rats, mice as well as adult ones [5, 35, 36]. Nevertheless, the current studies have indicated that ZnO nanoparticles loaded with metformin could attenuate CCl4-induced oxidative stress in mice. The nanocomposite improves enzyme levels related to liver function in blood also. Additionally, histopathological analysis confirms that ZnO-MET nanocomposite exhibits similar protective effects on the liver

Research has been carried out to identify hepatoprotective chemicals [37, 38]. Metformin, a well-Among the well-known scavengers of free radicals is metformin, a substance which has demonstrated its importance in liver by reducing inflammation and death of cells, inhibiting the generation of free radicals and initiating anti-oxidation [1, 2, 12, 13, 39, 40]. Nonetheless, metformin has low solubility in water implying that large quantities have to be used for liver protection. Unfortunately, this is often accompanied by unwanted systemic effects [41-44].

In the present study we assessed whether combining ZnO (a nanocomposite) with metformin could enhance solubility as well as effectiveness and efficiency of the drug while reducing its negative effects during mouse model of CCl4-induced liver damage [45, 46]. Mice injected with CCl4 experienced oxidative stress due to elevation in their MDA levels (an index of oxidative destruction) and reduction in their SOD, GPx activities and GR.

Metformin decreased MDA concentrations and increased the activities of SOD, GPx, and GR in rat liver damage caused by CCl4, which is in agreement with previous findings [47, 48]. A ZnO-metformin nanocomposite caused a dose-dependent decrease in MDA levels and an improvement in the activities of SOD, GPx, and GR in CCl4-treated rats at lower concentration than metformin alone. As a result, the nanocomposite enhanced the efficiency while reducing some side effects as well as increasing the potency of the drug metformin; all these are very important considerations to be made when developing medicines [16, 49].

However, regardless of numerous available data on this issue, there is no clear understanding about what exactly metformin nanocomposites can do for CCl4-induced liver damage. On the contrary, this research work revealed that ZnO-metformin nanocomposite had an equal therapeutic effect against CCL4-induced liver damage compared to using only metformin but less doses with reduced negative impacts

CONCLUSIONS

According to our findings, combining ZnO with metformin resulted in a nanocomposite that effectively counteracted liver damage caused by CCl4. This was achieved by enhancing the effectiveness of metformin. Both the ZnO-metformin nanocomposite and metformin alone were able to suppress liver toxicity induced by CCl4. This was demonstrated by a decrease in the activity of various liver enzymes and the prevention of liver cell degeneration. Notably, the ZnO-metformin nanocomposite exhibited greater potency compared to metformin alone. Consequently, it has the potential to replace metformin as a candidate drug for protecting the liver

CONFLICT OF INTEREST

None.

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None

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Use and Care Committee of the Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Research Protocol and Ethical Code Number =IR.SBMU.NRITLD.REC.1402.213).

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