

Therapeutic role of bone marrow-derived stem cells and zinc sulfate for reduction of liver fibrosis

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ABSTRACT

Introduction: Hepatic fibrosis is considered as the initial state of any chronic hepatic disease; progression causes accumulation of extracellular matrix (ECM) and develops into cirrhosis. In case of liver failure, transplantation was considered as the only suitable therapy but due to the hurdles in transplantation, therapy using stem cells was promoted. Mesenchymal stem cells (MSCs) are widely used for the treatment of a variety of diseases. Oxidative stress at the damage site causes poor MSC proliferation and engraftment. Thus, it is necessary to induce antioxidants with MSCs to enhance potency. **Methods:** To explore the therapeutic potential of zinc sulfate (ZnSO₄) and MSCs on carbon tetra chloride (CCl₄)-induced hepatic toxicity, 6-8 week-old female albino mice (BALB/c) were used. Initially, mice were infected with CCl₄ and then intra-peritoneally injected with MSCs only, ZnSO₄ only, or a combination of MSCs and ZnSO₄. MSCs were isolated from femur and tibia of mice, and were cultured under control conditions. **Results:** The morphological results revealed that in contrast to MSC-only therapy, ZnSO₄ enhanced the therapeutic potential of MSCs when administered to CCl₄-injured mice. Biochemically, level of serum Alanine Transaminase (ALT) and total bilirubin was found to be significantly decreased in ZnSO₄ + MSC transplanted mice. Histopathological examination also revealed that ZnSO₄ + MSC transplantation induced a strong anti-apoptotic effect on CCl₄-injured liver. Reverse transcription polymerase chain reaction (RT-PCR) detected a noteworthy anti-fibrotic effect of MSCs in the presence of ZnSO₄, down-regulation of apoptotic marker (*Bax*), and up-regulation of anti-apoptotic (*Bcl-xl*) and hepatic (*Albumin*) markers. **Conclusion:** Thus, it was concluded that the presence of ZnSO₄ reduced oxidative stress, enhanced the proliferation rate of MSCs, and significantly attenuated hepatic fibrosis.

Key words: Albumin, Bax, Bcl-xl, Liver Fibrosis, Mesenchymal stem cells, Zinc sulfate, β -Actin

INTRODUCTION

Liver is one of the largest organs and central metabolic hub of the human body. Hepatocytes are the main building block of the liver and perform a variety of functions, such as protein synthesis, detoxification, carbohydrate, lipid metabolism and bile production. Hepatic fibrosis is the trademark of any chronic liver disease and is the major cause of morbidity and mortality. Cirrhosis is the final-stage of hepatic disease which occurs when the fibrotic tissues replace the healthy tissues¹. In liver fibrosis, repeated injury causes tissue alteration, which is observed by extreme production of extracellular matrix (ECM) with the formation of scar tissue covering the region of disease². The main causes of liver fibrosis are alcohol usage, viral hepatitis B and C (HBV and HCV), hepatitis due to autoimmune disorder, obesity, diabetes, parasitic disorder, metabolic disorder, biliary disease, as well as vulnerability to toxins, chemicals and drugs³. It was reported globally that liver cirrhosis was a major cause of over one million deaths in 2010, equivalent to 2% of all deaths globally⁴. Chronic liver dis-

eases account for 1 in 40 deaths worldwide⁵. Hepatocytes have a remarkable efficiency to repair the infected liver and restore its normal functionalities; when the inherent power of repairing hepatocytes is lost, liver transplantation remains the only efficient treatment. This procedure is highly invasive and limited due to the scarcity of donors, it is not exempt of complications⁶.

Nowadays, stem cells (SCs) are broadly used for the regeneration of hepatocytes. The high potency of cell multiplication and multipotency are the primary characteristics of a stem cell. The proliferation and differentiation capability of stem cells depends on the source and type of stem cells, and is referred to as "stemness"⁷. If suitable conditions with accurate signals are provided, stem cells can differentiate into variety of cell types and lead to formation of organs and, finally, a complete organism. Therefore, SCs have valuable properties for regenerative medicine⁸. Based on differentiation, SCs are classified into totipotent, pluripotent, multipotent or unipotent⁹, while based on source and properties,

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they are categorized into embryonic SCs (ESCs), induced pluripotent SCs (iPSCs), and non-embryonic SCs/adult stem cells (ASCs)¹⁰.

ESC therapy has some limitations; these include safety and ethical issues, as their acquirement involves the destruction of embryos due to which ethical concerns are raised. Due to this limitation of ESCs, ASCs provide another platform for therapy of liver diseases, especially bone marrow derived stem cells (BMSCs)¹¹. ASCs also have the ability of proliferation, differentiation and regeneration of tissues¹². ASCs are classified into hematopoietic stem cells (HSCs) which are blood-forming cells, and Mesenchymal Stem Cells (MSCs), which are somatic-forming cells. It had been thought that HSCs in the bone marrow could not differentiate into the variety of cells, such as nerve cells in the brain.

MSCs have the ability of self-renewal and differentiation into a variety of cell types. MSCs have fibroblastic appearance with multipotent nature and can be isolated from a wide range of tissues but bone marrow and gingiva are the key sources of MSCs^{13,14}. There, MSCs are responsible for the maintenance of the microenvironment (niche) and provide support to HSCs in the bone marrow¹⁵. MSCs can also be differentiated into hepatocytes to treat different types of liver disease^{16,17}.

Oxidative stress is a pathogenic mechanism, whereby exposure of the liver to oxidative stress damages the liver and leads to hepatic fibrosis. Cell damage increases with the increase in concentration of reactive oxygen species (ROS) in the liver. The use of antioxidants reduces the amount of these free radicals¹⁸. Enzymes involved in the synthesis and breakdown of carbohydrates, lipids, proteins and nucleic acids, and in the metabolism of other micro-nutrients, contain zinc as one of their key components. Zinc has been demonstrated to protect against hepatotoxicity produced by a variety of chemicals¹⁹.

The aim of the present study was to investigate the therapeutic potential of MSCs, in the presence of Zn, against CCl₄-induced liver toxicity in mice.

METHODS

Preparation of animals

Six to 8 week-old female albino mice (*BALB/c*), with weight ranging from 25-30 g, were bought from the Directorate of Veterinary Research Institute, Peshawar, Pakistan. All mice were housed in a pathogen-free environment at optimum temperature (20-25 °C) and were provided a standard rodent diet with appropriate cycle of light and dark. The Bioethics

Committee of the Biochemistry Department, Abdul Wali Khan University, Mardan (AWKUM) approved all the laboratory procedures. For therapeutic treatment of liver fibrosis, different mouse treatment groups were assigned, such as positive control group (only infected with CCl₄), negative control group (untreated group), MSC-only treated group, Zn-only treated group, and MSC + Zn treated group. Three mice were used for each group (triplicates) and a few were used to isolate bone marrow cells. Initially, CCl₄ was injected in the body of mice via intra-peritoneal route, at a dose of 1 µl/g (do you mean 1 µg/ml) per gram body weight, dissolved in an equal volume of olive oil (1:1 ratio). The injection was given with 1 ml insulin in syringe- twice a week for 4 weeks- to induce hepatic fibrosis.

Isolation and characterization of MSCs

MSCs were isolated from femur and tibia of *Balb/C* mice according to the protocol described by Khan *et al.*²⁰, and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France) and 100 IU/ml penicillin and 100 µg/ml streptomycin (both from Capricorn Scientific, Ebsdorfergrund, Germany) in 25-mm culture flasks. The culture was maintained in a humidified incubator supplied with 5% CO₂ at 37°C. The stem cells were characterized as MSCs by the method described by Oyagi *et al.*²¹. After 3 days of culturing, in each flask the medium was removed completely and washed with PBS (twice) to remove non-adherent cells (HSCs) while the adherent cells (MSCs) remained attached. After washing, fresh medium was replenished in the culture flask, which was then placed back into the incubator for proliferation of MSCs.

Transplantation of MSCs and Zn in mice

For transplantation of MSCs, firstly, the cells were isolated from the cultured flasks. For cell detachment trypsin (1x) was added into the culture flask for 5-10 minutes. Then, the cells were centrifuged at 5000 rpm for 10 minutes. After centrifugation, the supernatant was discarded and the pellet diluted in 100-200 µl of PBS. The pellet containing about 1x10⁶ MSCs in 1ml syringe was used for transplantation.

Mice from MSC-treated group received MSCs (1 × 10⁶ cells in 1 mL PBS) at a dose of 1x10⁶ cells/100 µl PBS/mouse through the mouse tail portal vein. Zinc-treated mice were injected with 100 µl/mice

ZnSO₄.7H₂O intra-peritoneally, whereas mice from MSC + Zn treated group receive a dose of 100 µl/mice ZnSO₄.7H₂O with MSCs (1x10⁶ cells/100 µl PBS/mouse), also through the tail portal vein.

Biochemical analysis

After dissection of the mice, blood samples were collected from the hearts of mice of each group through 1 ml syringe. The blood was centrifuged at 8000 rpm for 10 min to isolate the serum. Serum ALT and bilirubin levels were determined through spectrophotometer using commercial kits (Vitro Scient, Inc., Cairo, Egypt).

Histopathological analysis

After excising the mouse liver from each animal, the hepatic tissues were fixed in 10% formalin. After 24-72 h of fixation, the tissues were moved to different degrees of alcohol for dehydration. Then, the paraffin sections were prepared and cut into 5-µm sections by a rotary microtome (ROBUS). After bathing in gelatin-containing ultrapure water, the sections were made with the help of microscopic slides and allowed to dry at 37°C overnight. Then, the sections were stained with hematoxylin (H) and eosin (E) reagents. The sections were studied through a microscope at 10X magnification for histological changes (*i.e.* apoptosis and collagen deposition).

qPCR analysis for gene expression

RNA was isolated from liver tissue homogenate using Trizol kit (Invitrogen, Carlsbad, CA, USA). After isolation of RNA, cDNA was synthesized through RT-PCR using 2 µg of RNA and oligo-dT primers at 42°C for 60 minutes using a kit (Invitrogen). The purity/concentration of RNA and cDNA was checked using Nanodrop spectrophotometer (COLIBRI, TITERTEK BERTHOLD). Next, 100–500 ng/ml of cDNA was amplified through PCR using a standard PCR kit. The sequences of the primers are indicated in **Table 1**. The qPCR protocol consisted of 40 cycles at 94°C for 4 minutes, 56°C–58°C for 45 sec, and 72°C for 30 sec, followed by a final extension step at 72°C for 10 minutes. Gene expression levels of hepatic marker (*Albumin*) and apoptotic marker (*Bax*) were evaluated at the mRNA level by RT-PCR in all mouse samples using Biorad CFX96 Software. In this study, *β-actin* was used as a reference marker. Expression levels of *Bax*, *Bcl-xl*, and *Albumin* in the different mouse groups were different, whereas the expression level of *β-actin* was constant in all groups.

Therefore, *β-actin* was used as standard and the expression level of each of the other markers was compared to it; the relative expression of the target genes was determined by comparing to the reference gene (*β-actin*).

Bradford assay analysis

Whole protein was isolated from liver using Trizol method (Invitrogen) and BSA was used as a standard protein. Six different dilutions of BSA were prepared in PBS, along with the sample protein. Each cuvette contained 1 ml of standard solution and 1.5 ml of Bradford reagent. This was followed by incubation at room temperature for 10 min. Then, the absorbance of each cuvette was measured at 595 nm. The graph for standard curve values was plotted (the x-axis represented the micrograms of standard protein and the y-axis represented the absorbance reading at 595 nm). The sample protein absorbance was quantified using the same above method and the unknown protein concentration was calculated by comparison to the standard protein.

Statistical analysis

Standard errors (±) and mean values of various treatments were determined by analysis of variance (ANOVA). Significant difference was separated using Duncan's multiple range test, set at $P \leq 0.05$.

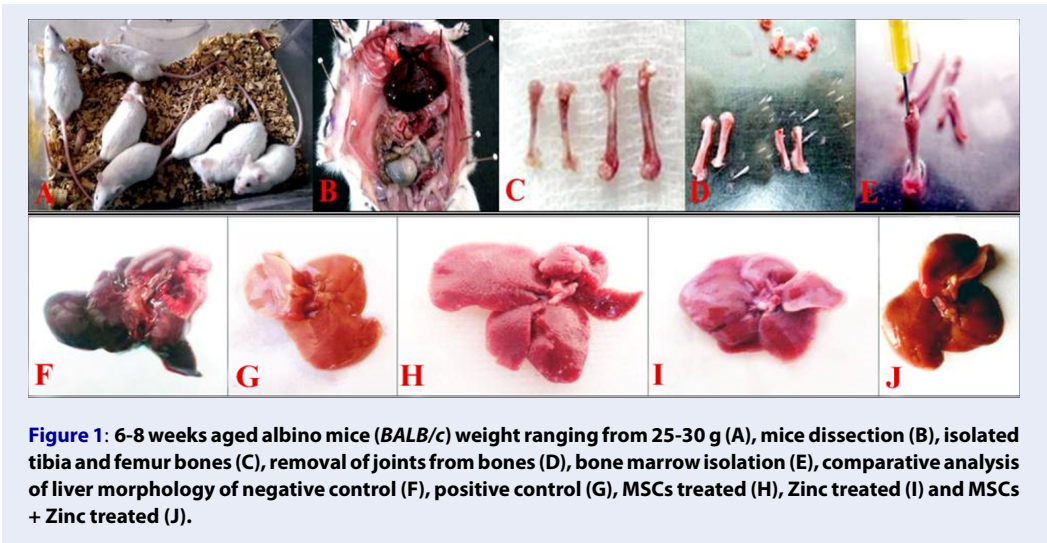
RESULTS

Isolation and characterization of MSCs

After the removal of tibia and femur bone from the dissected mice, bone marrow cells were isolated by flushing prepared media through it (**Figure 1 B-E**). Bone marrow cells were centrifuged at 8000 rpm for 10 min along with media to collect the cell pellet formed at the bottom. Bone marrow mainly consists of two types of cells: HSCs and MSCs. The adherent property of MSCs allow them to become attached to the surface of culture flasks. After 3 days, the medium was discarded and the flask was washed with PBS to remove non-adherent cells (*i.e.* HSCs); adherent cells were fed with fresh DMEM. This process was carried on until the cells were between 70-90 % confluence. Hepatocyte growth factor (HGF) was not added to the medium, therefore the MSCs were not differentiated into hepatocytes, specifically. The successful proliferation of MSCs was investigated through a hemocytometer; moreover, about 1x10⁶ MSCs were taken into a 1ml syringe and transplanted into the tail vein of fibrotic mice. Mice were dissected after 10 days

Table 1: Primers with their sequence, annealing temp and product size

PCR primer	Sequence (5'-3')	Annealing temperature	Size in bp
Albumin (F)	GCTGTAGTGGATCCCTGGTG	56°C	196
Albumin (R)	GCTGTAGCCTTGGGCTTG		
Bax (F)	TGGAGATGAACTGGACAGCA	58°C	152
Bax (R)	CAAAGTAGAAGAGGGCAACCAC		
Bcl-xl (F)	TTCGGGATGGAGTAAACTGG	58°C	150
Bcl-xl (R)	AAGGCTCTAGGTGGTCATTTCAG		
β -actin (F)	GCTGTGTTGTCCCTGTATGC	58°C	106
β -actin (R)	GAGCGCGTAACCCCTCATAGA		



of transplantation. After dissection, liver tissues and blood were collected to assess the level of hepatic fibrosis regeneration.

Comparative analysis of hepatic morphology

The hepatic morphology of all the groups of mice was studied. In MSC + Zn treated group of mice, the liver morphology was more similar to that of the negative control mouse group (Figure 1 F), than to the other treatment groups. The livers of MSC + Zn treated mice were reddish in color, with smooth surface and less scarring (Figure 1J). The liver morphology of the positive control mice was more brownish and pale in color, and shrinkage in structure could be seen (Figure 1G). However, the morphological response of the livers of the mouse group treated with MSCs only or Zinc only, revealed a moderate reduction in hepatic fibrosis with less reddish black color, rough sur-

face and few fibrotic scars. Hepatic morphological results clearly indicated that MSCs- in the presence of Zinc- was more effective in reducing by fibrosis.

Biochemical tests of hepatic function

Serum levels of ALT and bilirubin were compared across all groups of mice using ALT and bilirubin kits (Vitro Scient). The serum level of ALT in mice treated with MSCs + Zinc was significantly lower (66.63±9.24 units/L) as compared to the positive control group (297.03±10.3 units/L), MSC-treated group (indicate the values), or Zinc-treated group (indicate the values) (Figure 2A). The ALT value in the positive control mouse group revealed that there was remarkable incline in ALT (liver enzyme) level. ALT value of MSC + Zn treated group of mice was closer to the mean value of the negative control (56.43±6.8 units/L), indicating normalization of hepatic functions (Figure 2 A). Serum bilirubin level was also analyzed in all groups of mice. The level of total bilirubin is an indicator for

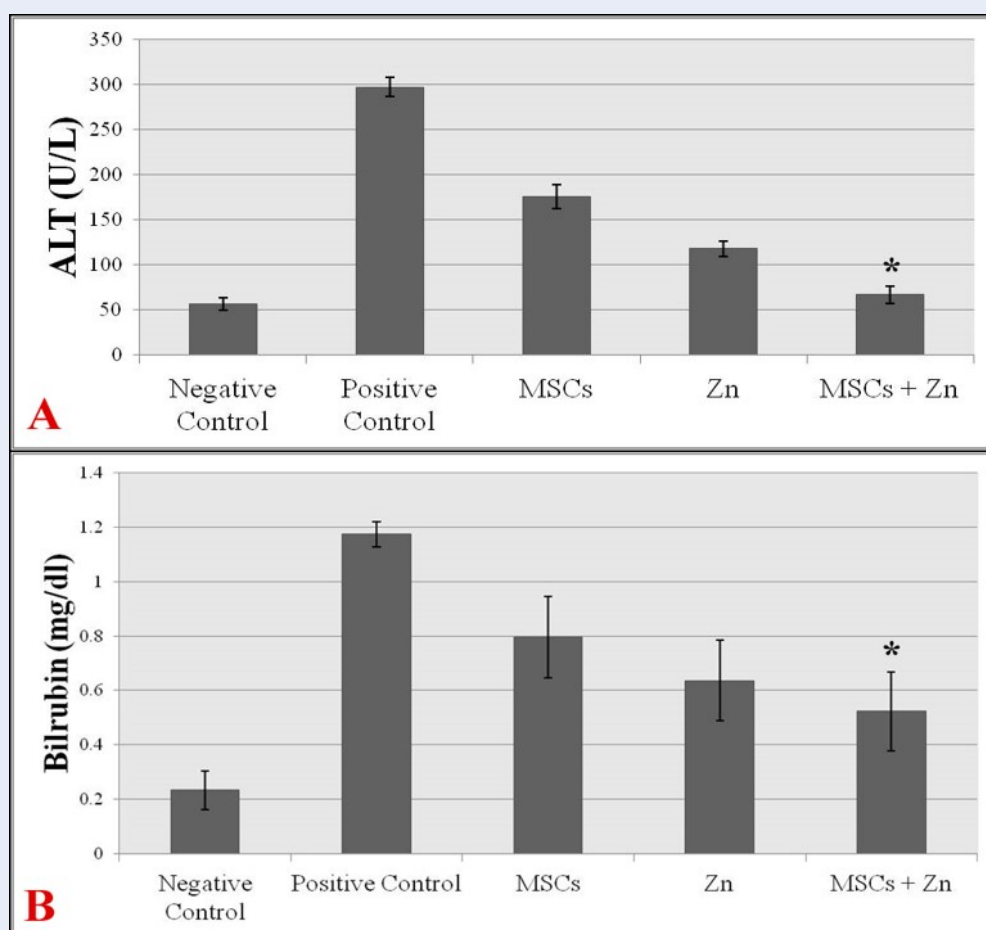


Figure 2: Liver function analysis; level of serum ALT in all treatments (A), level of serum total bilirubin in all treatments (B).

apoptosis; the higher the level of bilirubin, the higher the injury status. This sort of trend was observed when total bilirubin level was detected in all samples. The highest bilirubin level was recorded in the positive control mouse group. However, a significant decline in the serum level of bilirubin was recorded in MSC-only and Zinc-only treated groups of mice. The lowest level of serum bilirubin was recorded in MSCs + Zinc treated group of mice (0.52 ± 0.14 mg/dl), which was even notably lower than that of the positive control group (1.17 ± 0.04 mg/dl) (Figure 2 B). These results clearly indicate that MSCs, in the presence of Zinc, have high ability to reduce liver fibrosis by reducing the apoptotic effect.

Histopathological analysis of hepatic fibrosis

Liver sections of all groups of mice were stained with standard hematoxylin and eosin (H&E) stain, as

shown in Figure 3. The liver sections of the positive control mice had an abundance of hepatocytic apoptosis, which was represented by the irregularly-shaped individual cells with hyper-eosinophilic cytoplasm and fragmented nuclei with more central vein congestion and higher liver collagen deposition (Figure 3B). Mice treated with only MSCs and only zinc revealed a moderate level of reduction in apoptosis, vein congestion and collagen deposition (Figure 3 C and D). The livers of MSC-only and zinc-only treated mice showed a remarkable decrease in liver collagen, central vein congestion, and number of apoptotic hepatocytes. A comparative study demonstrated that the liver sections of MSC + Zn treated mouse group were more similar to that of the negative control mouse group, indicating that the liver had showed signs of restoring its normal functioning (Figure 3).

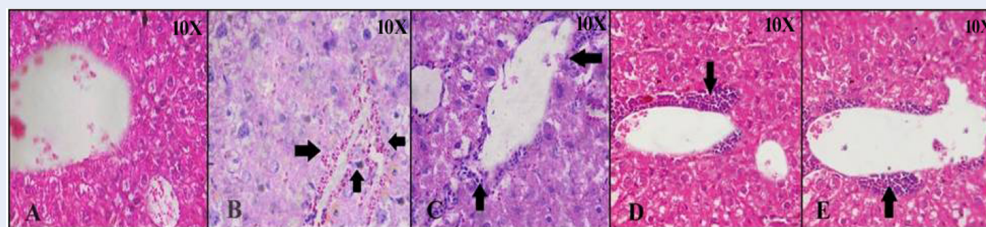


Figure 3: Histopathological analysis of fibrotic liver of treated and untreated mice (10X). Negative control (A), Positive control (B), MSCs treated (C), Zinc treated (D), MSCs + Zinc treated (E).

Gene expression analysis

The levels of apoptotic, anti-apoptotic, and hepatocyte marker expression were analyzed for all the samples by real-time PCR. In this study, β -actin was used as a reference marker. The expressional levels of *Bax*, *Bcl-xl* and *Albumin* across the different groups were different, whereas the expression level of β -actin was constant in all groups. Therefore, β -actin was used as a standard, and the expression level of the other markers was compared to it.

The expression of *Bax* marker was down-regulated in MSC + Zn treated group (1.54 ± 0.15), as compared to the positive control group (4.7 ± 0.3), MSC-treated group (3 ± 0.3), and Zinc-treated group (2.9 ± 0.2). However, the reduction in expression of *Bax* marker in MSC-only or Zinc-only treated groups of mice was quite similar to that of the positive control group; however, a significant reduction was detected in MSC + Zn treated mice.

Bcl-xl is an anti-apoptotic marker; higher expression of this marker indicates normal functioning of the liver. The expression of *Bcl-xl* marker was upregulated in MSC + Zn treated group (5.80 ± 0.4), similar to that of the negative control group (6.36 ± 0.3), whereas the lowest expression of *Bcl-xl* marker was recorded in the positive control group. Significant levels of *Bcl-xl* expression were also observed for MSC-only or Zinc-only treated groups (Figure 4 C).

Maximum regulation of albumin (a hepatocyte marker) was recorded in the MSC + Zn treated group of mice (1.86 ± 0.1). Similar to the *Bcl-xl* marker, the albumin marker also displayed an elevated expression in the MSC-only and Zinc-only treated groups, as compared to the positive control group (Figure 4 D).

After analyzing the expression of β -actin through RT-PCR, the results showed that β -actin expression in all experimental groups was similar and, thus, it was used as a standard (mean of 16.46 ± 1.36). The expression levels of all other markers were compared with β -actin to determine expression level of *Bax*, *Bcl-xl* and

albumin, in the different groups to analyze the effect of CCL₄, MSCs only, Zinc only, and MSC + Zn on the experimental mouse groups.

Bradford assay analysis

The protein concentration was greater in the positive control group due to deposition of ECM proteins, including collagen fibers. Free radicals were formed due to induction of CCL₄, which was involved in the depletion of glutathione (GSH). The absorbance of samples from MSC + Zn treatment was 0.28 nm, which was close to the absorbance for negative control (Figure 5). The absorbance values of the unknown concentration samples were plotted on the graph to determine the concentration. The concentration of positive control (50 μ g/ml) was greater than the treated groups due to degradation of ECM in the treated groups. The concentration of negative control was 10 μ g/ml, MSC-treated group was 40 μ g/ml, Zn-treated group was 30 μ g/ml, and MSC + Zinc treated group was 20 μ g/ml.

DISCUSSION

Excessive accumulation of fibrillar ECM leads to development of hepatic fibrosis. This ECM is gradually replaced by interstitial collagens, particularly collagens I and III, proteoglycans, and fibronectin²². Due to excessive production of fibrillar collagen and fibronectin, the normal architecture of the liver can become disrupted; these changes can lead to abnormal functioning of hepatocytes and parenchymal cell damage²³. Previous studies have shown that MSCs have been used for the treatment of both acute and chronic liver diseases. There is a body of clinical and experimental evidence that confirms that transplantation of MSCs can restore the liver's normal functions in acute and chronic hepatic diseases²⁴.

Besides its other functions, the liver also plays a vital part in the maintenance of zinc homeostasis- as a rapid exchanging repository for zinc storage. Hepatic diseases are also a cause of alteration in Zn levels;

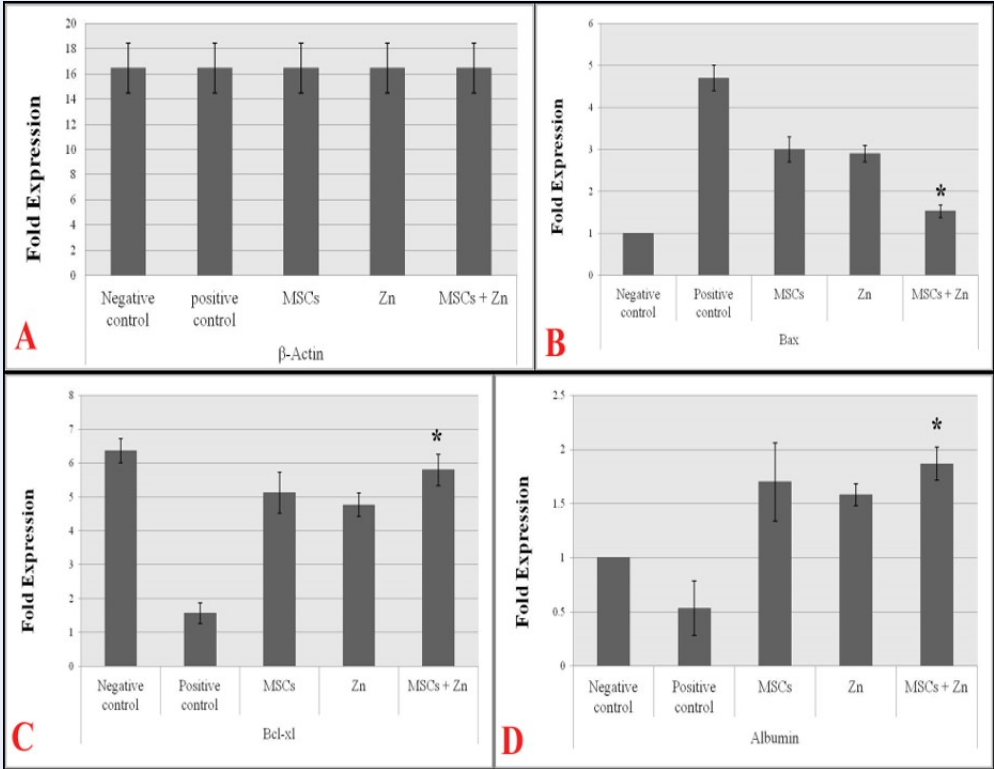


Figure 4: Comparative analysis of different genes expression in treated and untreated mice. Comparison of β -actin expression (A), Comparison of Bax gene expression (B), Comparison of Bcl-xl expression (C), Comparison of albumin expression (D).

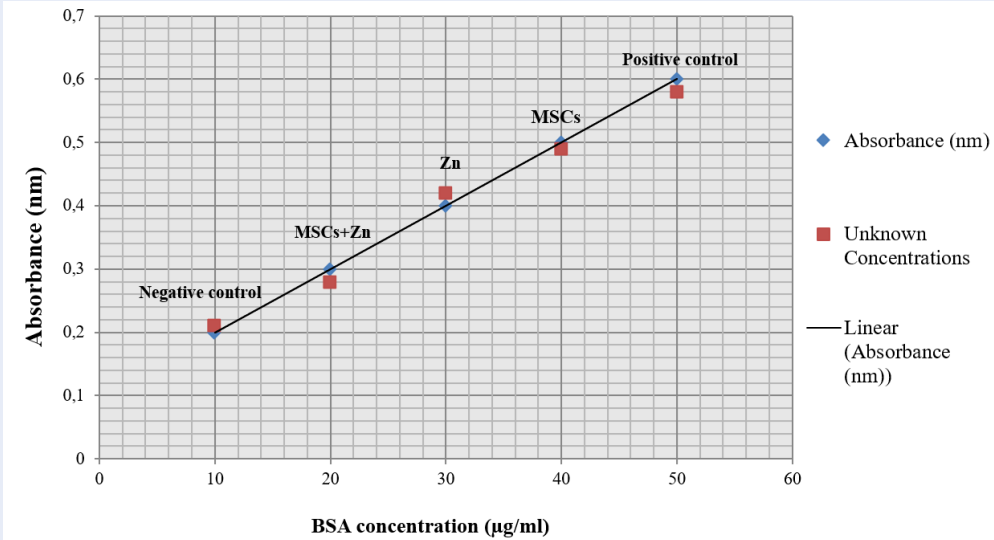


Figure 5: Graph between absorbance and concentration, determination of total protein concentration in unknown sample using Bradford assay.

low Zn level in the liver is related with disturbed liver function and regeneration. Zinc deficiency was found to be increased in an animal model of acute liver injury which was stimulated by a hepatotoxic agent (*i.e.* carbon tetrachloride; CCl₄)²⁵. The properties of Zn are protection against vitamin E depletion, stabilizing membrane structure, inhibiting endogenous free radical production, contribution to the structure of extracellular superoxide dismutase, and protection of DNA from damage (as it is a component of many DNA repair proteins)²⁶.

Membranes of Zn-deficient cells were found to be rich in concentrations of malondialdehyde (MDA), and it has clearly been indicated that peroxidation of membrane phospholipids take place more in those cells which have less zinc concentration. Hepatic lipid peroxide development and proline hydroxylase activity can be down-regulated by the injection of Zn this is because liver has an active role in Zn metabolism and tissues mount up Zn in the form of Zn metallothionein (MT)²⁷.

These two mechanisms are implicated in the defensive effect of Zn against CCl₄ toxicity. Zinc inhibits the expression of CYP2E1, preventing toxic CYP-derived CCl₄ metabolites formation; this mechanism was shown in a previous study in which 17% repression of CYP2E1 mRNA expression by Zn was reported. The second possible mechanism is that Zn is the central component of metallothionein, which shows antioxidant action against reactive oxygen species by removing the free radicals²⁸.

Therefore, the present work follows up on previous data; in this study, we hypothesized that the combined effect of MSCs and Zn would minimize oxidative stress and also enhance the regeneration of the fibrotic liver. In the present study, MSCs were isolated and transplanted into infected mice- in presence of Zn. Zinc enhanced the proliferation frequency of cells and caused considerable beneficial effects on liver fibrosis (induced by CCl₄) in mice.

ROS reacts with sulfhydryl groups, such as GSH, protein thiols and antioxidant enzymes, and causes deleterious effects on them. The excessive production of free radicals causes membrane lipid peroxidation and they bind to larger biological molecules²⁹. In CCl₄-treated rats, the hepatic content of GSH was extremely reduced, while MSCs increased hepatic GSH content and entirely stopped their inhibition³⁰. An antioxidant with hepatoprotective activity can be effective against hepatic damage, such as *Careya arborea* against CCl₄ induced liver injury in rats³¹. In another

study, CCl₄-induced injury in mice was efficiently reduced by the pre-treatment of Zn²⁸. The present results are in correlation with these findings; the optimum dose of zinc strongly enhances the antioxidant effect and proliferative ability of MSCs by increasing the hepatic MT content to normal levels in the liver tissue. This significantly reduces the concentration of free radicals and stops the succession of liver fibrosis. Rungruang *et al.* reported that normal mouse liver is reddish black in color, compared to normal mouse liver. Indeed, the liver of diseased mice is changed from a reddish black to a brownish black or pale color³². Similar morphological results were observed in our studies. The liver morphology of MSC + Zinc treated group of mice showed more resemblance with the negative control mice- with reddish black color, in contrast to MSC-only or Zn-only treated groups of mice. The liver morphology of positive control mice was brownish pale in color (**Figure 1**). These morphological results demonstrate that MSCs- when combined with Zinc- have a remarkable antifibrotic effect on CCl₄, as compared to MSC-only treatment.

AST and ALT activities were increased in plasma, with lipid accumulation and hepatocytic necrosis reported when rats were exposed to CCl₄³³. In the present work, compared to the positive control group, the liver enzymatic activity in MSC-only treated group was decreased. However, a significant reduction of liver enzymes to the normal levels was observed in MSC + Zinc treated group (**Figure 2 A and B**).

Histological changes demonstrated that MSC transplantation recovered liver function and reversed hepatic necrosis, deposition of collagen, and inflammation³⁴. The histopathological examination of CCl₄-damaged livers exhibited a noteworthy increase in liver collagen adjacent to hepatic lobules, leading to large fibrous septa with central vein congestion and apoptotic hepatocytes. The combination of MSCs and Zinc showed considerable anti-apoptotic effects on liver with less collagen deposition (**Figure 3**).

Zinc sulfate increases the hepatic conversion of amino acids into urea, which indicates clinical and biochemical improvements. In alcoholic and hepatitis patients, the concentration of amino acids are high in contrast to control subjects³⁵. The Bradford assay resembles in this study are in accordance with previous findings; that is, there was a clear reduction of total protein observed in the MSC + Zn treated group of mice, while the concentration of total protein was high in the positive control group.

The qPCR analysis demonstrated that the expression of apoptotic markers, such as *Bax*, was up-regulated in CCl₄-infected hepatocytes, while the ex-

pression of anti-apoptotic marker, *Bcl-xl*, was down-regulated. In the same manner, it has been shown that the expression of hepatocyte marker, *albumin*, was inversely proportional to the concentration and duration of CCl₄ treatment³⁶, which are similar results to those observed in our present study. Zn and MSC transplantation significantly restored the abnormal expression of *Bax*, *Bcl-xl*, and *albumin*, as compared to MSC-only transplantation in CCl₄-infected mice. Thus, the combination of Zn and MSCs facilitated high proliferation and regenerative capability of MSCs, as compared to MSC-alone transplantation, to diminish liver fibrosis.

CONCLUSION

Overall, from morphological, biochemical and histopathological results- along with Bradford assay and RT-PCR results- it can be concluded that BMSCs, in combination with zinc, had a high capacity to regenerate fibrotic liver and restore some of its normal functions. Zinc enhanced the regenerative ability of MSCs by increasing the proliferation rate of these cells for the reduction of liver fibrosis. Additionally, zinc displayed a strong antioxidant activity which regulated the enzymes present in the liver, such as glutathione and metallothionein, which neutralized free radicals and reduced the rate of hepatocyte damage. Due to these two reasons, the combination of zinc and MSC represents a potentially promising stem cell-based therapy for the treatment of liver fibrosis.

CONFLICT OF INTEREST

All the authors declared that they have no conflict of interest.

ACKNOWLEDGMENT

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ETHICAL STATEMENT

The bioethics committee of Biochemistry Department, Abdul Wali Khan University, Mardan (AWKUM) approved all the laboratory procedures.

DATA AVAILABILITY

All the data of this research work are available in this manuscript.

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