

Original Article

Therapeutic potential of stem cell and melatonin on the reduction of CCl₄-induced liver fibrosis in experimental mice model

Potencial terapêutico de células-tronco e melatonina na redução da fibrose hepática induzida por CCl₄ no modelo de ratos experimentais

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Abstract

Liver fibrosis is initial stage of any chronic liver disease and its end stage is develops into cirrhosis. Chronic liver diseases are a crucial global health issue and the cause of approximately 2 million deaths per year worldwide. Cirrhosis is currently the 11th most common cause of death globally. Mesenchymal stem cell (MSCs) treatment is the best way to treat acute and chronic liver disease. The aim of this study is to improve the therapeutic potential of MSCs combined with melatonin (MLT) to overcome CCl₄-induced liver fibrosis and also investigate the individual impact of melatonin and MSCs against CCl₄-induced liver impairment in animal model. Female BALB/c mice were used as CCl₄-induced liver fibrotic animal model. Five groups of animal model were made; negative control, Positive control, CCl₄+MSCs treated group, CCl₄+MLT treated group and CCl₄+MSCs+MLT treated group. Cultured MSCs from mice bone marrow were transplanted to CCl₄-induced liver injured mice model, individually as well as together with melatonin. Two weeks after MSCs and MLT administration, all groups of mice were sacrificed for examination. Morphological and Histopathological results showed that combined therapy of MSCs+MLT showed substantial beneficial impact on CCl₄-induced liver injured model, compared with MSCs and MLT individually. Biochemically, considerable reduction was observed in serum bilirubin and ALT levels of MLT+MSC treated mice, compared to other groups. PCR results shown down-regulation of Bax and up-regulation of Bcl-xl and Albumin, confirm a significant therapeutic effect of MSCs+MLT on CCl₄-induced liver fibrosis. From the results, it is concluded that combined therapy of MSCs and MLT show strong therapeutic effect on CCl₄-induced liver fibrosis, compared with MSCs and MLT individually.

Keywords: mice, liver, fibrosis, stem cell, melatonin.

Resumo

A fibrose hepática é a fase inicial de qualquer doença hepática crônica, e em sua fase final desenvolve-se para cirrose. As doenças hepáticas crônicas são uma questão de saúde global crucial e a causa de aproximadamente 2 milhões de mortes por ano em todo o mundo. A cirrose, hoje em dia, é a 11^a causa mais comum de morte globalmente. O tratamento da célula-tronco mesenquimal (MSCs) é uma maneira eletiva de tratar a doença hepática aguda e crônica. O objetivo deste estudo é melhorar o potencial terapêutico dos MSCs combinados com a melatonina (MLT) para superar a fibrose hepática induzida por CCl₄ e também investigar o impacto individual da melatonina e MSCs contra o comprometimento do fígado induzido por CCl₄ no modelo animal. Os ratos BALB / C fêmeas foram usados como modelo de animal fibrótico de fígado induzido por CCl₄. Cinco grupos de modelo animal foram feitos: Controle Negativo, Controle Positivo, CCl₄ + MSCs Tratados Grupo, Grupo Tratado CCl₄ + MLT e Grupo Tratado CCl₄ + MSCs + MLT. MSCs cultivados da medula óssea dos ratos foram transplantados para o modelo de camundongos de fígado induzido por CCl₄, individualmente, bem como em conjunto com a melatonina. Duas semanas após a administração MSCs e MLT, todos os grupos de camundongos foram sacrificados para o exame. Os resultados morfológicos e histopatológicos mostraram que a terapia combinada do MSCs + MLT mostrou impacto benéfico substancial no modelo ferido no fígado induzido pelo CCl₄, em comparação com o MSCs e o MLT individualmente. A redução bioquimicamente considerável foi observada em bilirrubina sérica e níveis ALT de ratinhos tratados com MLT + MSCs, em comparação com outros grupos. Os resultados de PCR mostraram regulação negativa do BAX e regulação positiva do BCL-XL e da albumina, confirmando um efeito terapêutico significativo do MSCs + MLT na fibrose hepática induzida por CCl₄. Dos resultados, conclui-se que a terapia combinada de MSCs e MLT mostram um forte efeito terapêutico na fibrose hepática induzida por CCl₄, em comparação com MSCs e MLT individualmente.

Palavras-chave: ratos, fígado, fibrose, células-tronco, melatonina.

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1. Introduction

Liver fibrosis is the hallmark of any chronic liver disease and is the major cause of mortality. Cirrhosis is the final stage of hepatic disease which occurs when the fibrotic tissues replace the healthy tissues (Byass, 2014). Hepatic fibrosis accompanied by extracellular matrix accumulation (ECM) in response to over activation of hepatic stellate cells (HSCs) and increased expression of transforming growth factor $\beta 1$ (TGF- $\beta 1$) (Tu et al., 2015). Worldwide, liver fibrosis is the cause of sever disease and deaths. Liver fibrosis due to alcoholic liver disease (ALD) in 2010 killed 493,300 populations worldwide. Liver transplantation therapy is the end stage of cirrhosis (Kang et al., 2020).

Mesenchymal stem cells are the main cell source for tissue regeneration. MSC have great potential for application in cell therapy and tissue engineering procedures because of their plasticity and capacity to differentiate into different cell types (Nascimento et al., 2021). The administration of MSCs as a therapy for liver disease holds great promise (Lou et al., 2017). MSCs can differentiate into nearly any end-stage lineage cells to empower their seeding in specific scaffolds, Anti-inflammatory, immune regulatory, and immunosuppressive capacities, are immunological properties which contribute to their potential role as immune tolerant agents (Han et al., 2019). Multipotent MSCs based regenerative dose has gained considerable attention as a powerful treatment for various refractory diseases owing to the capability of MSCs to repair effected tissues and restore functionality by differentiate into multiple cells (Han et al., 2020). Due to their differentiation into hepatocytic cells, MSCs are hopeful sources of liver regeneration. MSCs can be induced to differentiate into hepatocytic cells by pretreatment with different growth factors; hepatocyte growth factor (HGF), fibroblast growth factor (FGF)-2/-4, epidermal growth factor (EGF), and dexamethasone, leukemia inhibitory factor and insulin-transferring-selenium. MSCs may be induced to differentiate into hepatocytic cell by co-culture with liver cells (Shams et al., 2021).

It has been reported that melatonin have numerous beneficial properties, such as anti-inflammatory, antioxidant, anti-fibrotic, anti-apoptotic, anticancer and hepatoprotective. Melatonin is a low molecular weight indole amine produced and secreted principally by the pinealocytes of pineal gland in vertebrate. A multifaceted biochemical pathway triggers the biosynthesis of melatonin from its precursor tryptophan. The biological catalysts that are involved in its biosynthetic pathway are tryptophan-5-hydroxylase, 5-hydroxytryptophan decarboxylase, Arylalkylamine N-acetyltransferase (AANAT), and hydroxyl indole-O-methyltransferase (HIOMT, currently the ASMT). Melatonin is also synthesized in extra-pineal sites. In the oral cavity, the salivary glands and the gingival tissues are documented sites of melatonin production. The receptors of melatonin are also present in the oral cavity and in the gingiva (Balaji et al., 2021). Melatonin has the competence to counteract free radicals and constrain the production of inflammatory cytokines. It minimizes the levels of pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α) and also activate the antioxidant defense systems, such as glutathione peroxidase, superoxide dismutase and glutathione in liver damage experimental animal model. In addition, it has

cytostatic impact on neutrophils and hepatic stellate cells, which may lead to the concealment of combative radicals and fibrogenesis (Sulimani et al., 2021).

2. Methodology

2.1. Bioethical committee approval

This study was approved by the bioethics committee from the department of Biochemistry, Abdul Wali Khan University Mardan, Pakistan.

2.2. Experimental animals

All experimental work were performed according to appropriate protocol for the care and use of laboratory animals. Female BALB /c mice, 6-7 weeks age and weight ranging from 25 to 30gm were obtained from University of Peshawar department of pharmacy, Pakistan. The experimental animals were kept in hygienic animal house with appropriate light/dark cycle at temperature (20-25 °C) and free access to standard rodent diet. Five mice were used for each experimental group.

2.3. Culturing of bone marrow-derived MSCs

Femur and tibial bone marrow (BM) were harvested from albino mice (Balb/c) and cultured in prepared culture medium that consist of Dulbecco's modified Eagle's medium (DMEM, GIBCO), 10% fetal bovine serum (FBS, BIOWEST), 1% penicillin (100 U/mL) and streptomycin (100 μ g/mL), according to protocol described previously (Khan et al., 2011). The culturing cells in 25 cm² culture flask were incubated in 5% CO₂ incubator and the temperature were maintained at 37 °C for 2 weeks, termed as primary culture. After primary culture confluency, it was passaged and subcultured to second passage.

2.4. CCl₄-induced liver fibrotic models and administration of MSCs and melatonin

Six weeks old female Albino mice were treated with CCl₄ and olive oil intra-peritoneally at a dose of 1ml/kg, twice a week for 4 weeks. For MSCs transplantation, cultured BM derived-MSCs in PBS (phosphate buffer saline) were transplanted to CCl₄-induced mice model at a dose of 1x10⁶ cells/100 μ L/ mice in their tail vein. Melatonin (sigma, USA), dissolved in a mixture of ethanol and saline, was administered to CCl₄-induced mice for 14 day at a dose of 0.30 mg/g/day. Similarly, for combined administration of BM-MSCs and melatonin follow the same procedure as described for individual administration of MSCs and MLT. Mice were sacrificed after two weeks of MSCs transplantation and MLT treatment.

2.5. Experimental groups

All mice were divided into five experimental groups, each group containing five mice (n=5); mice treated only with olive oil was considered as negative control while those treated with CCl₄ were termed as positive control, positive control group were treated with MSCs and were called as CCl₄+MSCs treated group while positive control

group treated with MLT were considered as CCl₄+MLT treated group, positive control group treated with MSCs and MLT combinedly were termed as CCl₄+MSCs+MLT treated group. CCl₄ treatment will be continued throughout the experiment.

2.6. Morphological studies

For study liver morphology, all experimental animal models were euthanized with right chloroform for dissection to isolate their liver. After liver isolation from each experimental animal model, it was observed immediately for comparative morphological studies. Later on, these livers were used for a histopathological analysis.

2.7. Biochemical tests

Blood serums were collected from all groups of mice and were screened through spectrophotometer for alanine transaminase (ALT) and total bilirubin analysis, according to manufacturer's protocol (Ecoline, Diagnostic system GmbH Germany).

2.8. Histopathological analysis

For histopathological study, the mice liver were cut into thin section (5µm) through rotary microtome (ROBUS) and then stained with Hematoxyline and Eosin (H&E staining) reagents using standard protocol. The liver thin sections were studied for apoptosis and collagen deposition microscopically (10X).

2.9. PCR analysis

To analyze the impact of MSCs and MLT on CCl₄ damaged liver at molecular level, mRNA expression of different hepatic markers were evaluated by semi-quantitative RT-PCR. The extracted mRNA from liver tissue homogenate using TRIZOL reagent (Invitrogen, USA), were converted into cDNA by Revert Aid H-Minus first strand cDNA

synthesis kit (Invitrogen, USA). Semi-quantitative RT-PCR was performed to evaluate the expression of distinctive gene markers such as Bax, Bcl-xl, and Albumin. β -Actin was used as housekeeping gene or internal control. The detail of all primer and their sequence were given in the Table 1.

2.10. Statistical analysis

All statistical data were Analyzed as Mean \pm SD. One-way ANOVA Dunnett's multiple comparison tests was used for comparison of data between five groups by using GraphPad Prism 8. P value less than 0.05 was considered significant.

3. Results

3.1. Comparative analysis of liver morphology

The comparative morphological studies of all experimental groups of mice were shown in Figures 1A-E. The positive control group liver (Figure 1B) is pale color with rough surface indicating significant fibrotic scar,

Table 1. Primer sequences for gene expression analysis.

Gene markers	Primer	Sequence
Albumin	Forward	GCTGTAGTGGATCCCTGGTG
	Reversed	GCTGTAGCCTTGGGCTTG
Bax	Forward	TGGAGATGAACTGGACAGCA
	Reversed	CAAAGTAGAAGAGGGCAACCAC
Bcl-xl	Forward	TTCGGGATGGAGTAACTGG
	Reversed	AAGGCTCTAGGTGGTCATTAG
β -Actin	Forward	GCTGTGTTGTCCTGTATGC
	Reversed	GAGCGCGTAACCTCATAGA

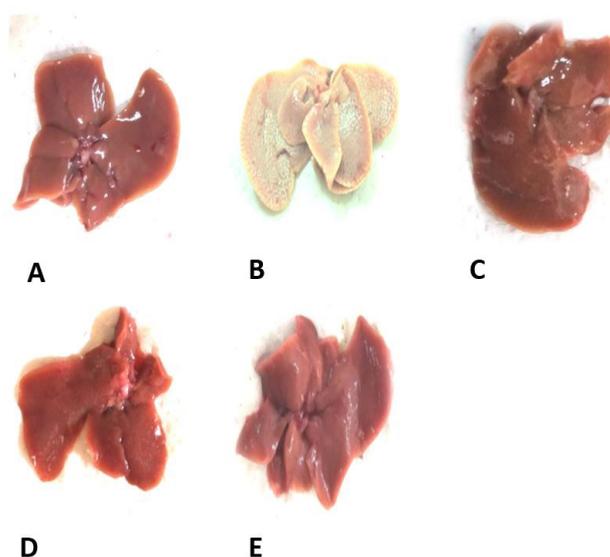


Figure 1. Comparative morphological analysis of experimental groups: negative control group (A), Positive control group (B), CCl₄+MSCs treated group (C), CCl₄+MLT treated group (D), CCl₄+MSCs+MLT treated group (E).

compared to negative control (Figure 1A). MSCs treated group (Figure 1C) liver were reddish color and shows fewer scars while MSCs+MLT treated group was closely related to negative control as compare to MSCs and MLT treated group individually. The morphological results of all groups indicate that MSCs+MLT treated mice liver show reduction in fibrosis as close resemblance with negative control mice liver, compared to other groups.

3.2. Evaluation of liver weight

Comparative liver weight analysis of all group of mice were shown in the table 2. The results of positive control group (1.86±0.40) show significant increase in liver weight due to fibrosis than negative control (2.68±0.54) while individual treatment of MSCs and MLT reduces this increase to some limited extent. Liver weight of MSCs+MLT treated group (1.95±0.79) show significant decrease due to strong therapeutic effect of MSCs and MLT, compared to their individual therapy

3.3. Biochemical results

Serum ALT and total bilirubin level was determined to evaluate the function of liver in experimental groups of mice, using human's kit of ALT and bilirubin. Positive control group of mice serum shows remarkable increase

in ALT and total bilirubin level as compared to normal mice serum. Serum ALT and bilirubin level were reduced in MSCs treated and MLT treated groups as compared to positive control but significant reduction were observed in MSCs+MLT treated group as compare to other groups. Biochemical results of serum ALT and total bilirubin in MSCs+MLT treated group is comparatively similar to negative control, which show normalization in the liver functions (Figure 2A-B).

3.4. Histopathology

Histopathological results of all animal groups were shown in Figure 3. Hepatic section of negative control (Figure 3A) show normal hepatic architecture whereas positive control group (Figure 3B) had large number of apoptotic cells which presenting irregularity of individual cell, increase in main vein congestion and also increase in collagen accumulation. Individual administration of MSCs and MLT in MSCs treated group (Figure 3C) and MLT treated group (Figure 3D) show decline in apoptosis and decrease main vein congestion and collagen accumulation, compare to positive control group. The histopathological results of MSCs+MLT treated group (Figure 3E) showed significant reduction in liver fibrosis as reduction observed in irregularity of individual cell structure, reduction in collagen deposition and thinning in septal fibrosis, compared to MSCs and MLT treatment individually.

3.5. Gene expression analysis

To investigate therapeutic potential of MSCs, MLT, and their combination on CCl₄ injured mice, expression level of Alb, Bax, and Bcl-xl, and Albumin markers were measured in liver tissue of all experimental animal model through semi quantitative PCR (Figure 4). The expression levels of all markers were compared with internal control (β-Actin). All of these markers were studied at the mRNA level. The expression level of Bax (Apoptosis marker) in

Table 2. Treatment effect on liver weight of mice (mean ± SD).

S.No	Treated Groups	Mean ± SD
1	Negative Control	2.09 ± 0.54
2	Positive Control	2.62 ± 0.40
3	CCl ₄ +MSC	2.53 ± 0.24
4	CCl ₄ +MLT	2.01 ± 0.42
5	CCl ₄ +MSC+MLT	2.03 ± 0.79

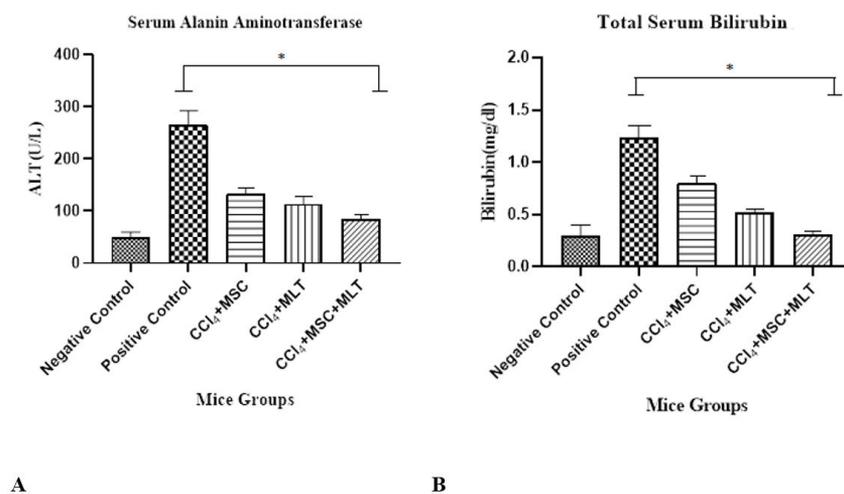


Figure 2. Biochemical results of liver function: Blood serum profile of ALT (A) and total Bilirubin (B). Values are expressed as mean ± SD. *P value < 0.05 was considered significant.

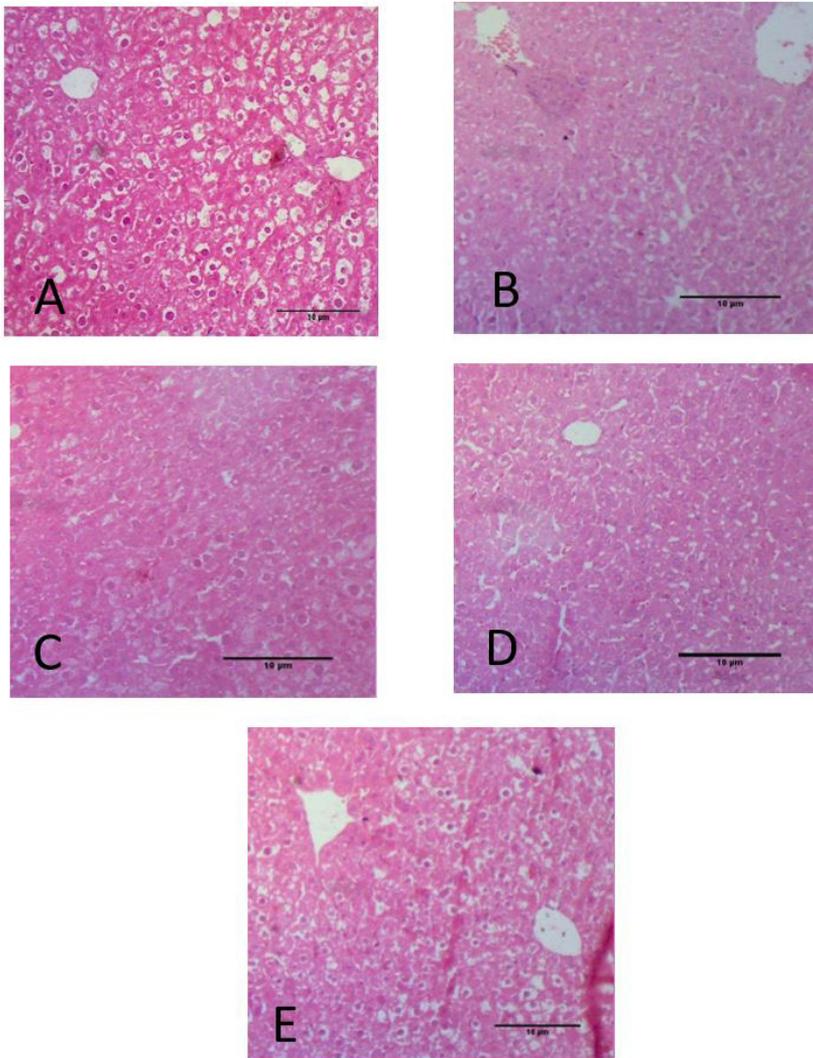


Figure 3. Histopathological examination of liver compared with positive control hepatic architecture was evaluated by H&E staining; negative control (A), positive control (B), CCl₄+MSC treated (C), CCl₄+MLT (D), CCl₄+MSC+MLT (E).

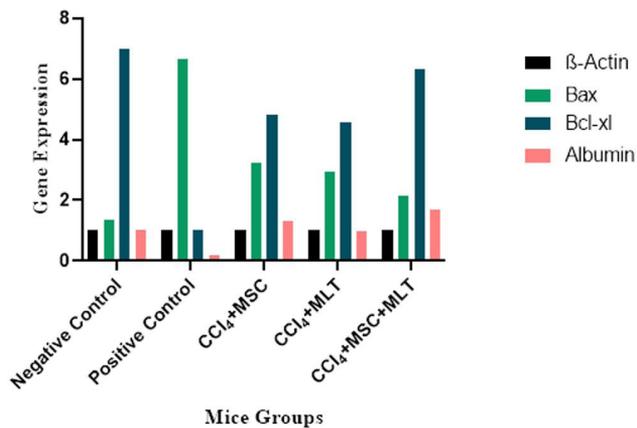


Figure 4. PCR analysis of the expression pattern of apoptotic, antiapoptotic, and hepatocyte markers in CCl₄ injured mice liver after transplantation of MSCs and MLT individually as well as combined.

positive control was significantly increased, compared with negative control group. Transplantation of MSCs and MLT individually decrease the expression of Bax in MSCs treated and MLT treated group respectively, but their expression level was significantly reduced in MSCs+MLT treated group. Conversely, Bcl-xl (anti-apoptotic marker) and albumin (hepatocyte marker) expression was significantly decreased in positive control group as compared with negative control. Administration of MSCs and MLT individually increased the expression level of Bcl-xl and albumin in MSCs and MLT treated group respectively, but their expression were significantly increased in MSCs+MLT treated group, close to negative control group. PCR result of MSCs+MLT treated group suggest that combined therapy of MSCs and MLT have significant therapeutic effect on CCl₄-induced liver fibrosis, compared to MSCs and MLT therapy individually.

4. Discussion

The major cause of hepatic fibrosis and cirrhosis is the excess buildup of fibrillar extracellular matrix. Collagen protein which are produced by the body in inflammation, are commonly accumulated protein in this ailment (Li et al., 2012). Previous studies demonstrate that MSCs is a new way of therapy for liver fibrosis. MSCs transplantation improves the function of various impaired organs such as lung, liver, heart and brain and also reduces different diseases such as fibrosis. Disintegration of collagen is very beneficial attribute of MSCs transplantation therapy in liver fibrosis (Rabani et al., 2010). In this study, mice BM derived-MSCs culture, combine with melatonin, was used as therapeutic agent against CCl₄-induced liver fibrosis. Tan et al. (2002) reported that melatonin acts as free radical scavenger and also a powerful antioxidant because of its small size and high lipophilicity which can cross biological membrane easily and reach all compartments within the cell thus protecting DNA. Melatonin also shows some beneficial effect on injured liver.

Previously studies reported that normal liver color is reddish while diseased liver is brownish black or pale in color (Rungruang et al., 2013). The morphological results of Rungruang et al. (2013), shows some similarities with present work. In the present study, morphological examination of MSCs treated group and MLT treated group reddish brown color with less scar on surface as compared to positive control mice liver which are brownish pale in color with fibrotic scare. MSCs+MLT treated group liver color (reddish color) showed more resemblance with negative control group (Figure 1). The result illustrated that MSCs+MLT have surprising anti-fibrotic impact on injured liver as compared to individual therapy of MSCs and MLT. Upon four weeks of CCl₄ administration, a fractionally increase in liver weight were observed which indicate increase in the degree of liver fibrosis (Tien et al., 2011). Liver weight results (Table 2) of MSCs+MLT treated group display decrease in liver weight as compare to positive control, the results agreed with Tien et al. (2011).

A large number of enzymes are present in liver for their biological functions. CCl₄-induced liver fibrosis in rats increases ALT and bilirubin level in plasma with lipid

accumulation and necrosis (Yachi et al., 2010). Currently, the effect MSCs and MLT were investigated on serum ALT and total bilirubin level in CCl₄-induced injured mice model. Liver enzymatic functions were increased in CCl₄ treated group as compared to negative control. Substantial decline of serum ALT and total bilirubin to normal level was observed in MSCs+MLT treated group, compared to positive control (Figures 2A and 1B).

Histopathological examination revealed that liver activities were restored after MSCs administration byrecovering liver fibrosis, fat changes and inflammation (Cho et al., 2012). Histopathological study of positive control group shows significant increase in liver collagen, central venous congestion and apoptotic hepatocyte. MSCs+MLT treated group showed decrease in collagen deposition and reduction apoptotic hepatocyte due to combined therapeutic effect of MSCs and MLT on CCl₄-induced liver injury, compared to MSCs and MLT individual treatment (Figure 3). The Histopathological results indicate that combined therapy of MSCs and MLT showed considerable anti-fibrotic effect on CCl₄-induced liver fibrosis.

Gene expression analysis indicated that Bax markers expression was high in CCl₄-treated hepatocyte, while the expression of Bcl-xl marker was down regulated in these cells (Nasir et al., 2013). These markers expression are used as indicators of liver fibrosis. In the current results, these markers studies showed that transplantation of MSC and MLT to CCl₄-injured model reversed liver fibrosis problem. This study revealed that combined therapy of MSC and MLT increases Bcl-xl expression and decreases Bax expression in MSCs+MLT treated group, compared to the MSCs treated group and the MLT treated group individually (Figure 4). It is therefore apparent that combined therapy of MSCs and MLT has a high therapeutic effect on CCl₄ -induced liver fibrosis.

5. Conclusion

It was concluded from the morphological, biochemical, histological and RT-PCR results, that transplantation of MSCs and MLT combinedly have significant antifibrotic effect on CCl₄-induced liver fibrosis. From the above results it was concluded that the combined therapy of MSCs and MLT have significant hepatoprotective effect on liver fibrosis, comparing with MSCs therapy alone.

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