Additive Effects of Mesenchymal Stem Cell-Conditioned Medium and Silymarin in Carbon Tetrachloride-Induced Liver Injury in Rats

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Abstract

Liver diseases remain one of the leading global causes of death, with increasing mortality over recent decades. Mesenchymal stem cells-conditioned medium (MSCs-CM), rich in cytokines, growth factors and extracellular vesicles, has shown anti-inflammatory and regenerative potential. This study evaluates the effect of MSCs-CM on carbon tetrachloride (CCl_4)-induced liver toxicity in Wistar rats and explores the combined effect of MSC-CM and silymarin using liver enzyme and antioxidant markers. This experimental animal study was conducted with sixty adults female Wistar rats. Liver toxicity was induced by intraperitoneal carbon tetrachloride (CCl_4, 1 ml/kg) thrice weekly for 28 days. Rats were randomly divided into six groups (n=10 each): normal control, CCl_4-only toxic control, CCl_4 + low-dose MSCs-CM, CCl_4 + high-dose MSCs-CM, CCl_4 + silymarin (100 mg/kg orally) and CCl_4 + silymarin + high-dose MSCs-CM. Low and high doses of MSC-CM (1 ml and 2 ml, respectively) were administered intraperitoneally over 4 weeks after hepatotoxicity induction. Therapeutic effects were evaluated by liver enzymes, antioxidant markers and histopathology.

Treatment with low-dose MSCs-CM (Group 3) and high-dose MSCs-CM (Group 4) significantly reduced liver enzymes compared to the toxic control ($p < 0.05$). Silymarin (Group 5) and the combination of silymarin with MSCs-CM (Group 6) further reduced levels with group 6 showing the greatest reduction. Histological analysis of the normal control group showed intact hepatic architecture. CCl_4-treated rats exhibited periportal mononuclear cell infiltration and sinusoidal dilation. Low-dose MSC-CM treatment led to mild perivenular inflammation while high-dose MSC-CM showed reduced inflammation and improved hepatocyte structure. Silymarin-treated rats displayed moderate sinusoidal dilation. Notably, combined treatment with high-dose MSC-CM and silymarin restored near-normal liver architecture. MSCs-CM showed modest anti-hepatotoxic effects in CCl_4-induced model, improving liver enzymes and histology. These findings suggest its potential as a supportive therapy, especially in combination with other agents.

Keywords: mesenchymal stem cells, conditioned medium, mesenchymal stem cells derived conditioned medium, hepatotoxicity in rats, conditioned medium- silymarin,

Introduction

Liver cirrhosis characterized by progressively deteriorating malfunction due to chronic injury continues to be among the leading global causes of disease and death.[7] The proportion of deaths due to cirrhosis is substantially increasing in the last decades.[7] Deaths due to cirrhosis have shown progressive increase in the past decades.[7] Although liver transplantation is considered a viable treatment option, it remains costly and is limited by the global shortage of suitable donors.[12] The mesenchymal stem cell transplantation has demonstrated anti-apoptotic and regenerative potential in preclinical models of liver injury.[1,4,21,24] However, the therapeutic effect induced by stem cell therapy is primarily mediated through paracrine factors secreted by the cells.[3,14,18]

Several studies have identified growth factors in conditioned media from mesenchymal stromal cells and are attributed for these paracrine effects.[8,9,15,22] Mesenchymal stem cells culture medium (MSCs-CM) contains a spectrum of bioactive molecules secreted by the MSCs such as cytokines, growth factors and extracellular vesicles which are hypothesised to mediate many of the therapeutic effects of MSCs through paracrine signaling.[10] The MSCs-CM demonstrated a potent anti-inflammatory effect on macrophages and may be considered as a therapeutic agent against inflammation.[11] The stem cell secretome includes a variety of bioactive components such as extracellular

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vesicles, particularly exosomes as well as membrane particles, peptides and small proteins like cytokines.

Increasing evidence suggests that the paracrine activity of stem cells, rather than their direct differentiation, plays a central role in mediating the therapeutic effects of stem cell-based therapies, especially in the treatment of degenerative and inflammatory diseases.[5,20] Hence, the study was designed to evaluate the effect of mesenchymal stem cells-conditioned medium (MSCs-CM) on carbon tetrachloride toxicity in Wistar rats. Carbon tetrachloride (CCl_4) is the time-tested and most widely used toxin for experimental induction of liver toxicity in animals. Also, the combination of silymarin and MSCs-CM in CCl_4-induced liver toxicity among Wistar rats is assessed by liver enzymes and antioxidants markers.

Material and Methods

The experimental animal study was planned in the RAKMHSU Central Animal Research Facility. Sixty adults female Wistar rats were studied. The therapeutic effect was assessed by liver function tests, antioxidants markers and histopathological examination. The rats were randomly assigned into six groups, each consisting of ten animals. Liver toxicity was induced by intraperitoneal injection of CCl_4 (1ml/kg body weight) three times a week for 28 days. Group 1 (n=10) served as the normal control. Group 2 (n=10), the toxic control, was treated with CCl_4 only. In group 3 (n=10), rats received low-dose MSC-conditioned medium (MSCs-CM) intraperitoneally after the CCl_4 schedule. Group 4 (n=10) was treated with high-dose MSCs-CM intraperitoneally, in addition to CCl_4. Group 5 (n=10) received silymarin orally at a dose of 100 mg/kg body weight daily for 28 days, after CCl_4.

Group 6 (n=10) was treated with both silymarin (100 mg/kg body weight daily) and high-dose MSCs-CM for 28 days after CCl_4. The doses of MSCs-CM used in this study are based on the dose used by previous studies.[13,14] The low dose consisted of 1 ml of reconstituted MSCs-CM and high dose consisted of 2 ml of reconstituted MSCs-CM. Both doses of CM derived from MSCs were formulated in 0.5 ml normal saline and injected intraperitoneally into the rat. The total study duration was sixty days.

Mesenchymal stem cells-conditioned medium (MSCs-CM) was obtained from Mothercell Regenerative Centre, Trichy, India. Human bone marrow-derived mesenchymal stem cells (MSCs) were cultured in flasks of appropriate sizes using Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal calf serum (FCS) and antibiotics until they reached optimal confluency (80-100%). The growth medium was then aspirated and the cells were washed with sterile phosphate-buffered saline (PBS) before being replenished with serum-free DMEM. After incubation for approximately 48 hours, the conditioned medium was collected, filtered using a 0.22-micron filter and subjected to ultrafiltration and lyophilization before use in the study. Upon reconstitution with 5 ml of normal saline, each vial yielded VEGF (940.0 ± 139.0 pg/ml), FGF (0.43 ± 0.14pg/ml), IL-8 (189.0 ± 37.5pg/ml) and IL-6 (135.8 ± 32.8pg/ml).

Drugs and reagents: Silymarin, carbon tetrachloride (CCl_4) and other chemicals required for the study including those of assay kits for Superoxide Dismutase (SOD), Malondialdehyde (MDA) ELISA, total protein, albumin, aspartate transaminase (AST) and alkaline phosphatase (ALP) were sourced from commercially available suppliers [Algenome International Scientific and Laboratory Products, UAE]. These kits were used as per the manufacturer's instructions to ensure accurate and reliable biochemical estimations. All reagents used were of analytical grade and stored at 4°C. Prior to analysis, they were brought to room temperature by equilibration for 30 minutes.

Experimental Animals: Female Wistar albino rats (aged 4–5 months, weighing 150–200 g) were used in this study. The animals were locally bred at the Central Animal House of RAK Medical and Health Sciences University. They were housed individually in polypropylene cages under standard laboratory conditions including a controlled temperature of 22–24°C, a 12-hour light/dark cycle and relative humidity maintained between 40–60%. All rats had *ad libitum* access to a standard calorie rodent pellet diet and tap water. Prior to the commencement of the experiment, the animals were acclimatized to the laboratory environment for one week. The study protocol was approved by the Research and Ethics Committee of RAKMHSU (RAKMHSU-REC-132-2020/21-F-M).

Experimental model: Fifty rats were injected with CCl_4 for four weeks to induce liver toxicity. A baseline evaluation of liver enzymes was conducted after hepatotoxicity induction, confirming elevated liver function test (LFT) values. MSCs-CM was administered according to group allocations for four weeks, with two doses per week. After the treatment period, the animals were sacrificed and serum and liver samples were collected for analysis, with all biochemical samples stored at -20°C. The stored serum samples were scheduled for biochemical analysis of AST, ALP, malondialdehyde and superoxide dismutase.

Liver Enzyme Analysis: Blood samples were collected on day 0 (baseline) and at the end of the 15th and 30th days after the treatment. Following a 12-hour fasting period, the animals were anesthetized with ketamine (80 mg/kg; i.p.) and blood was drawn via orbital puncture into small centrifuge tubes. The collected blood was allowed to clot and then centrifuged at 3000 rpm for 10 minutes to separate the serum which was immediately used for liver enzyme estimations. Levels of alanine transaminase (ALT), aspartate transaminase (AST), albumin, total protein malondialdehyde and superoxide dismutase in the serum were measured using commercially available kits.

Table 1
Comparison of liver parameters and oxidative stress markers among the groups

Groups	AST (U/L)	ALP (U/L)	Albumin	Total protein g/dL	MDA Malondialdehyde (nmol/ml)	Superoxide dismutase (U/ml)
Normal control	102.22±0.98	248.36± 1.11	3.68±0.28	6.28±0.32	2.48±0.30	93.48±0.28
CCl_4 (positive control)	147.75± 0.62$^{\#}$	360.42±2.20$^{\#}$	2.38±0.28$^{\#}$	4.53±0.29$^{\#}$	8.03±0.14$^{\#}$	48.90±1.92$^{\#}$
CCl_4 + low dose MSCs-CM Group	117.36±0.60*$	334.78±3.37*$	3.20±0.24*	5.16±0.24*$	5.09±0.24*$	53.06±0.27*$
CCl_4 + high dose MSCs-CM group	113.14±0.76*$	319.29±2.49*$	3.47±0.31*	5.40±0.33*	3.33±0.16*	69.82±0.36*
CCl_4 + Silymarin	114.19±0.78*$	336.98±4.55*$	3.09±0.21*	5.38±0.32*	3.41±0.22*	65.54±1.26*
CCl_4 + silymarin + MSCs-CM group	104.28±0.49*	294.11±4.63*	3.48±0.22*	5.88±0.27*	3.20±0.13*	73.21±0.43*

Data are expressed as mean ±SD. One-way ANOVA followed by Tukey's post hoc test indicated statistically significant differences between the groups. $^{\#}$, normal control vs. CCl4 group (P<0.05); *, CCl4 group vs. all treatments (P<0.05); $, MSCs CM + silymarin vs. CCl4 + low dose MSCs-CM Group, CCl4 + high dose MSCs-CM group, CCl4 + Silymarin (P<0.05). AST, aspartate transaminase; ALP, alkaline phosphatase; SD, standard deviation; CCl4, carbon tetrachloride; MSCs CM, bone marrow-derived mesenchymal stromal cells conditioned medium; ANOVA, analysis of variance.

Histopathological Examination: Liver tissue samples from each group of rats were fixed in 10% formalin followed by cutting and dehydration in ascending grades of alcohol. The samples were then defatted in xylene and embedded in paraffin. After 24 hours of block preparation, paraffin sections were obtained using a microtome and mounted on clean glass slides. The sections were stained with Hematoxylin and Eosin (H and E) and examined under a light microscope for histopathological changes. Relevant microphotographs were captured and the sections were analyzed for signs of toxicity including inflammation, fibrosis and regenerative nodules. The data was analyzed using SPSS (Version 24) software and values are stated as mean ± SEM. Means of different groups were analyzed by using one-way ANOVA followed by Tukey test. $P < 0.05$ was considered significant.

Results

The study assessed the impact of MSCs-CM on CCl4-induced liver injury by evaluating liver function markers, oxidative stress parameters and hepatoprotective effects. ANOVA revealed a significant difference in AST levels among the groups ($p < 0.05$). The toxic control group (Group 2) exhibited a significant increase in AST compared to the normal control (Group 1) ($p < 0.05$). Treatment with low-dose MSCs-CM (Group 3) and high-dose MSCs-CM (Group 4) significantly reduced AST levels compared to the toxic control ($p < 0.05$). Silymarin (Group 5) and the combination of silymarin with MSCs-CM (Group 6) further reduced AST levels, with group 6 showing the greatest reduction (Table 1).

A significant difference in ALP levels was observed across groups ($p < 0.05$). The toxic control had markedly elevated ALP levels compared to the normal control ($p < 0.05$). MSCs-CM treatment significantly reduced ALP levels in a dose-dependent manner. The combination treatment (Group 6) resulted in the most significant reduction in ALP. Albumin and total protein levels were significantly decreased in the toxic control group compared to the normal control ($p < 0.001$). MSCs-CM treatment increased albumin and total protein levels, with group 6 showing the highest improvement. MDA levels, indicative of lipid peroxidation, were significantly elevated in the toxic control group compared to the normal control ($p < 0.001$). MSCs-CM administration significantly reduced MDA levels, with the greatest reduction observed in the combination treatment group ($p < 0.001$) (Table 1).

SOD activity was significantly lower in the toxic control group than in the normal control ($p < 0.001$). MSCs-CM treatment significantly improved SOD activity, with the highest restoration observed in group 6 ($p < 0.001$). Post-hoc Tukey analysis confirmed that MSCs-CM, especially at high doses and in combination with Silymarin, significantly attenuated CCl_4-induced hepatotoxicity. The combination therapy (Group 6) exhibited superior hepatoprotective effects, demonstrating significant improvements across all biochemical markers compared to individual treatments. MSCs-CM exhibited hepatoprotective effects against CCl_4-induced liver injury as evidenced by improved liver function markers and oxidative stress parameters. The combination of MSCs-CM with Silymarin demonstrated the most significant protective effects, suggesting a potential synergistic effect (Table 1).

Effect on liver histological morphology: The histopathological examination of the normal control (Fig. 1A) showed normal hepatic architecture with hepatocytes arranged in hepatic cords radiating from the central vein. In contrast, the liver sections from the CCl_4-intoxicated control group (Fig. 1B) revealed moderate periportal infiltration of mononuclear cells, primarily macrophages and lymphocytes and dilation of the sinusoids and central vein.

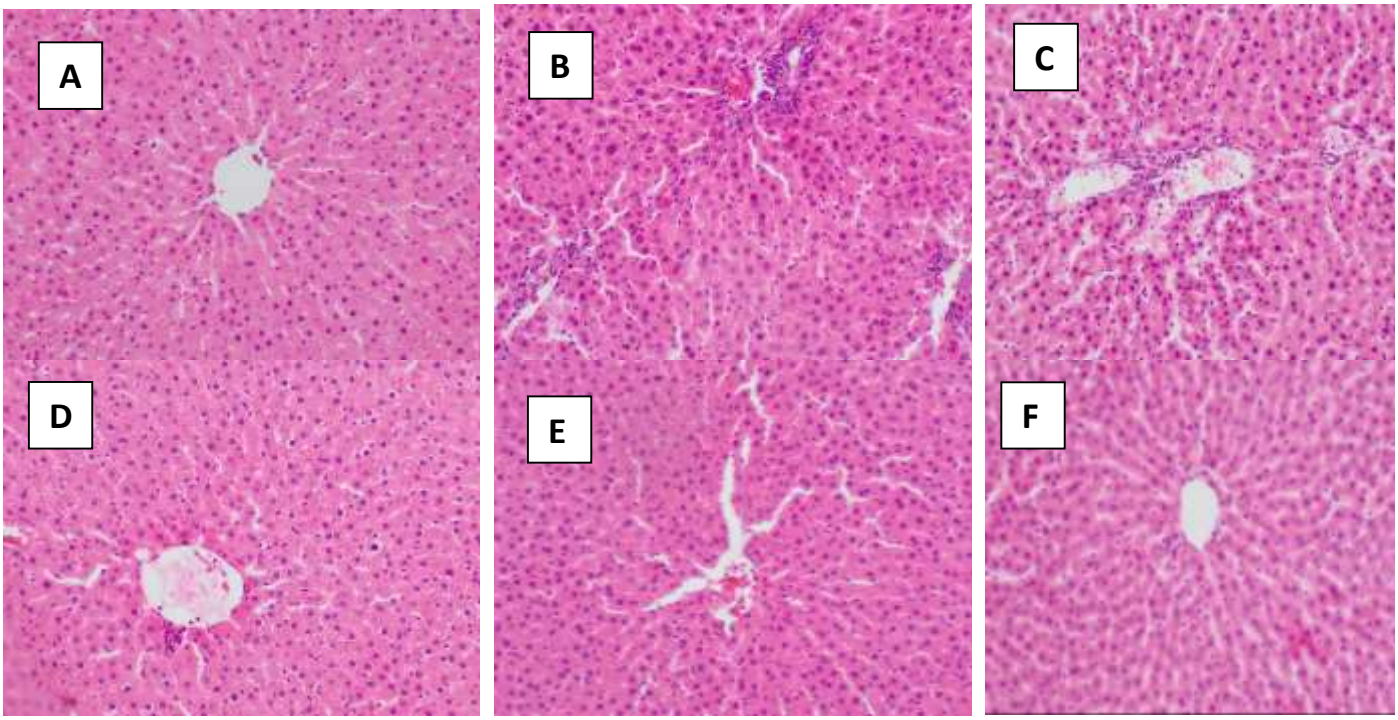

Figure 1: Qualitative histopathological examination of Liver (Stained by H&E, observed under 200x)

Meanwhile, hepatotoxic rats treated with low-dose MSCs-CM (Fig. 1C) displayed mild perivenular infiltration of mononuclear cells. *H*igh-dose MSCs-CM (Fig.1D) showed the infiltration of a few mononuclear inflammatory cells around the central vein, along with marked improvement in hepatocyte architecture. Silymarin-treated hepatotoxic rats (Fig. 1E) showed mild congestion of the central vein and moderate sinusoidal dilation. Notably, the liver sections of hepatotoxic rats treated with a combination of high-dose MSCs-CM and silymarin (Fig. 1F) exhibited hepatocellular architecture resembling that of the normal control group.

Discussion

In this study, we assessed the effects of MSCs-CM against CCl_4-induced liver injury. The results demonstrated that MSCs-CM produced anti-hepatotoxic effects as evidenced by reductions in liver enzyme levels (AST, ALP) and improvements in histopathological examination compared to the CCl_4-only group. The anti-hepatotoxic effects observed with MSCs-CM are in line with previous studies that have reported varying degrees of efficacy with antioxidant or anti-inflammatory properties in similar models of liver damage.[2,13,16,19,23] The anti-hepatotoxic activity is promising and warrants further exploration, especially its mechanistic pathways and combinations with other protective agents. The anti-hepatotoxic effects of MSCs-CM in this model may be related to its ability to reduce oxidative stress or inflammation.

Further, anti-hepatotoxic effects of MSCs-CM were dose dependent for reduction in AST and ALP. The CCl_4-induced hepatotoxicity is primarily driven by the formation of reactive oxygen species (ROS), leading to lipid peroxidation and oxidative damage in hepatocytes. MSCS-CM may act as an antioxidant, scavenging some ROS and thereby mitigating the extent of liver damage.

Further research is necessary to understand better these mechanisms, particularly focusing on whether MSCs-CM can modulate cellular functions in the liver and if its anti-inflammatory effects can be enhanced with different therapeutic strategies. Histological analysis of liver tissue revealed improvements in the group treated with MSCs-CM compared to the CCl_4-only group. While there was a reduction in inflammatory cell infiltration, these changes were not as substantial as those seen in studies of more potent hepatoprotective agents. These findings suggest that MSCs-CM dose and duration optimization may be required to achieve more pronounced effects.

These findings could still have clinical relevance, particularly in situations where minimal protection is sufficient or when used as part of a broader therapeutic regimen. For instance, MSCs-CM could serve as an adjunctive therapy in patients who are at risk of mild liver damage or as a maintenance treatment for individuals who cannot tolerate more potent hepatoprotective agents. Additionally, MSCs-CM may have a higher safety profile, making it suitable for long-term use with fewer side effects, though this remains to be confirmed through further study.

The statistical significance was seen between MSCs CM + silymarin vs. CCl_4 + low dose MSCs-CM Group, CCl_4 + high dose MSCs-CM group, CCl_4 + Silymarin for AST, ALP. The statistical significance was also seen between, CCL_4 +MSCs CM + silymarin vs CCl_4 + low dose MSCs-CM for total protein, MDA and SOD. However, these effects did not reach statistical significance across albumin.

Nevertheless, the potential trend seen in the values of albumin suggests beneficial effects of MSCs CM.

However, there are studies which have no benefit of MSCs-CM. For instance, a study evaluating liver regeneration after partial hepatectomy in Wistar rats found that MSCs-CM was less effective in promoting liver tissue repair compared to direct mesenchymal stem cell (MSC) transplantation or epidermal growth factor (EGF) treatment. The study concluded that while MSCs induced hepatocyte proliferation and EGF promoted angiogenesis, MSCs-CM did not match the regenerative outcomes observed with these treatments.[17] Similarly, MSCs-CM showed no benefit in ischemia /reperfusion liver injury model.[6] These findings highlight that while MSCs-CM possesses therapeutic potential, its effectiveness can vary depending on the specific injury model and may not always match the benefits observed with direct stem cell therapies.

Several limitations of this study should be noted. First, the anti-hepatotoxic effects observed could be due to the dose or treatment duration of MSCs-CM, which may not have been optimal for this model of liver injury. Additionally, the study used a single hepatotoxicity model (CCl_4-induced), which may not fully capture the potential of MSCs-CM to protect against other types of liver damage. Future studies should investigate a wider range of doses, treatment durations and different models of liver injury to better understand the full therapeutic potential of MSCs-CM.

To explore the full potential of MSCs-CM, further studies should focus on optimizing the dose and treatment regimen to enhance its hepatoprotective effects. In particular, dose-escalation studies could help determine whether higher doses of MSCs-CM might produce more substantial liver protection. Additionally, combining MSCs-CM with other known hepatoprotective agents may produce synergistic effects and should be explored future. Investigating the molecular mechanisms underlying its slight effects could also reveal ways to enhance its therapeutic potential, possibly through formulation modifications or combination therapies.

Conclusion

MSCs-CM demonstrated anti-hepatotoxic effects in this study with improvements in liver enzyme levels and histopathology in a CCl_4-induced hepatotoxicity model. While these effects were modest, they suggest that MSCs-CM may have potential as a hepatoprotective agent, particularly in scenarios where mild protection is sufficient or when combined with other therapies. Further research is needed to optimise dosing, to explore combination strategies and to elucidate fully the mechanisms responsible for its hepatoprotective activity.

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