Biochemistry Letters, 2(12) 2019, Pages 120-127

Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

Biochemistry Letters

Journal home page:

Effect of isolated mesenchymal stem cells on the liver injury of rats

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ARTICLE INFO

Colorectal cancer, HCT-116, Nanocurcumin, MTT assay, P53, BAX, BCL2, Caspase-9.

ABSTRACT

Background: This study aimed to evaluate the ability of mesenchymal stem to treat liver injury. Liver transplantation and surgical treatment may be one of the good available solutions for liver injuries. However, it is painful and limited due to shortage of donor organs and high medical costs. Mesenchymal stem cells (MSCs) can serve as an autologous treatment to the liver injury. This study aims to evaluate the ability of MSCs to treat liver injury. Rat MSCs were isolated, expanded for 4 passages. Then they were injected into the tail vein of Sprague Dawley rats which were previously inducted to liver fibrosis with carbon tetrachloride (CCL_4). After and before cell injection, the biochemical analysis of liver function tests and the immunohistochemistry were tested for the liver tissue after 6 weeks of disease induction. Conclusion: AD-MSCs have a promising effect against CCL4 induced liver fibrosis as well as enhancing liver function tests.

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INTRODUCTION

Chronic hepatic injury such as liver fibrosis is regarded as a fetal disease [(1)]. Liver fibrosis can result in biochemical deviations such as decrease albumin synthesis, cholesterol synthesis, and insulin resistance. Cholestasis is evidence for an increase in alkaline phosphatase (ALP) and Gamma-glutamyl (GGT) levels. Any type of liver injury may cause portal hypertension (an increase in blood pressure within the portal vein and its tributaries), since they may bleed profusely, frequently resulting in patient death [(2,3)]**.** Transplantation is currently the only reliable treatment for end stage of hepatic fibrosis [(4)]**.** But there are many complications are associated with transplantation as a shortage of organs and other transplantation complications, which forces us to find another therapeutic solution. Mesenchymal stem cells can help to solve that as it prevent the fibrotic lesions [(5)] or improve liver functions in experimental fibrosis models [(6)]. Adipose derived mesenchymal stem cells (AD-MSCs) are regarded as an adult mesenchymal stem cells source, presents a similar multiple-linage differentiating potential to bone marrow MSCs [(7)]. MSCs considered as an alternative improver for liver injury. Stem cells therapy plays an important role in regenerative medicine due to its benefits in the replacement and improvement of damaged tissue functions and understanding of cell development. It was expected that stem cells therapy provide an alternative source in the treatment of certain degenerative and genetic disorders such as neurological diseases, autoimmune disease, wound healing, cardiac disease, liver disease, metabolic disorders and bone disease [(8)]. In the current study, the hypothesis is that transplantation of AD-MSCs may revoke CCL_4 induced liver fibrogenesis in Sprague–Dawley (SD) rats and recover liver functions which were tested.

MATERIAL AND METHODS

Isolation and Expansion of AD-MSCs

AD-MSCs were isolated according to a method described by **Safford et al** [(9)]. Adipose tissue (100 mg) was obtained from the inguinal fat of SD rats, then was digested in Hank's balanced salt solution containing 0.075% collagenase type I (Gibco, Carlsbad, CA, USA) at 37 °C for 45 min. After dissociation, the digested solution was subsequently filtrated with a 100 μm cell strainer and centrifuged for ten minutes at 1800 rpm at room temperature. After that, complete media which was composed of dulbecco's modified Eagle's medium (DMEM; Gibco, USA) with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/ streptomycin was added to cells. Finally, the suspended cells were transferred to a tissue culture flask and incubated in 5% CO2 at 37 °C. After 3 days, the old complete medium was replaced with new one. When the cultured MSCs were reached to 80% confluence, they were seeded at a ratio of 1:2. This step was repeated again till third passage. At this point, the cells were spindle-shaped, displayed a fibroblast-like appearance and used for further experiments[(10)].

Induction of liver fibrosis

The required approvals were obtained from the ethical committee of Animal Experiment of the Tanta University. 8-week-old male Sprague–Dawley (SD) rats whose weights were around 180 gm were housed in a controlled temperature of 25 °C and humidity ~70%.After a period of adaptation on basal diet, healthy 30 Sprague Dawley rats were divided into 3 experimental groups: 10 animals for each group.

1. **Group (1):** 10 rats were served as a normal control group.
2. **Group (2):** 10 rats were given Intra peritoneal i.p. dose of carbon-tetrachloride ($CC1_4$) in olive oil (1ml/Kg) twice a week for 4 weeks to induce fibrosis in the liver [(11)].These rats were served as diseased group.
3. **Group (3):** 10 rats were injected with $CC1_4$ as in group 2 and then they were treated with intravenous injection of 3 x 10^6 cells of AD-MSCs.CCL_4 Intraperitoneal (i.p.) injections were performed twice a week for 12 weeks to induce liver fibrosis. The dose of CCL_4 (diluted in olive oil 1:1) was 1 mL/kg [(12)]. Liver function blood tests were performed to the rats after 12 weeks of CCL_4 administration. Ten normal rats were regarded as a negative control group to define the normal range of aspartate aminotransferase (AST),aminotransferase (ALT), gamma-glutamyl (GGT) and alkaline phosphatase activity (ALP). The rats with abnormal AST, ALT, GGT, and ALP were treated with AD-MSCs (treated group). AD-MSCs at 4th passage were used for treatment, as 3×10^6 cells were suspended at 0.5 ml saline per rat and injected through the tail vein. The untreated group was injected with saline. Recovery of administrated rats with CCL_4 was notable after AD-MSCs injection. The rats were sacrificed after 12 weeks from injection with CCL_4, blood and liver samples were collected

Transplantation of AD-MSCs

At confluence 90%, cells were sub-cultured from one flask into 2 flasks in a process called trypsinization (passage) and in this state cells became in passage one (P1). Cells took about one week till reached P1. The old media was removed by aspiration using sterile pipette then the cultured cells were washed by 10ml PBS.After that 10ml of (0.05% trypsin, 0.02% EDTA) was added and the flasks were checked under inverted microscope till cells were separated from each other but they were still attached to the flask. At this stage, the shape of the cells changed from spindle to spherical. The trypsin was removed and the cells were incubated in a CO_2 incubator for about 2 minutes **[(13)].** The incubated flasks were checked under an inverted microscope Twenty ml of complete media was added. The feeding process was performed after 3 days with daily examination under an inverted microscope till cells reached confluence 90% after about 4 days from P1 and at this point cells were trypsinized and became in P2. The culture of the cells was continued until passage 4 (P4). At p4 the cells were typsynized and collected for cell viability and count detection, then ($3x10^6$ per rat) in a volume of 0.2 ml PBS was injected via tail vein of by 26-gouge needle. Rats of groups (3) were used as cells recipient. The recipient rats were anesthetized with pentobarbital (50 mg/ml)

at a dose of 0.1 ml/100 gm of body weight intraperitoneally.

Biochemical analysis

Within one hour of blood collection after scarification, the serum was separated by centrifugation and stored at -80°C for further analysis. Using an Automated Chemical Analyzer (7600; Hitachi, Tokyo, Japan) according to the manufacturer's instructions to determine ALT, AST, GGT, and ALP serum levels.

Statistical analysis

Statistical analysis was performed using SPSS 13.0. Measurements were performed three times, and results were expressed as the mean ± SD. The difference regarded as statistically significant if the p-value was less than 0.05.

RESULTS

Morphological analysis:

On the 3rd day of isolation, small spindle-shaped MSCs started to appear adhered to the plastic surface of the flask with large numbers of non-MSCs present. On the 6th day, the number of MSCs increased, and the cells became larger and more spindle-shaped. From the 2nd feed until passage 1(P1) MSCs proliferated more and more till reaching a confluence of 90 **(Figure 1).**

Outcomes of biochemical parameters*:*

Serum ALT, AST, GGT and ALP activities showed highly significant elevation in rats induced liver fibrosis. Rats of treated group after 6 weeks of injection of AD-MSC showed a highly significant decrease *(P<0.0001)* in ALT, AST, GGT and ALP serum levels as compared with the treated group but still higher than that of the negative control group (**Table 1**).

Concealment of Liver Fibrosis after AD-MSCs Injection

Hematoxylin and eosin were used to confirm that the injection of AD-MSC to the tail vein of rats with fibrotic liver tissue caused improvement comparing to diseased with normal liver as control (**Figure 2A**). Analysis under microscope showed that there were scattered stem cells in liver tissue (**Figure 2B**). This refers to the injected stem cells reached the liver tissue. After 12 weeks of CCL_4 administration (untreated group), liver biopsy showed architecture with cloudy swelling of hepatocytes, fatty degeneration and portal fibrosis (**Figure 2C**). AD-MSCs injection significantly relieves necrosis and subs equently suppressed septal fibrotic defect. Hepatic tissues at six weeks after implantation of stem cells showed minimal portal fibrosis, mostly normal liver architecture (**Figure 2D**).

DISCUSSION

In the current study, we investigated the effect of AD-MSCs on liver fibrosis induced by carbon tetrachloride (CCL_4). Liver disease is still one of horrific issues in the world today. According to world health statistics, the strategies for liver disease treatments still have many limitations, because of the pathogenesis of liver diseases as well as the causative role of oxidative stress and inflammation [14,15].Liver diseases,

for instance, sever liver fibrosis MSCs are recently consider consecutive treatment for transplantation of liver. In other cases of severe liver injury such as hepatocellular carcinoma ischemia-reperfusion injury, MSCs have also been reported to be a potential solution **(16).** A large number of clinical researches have demonstrated that stem cell transplantation is a promising approach for the treatment of liver fibrosis **(17).** In the current study, we isolate MSCs from rat adipose tissue because it is enriched with multipotent MSCs and considered to be a good source for autologous stem cell transplantation. AD-MSCs have the ability of self-renewal, proliferation, and differentiate into multiple cell lineages. AD-MSCs can be easily isolated from subcutaneous fat tissue through a safe liposuction procedure and have a high frequency and proliferative rate than BM-MSCs **(18)**.

One of the most commonly used hepatotoxins is CCL_4 in many experimental studies of liver diseases. Its hepatotoxic effects are largely due to its active metabolite, trichloromethyl radicals. These activated radicals bind covalently to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides followed by pathological changes such as depression of protein synthesis and elevated levels of serum marker enzymes such as ALT, AST and ALP (20).

The results of the present study confirmed that CCL_4 is able to elevate the levels of serum marker enzymes ALT, AST and ALP in the diseased group which indicated that the liver tissue was damaged, but MSCs treatment showed nearly normal level of enzymes activities that proved MSCs can reduce the liver injury in the treated group. Moreover, Histological examination illustrated the same propensity, which stands with the **Rabani *et al*** study (20), which reported that MSCs tail vein injection helped in liver fibrosis treatment.

The tail vein injection was reported to be more effective than portal vein injection that has been reported by **Kim and co-workers** (21).

In conclusion the recent results suggest that AD-MSCs were easily accessible and enhance microcirculation and ameliorate liver fibrosis.

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Table 1 Serum ALT, AST, GGT and ALP activities in rats induced fibrosis and treated with MSCs.

Group	ALT	AST	GGT	ALP
Normal control	50.4 ± 6.75^{dt}	61.5 ± 10.05^{dt}	11.0 ± 2.13^{dt}	122.2 ± 13.95^{dt}
CCL_4-injected	400.3 ± 18.59^{ct}	448.2 ± 22.83^{ct}	55.9 ± 5.37^{ct}	520.7 ± 48.2^{ct}
Stem cells treated	135.5 ± 16.6^{cd}	154.9 ± 14.07^{cd}	15.4 ± 3.2^{cd}	233.3 ± 27.16^{cd}

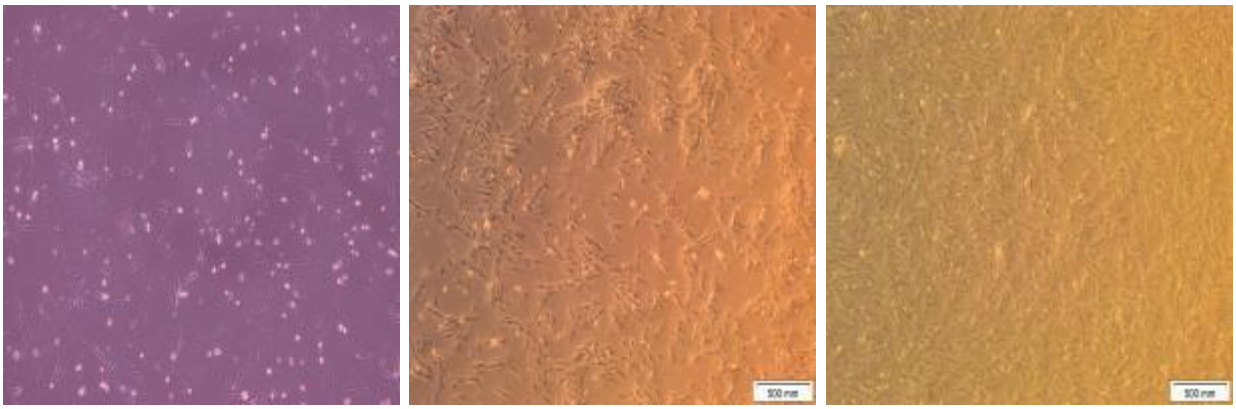

Figure 1: Inverted phase contrast microscopic images with 4x magnification power showing spindle AD-MSCs adhered to the plastic surface of the flask after 3 days from isolation (A), 6 days from isolation (B), and after 10 days of isolation with high confluence (C).

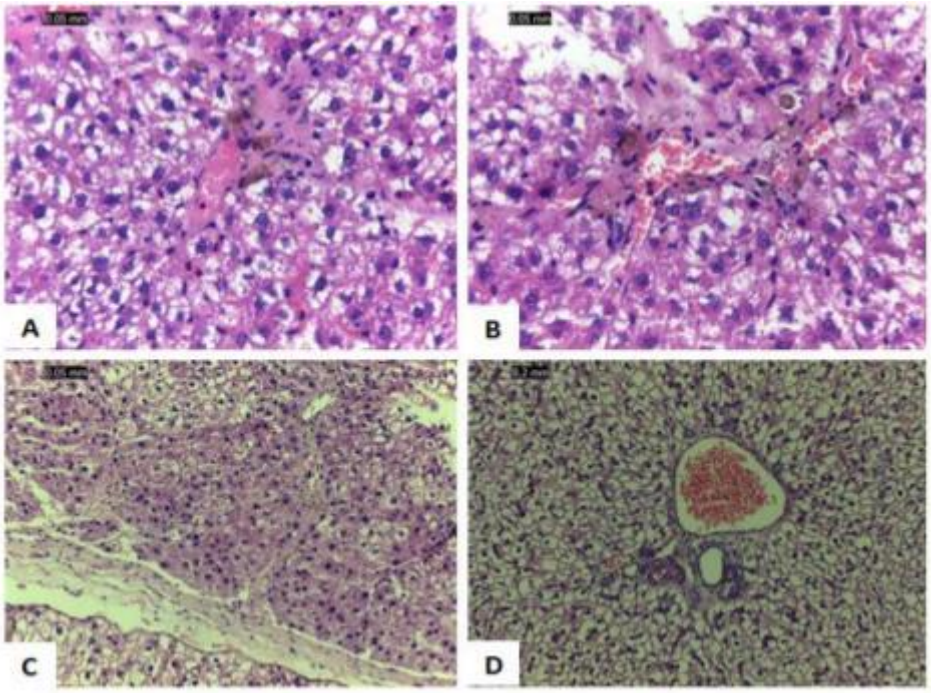

Figure 2: relieve of liver fibrosis after AD-MSCs injection. A: negative control liver shows that there was no pathological change in hepatic tissues. B: hepatic tissue revealed the presence of injected stem cells. C: hepatic tissues of untreated shows disturbed liver architecture with cloudy swelling of hepatocytes, fatty degeneration, and portal fibrosis. D: hepatic tissues of the treated group show minimal portal fibrosis (H&E, X100).