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Effect of Stem Cell and Vitamin E for the Reduction of Liver Fibrosis

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ABSTRACT

Liver disease is seventh leading cause of death worldwide. In the past, liver transplantation was thought to be the only treatment for the last stage liver disease but currently stem cells therapy is an alternative method for the treatment of liver disease. So mesenchymal stem cells (MSCs) transplantation is one of the best tool for treatment of liver disease. The aim of the current study was to investigate the combined effect of vitamin E (Vit E) and MSCs on liver fibrosis. Liver damage was induced in male albino mice intraperitoneally with carbon tetrachloride (CCl₄) twice a week for six weeks. Mice bone marrow derived MSC was cultured in vitro and then transplanted to CCl₄ injured mice through their tail vein injection. Two weeks after MSCs transplantation, all group of mice were examined. The morphological result showed that the combined therapy of Vit E (orally) and MSCs transplantation have significant therapeutic effect on CCl₄ injured mice as compared to Vit E and MSCs individually. Biochemical data also showed that serum ALT and bilirubin level were found to be significantly decreased by the combined treatment of Vit E and MSCs as compared to those mice which received MSCs and Vit E separately. MSCs and Vit E treated mice combined showed a significant decrease in liver weight, closely to normal. Reverse transcriptase PCR result also confirmed a significant anti fibrotic effect of Vit E combined with MSCs transplanted mice on liver fibrosis as showed by down-regulating apoptotic marker (Bax) expression and increasing the expression of anti-apoptotic marker (Bcl-xl). Therefore Vit E along with MSCs have strong therapeutic potential on liver fibrosis in CCl₄ injured mice.

KEYWORDS: Stem Cells, Liver, Fibrosis, Vitamin E, Mice

INTRODUCTION

Mesenchymal stem cells are one of the main type of adult stem cells, which reside in cord blood, bone marrow and adipose tissue. Recently MSCs originate as an promising applicant for hepatic regeneration [1,2]. The stem cells research has a great impact on human community because they could generate cures and will provide treatment for everything from heart disease to cancer. MSCs elucidate their therapeutic potential from both pre-clinical and clinical studies [3]. Other therapeutic uses of stem cells include neuron regeneration, bone repair, drug examination, repair of damage muscle, treatment of spinal cord injury, cancer therapy and other cell based therapy, etc. Stem cells might brought a bright future for the therapeutic world, as a regenerative medicine, for various diseases that are considered as incurable today. Due to their high regenerative ability and funding stem cell research, it will be possible to open a new way of stem cell therapy in the form of organ development and replacement of lost tissue such as hairs, tooth and retina cells [4].

The liver is a vital organ in the body which perform important role in the body as homeostasis, manufacturing and storing of glucose and protein, detoxification and immune defense [5]. Thus the liver has a significant regenerative ability but due to long time liver injury, it finally lead to liver fibrosis [6]. Liver fibrosis is a therapeutic response to chronic liver damage in which the accumulation of extracellular matrix (ECM) occur, mainly in liver parenchyma cells. Different substances like virus, cholestasis, toxic or metabolic diseases, autoimmune and nonalcoholic steatohepatitis may cause liver injury [7]. In advance phases of liver fibrosis, liver contains six times more ECM compared to normal liver, which contain different types of collagens (types I, II and IV), hyaluronic, laminin, Fibronectin, elastin, undulin, and proteoglycans [8]. The operational treatment for liver fibrosis was orthotopic liver transplantation (OLT). With the passage of time organ donation was not under the demand due to which the annual number of deaths and sickness increased and OLT become ineffective [9]. Besides organ donation there were some others limitations like the risk of operation, rejection of post transplantation, recurrence of already

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existing liver diseases and high cost of organ. So cell based therapy was suggested as alternative to OLT [10]. MSCs was suggested as potential therapeutic options for liver degenerative diseases like fibrosis and cirrhosis due to their differentiation and immune regulatory properties. MSCs has also some interesting features like the secretion of antifibrotic molecules such hepatocytes growth factors (HGF). Besides this, MSCs have no ethical issues and have a safer profile in term of oncogenicity as compared with embryonic stem cells (ESCs) [11].

In a series of liver diseases, liver injury is caused by direct attack of reactive oxygen species (ROS) on important biomolecules, affecting their functions and sustainability of cells. ROS disrupt membrane structure and function by lipid peroxidation of these membrane polyunsaturated fatty acid [12]. Various types of antioxidant have been reported to treat and prevent liver diseases in case of oxidative stress. The therapeutic potential of antioxidants as adjuvants including Vit E has shown some useful effects in the treatment of liver ailments like hepatitis C, liver fibrosis and cirrhosis [13, 14]. Vit E is a strong antioxidant, commonly found within the phospholipid bilayer of the plasma membranes where it has a key biological role in defending polyunsaturated fats and other parts of the cell membrane from free radicals oxidation. Vit E is a good antioxidant due to its structure, it provides hydrogen to free radicals from the hydroxyl group situated on its ring structure and rendering them inactive [15]. It also acts as scavenger for ROS distracting their activity in different tissues. Vit E has the ability to prevent liver damage from oxidative stress produced by ROS and other chemicals [16]. We employed CCl₄ induced liver injured model and examined the combined effect of Vit E and MSCs in liver fibrosis reduction. The current research was performed to determine the therapeutic potential of Vit E and MSCs on CCl₄ induced liver fibrosis in animal model.

MATERIALS AND METHODS

Animals

Female albino mice (Balb/c), six to eight weeks age, weighting between 26-30 gm were bought from university of Peshawar (department of pharmacy), Pakistan. All the experimental mice were kept in a permitted animal facility with 12h light/dark cycle at constant temperature (25°C).

Ethical approval

All the animal handling and experimental procedure were approved from the bioethics committee from the department of Biochemistry Abdul Wali Khan University Mardan Pakistan.

Preparation of BM-derived MSCs culture

The mice were anesthetized with a lethal dose of chloroform and the bone marrow was harvested from the femer and tibial bone of six weeks male albino mice. These BM-derived MSCs was cultured prepared culturing medium called Dulbecco's modified Eagle's medium (DMEM, GIBCO) along with 100 units/ml penicillin and 100 ug/ml streptomycin (CAPRICON) and 10% fetal bovine serum (BIOWEST). Cell culture were maintained in CO₂ incubator (5%) at 37 °C for 3 days. On 3rd days of incubation, removed all old media and wash with PBS (two times) to remove non adherent cells (hematopoietic stem cells) and allow the adherent cells (mesenchymal stem cells) attach and add fresh medium to 25 mm culture plate and return to incubator. Examine the cultured cells daily under inverted phase contrast microscope. Continue this process until the MSCs reached to 70%-90% confluency. These cultured MSCs were transplanted to CCl₄ injured mice through injection in their tail vein.

Establishment of animal model

In this study, five different group of mice were prepared, each contains six mice. Group I were injected olive oil alone (intraperitoneally) two time a week for 6 weeks, at a dose of 1 ul/g body weight. Group II received a mixture of CCl₄ and olive oil (1:1 ratio) intraperitoneally at a dose of 1 ul/g body weight twice a week for 6 weeks. Group III that received CCl₄ by the same way as mention above, were transplanted MSCs at a dose of 1x10⁶ cells/100ul PBS/mice through their tail vein. Group IV in addition to receiving CCl₄, were given Vit E orally at a dose of 16mg/100g body weight for two weeks. Group V also involved CCl₄ treated mice, that received both Vit E orally and MSCs through their tail.

Vitamin E and MSCs administration

For MSCs transplantation, first the cells were detached from culture flask with trypsin EDTA and then centrifuged. The centrifuged pellet (MSCs) were diluted in about 100-200ul of PBS. This diluted MSCs in 1ml syringe were transplanted to CCl₄ injured mice through their tail vein, at a dose of 1x10⁶ cells/ 100ul PBS/ mice. Vit E was administrated orally to CCl₄ injected mice at dose of 16mg/100g body weight of the mice.

Mice Scarification

After 15 days of transplantation, the mice was sacrificed, blood and liver were collected for further analysis. The intensity of liver fibrosis was measured morphologically, biochemically and at molecular basis by physical examination of liver, blood serum tests and at RNA level by PCR analysis.

Alanine transaminase (ALT) and bilirubin examination

Animals were given anesthetic with pentobarbital for collection of blood sample from their hearts of each group of experimental mice. For serum isolation, the blood was centrifuged at a speed of 8000 rpm for 10 mints. The serum was analyzed for ALT and bilirubin level determination through spectrophotometer, using Vitro scient kit.

PCR analysis

From the homogenates of liver tissue total RNA was isolated using TRizol kit (INVITROGEN) and cDNA were synthesized with reverse transcriptase PCR using 2 μ g of RNA (Invitrogen kit). cDNA was then amplified through PCR using standard PCR kit with specific pairs of primer. In this study specific pair of primer was used to determine the expression level of different gene marker such as Bax (apoptotic marker) and Bcl-xl (antiapoptotic marker), in mice model. β -actin (housekeeping gene) was used as a reference gene and their expression was checked in all group. The sequence of the primers, their temperature and product size were shown in the table below (Table 1). The PCR protocol consisted of 95°C for 5 minutes (35 cycles), 56-58°C for 30 sec, and 72°C for 30 sec, followed by a final extension for 10 minutes at 72°C. Gene expression levels of apoptotic marker (Bax) and anti-apoptotic marker (Bcl-xl) were analyzed by running PCR product on agarose gel and detected with ethidium bromide. β -actin was used as reference gene.

Table 1: Primer list with their sequence, annealing temperature and product size

PCR primer	Sequence	Annealing temperature	Size in bp
Bax(F)	TGGAGATGAACTGGACAGCA	58°C	152
Bax(R)	CAAAGTAGAAGAGGGCAACCAC		
Bcl-xl(F)	TTCGGGATGGAGTAAACTGG	58°C	150
Bcl-xl(R)	AAGGCTCTAGGTGGTCATTAG		
β -actin(F)	GCTGTGTTGTCCCTGTATGC	58°C	106
β -actin(R)	GAGCGCGTAACCCTCATAGA		

Statistical Analysis

Data were statistically analyzed using SPSS version 16.0. $P \leq 0.05$ is considered statically significant.

RESULTS

Comparative anatomy of liver morphology

Comparative liver morphology showed that the color of group II mice liver were more scar, brownish black color and shrink architecture in appearance (Fig. 1b). Groups V mice liver showed more similarities to group I (normal mice liver). The liver colors of group V mice were closed to radish black with minorscar as compared to group III and IV (Fig. 1). These morphological results described that group V mice liver presented high reduction in fibrosis on CCl₄ injured mice.

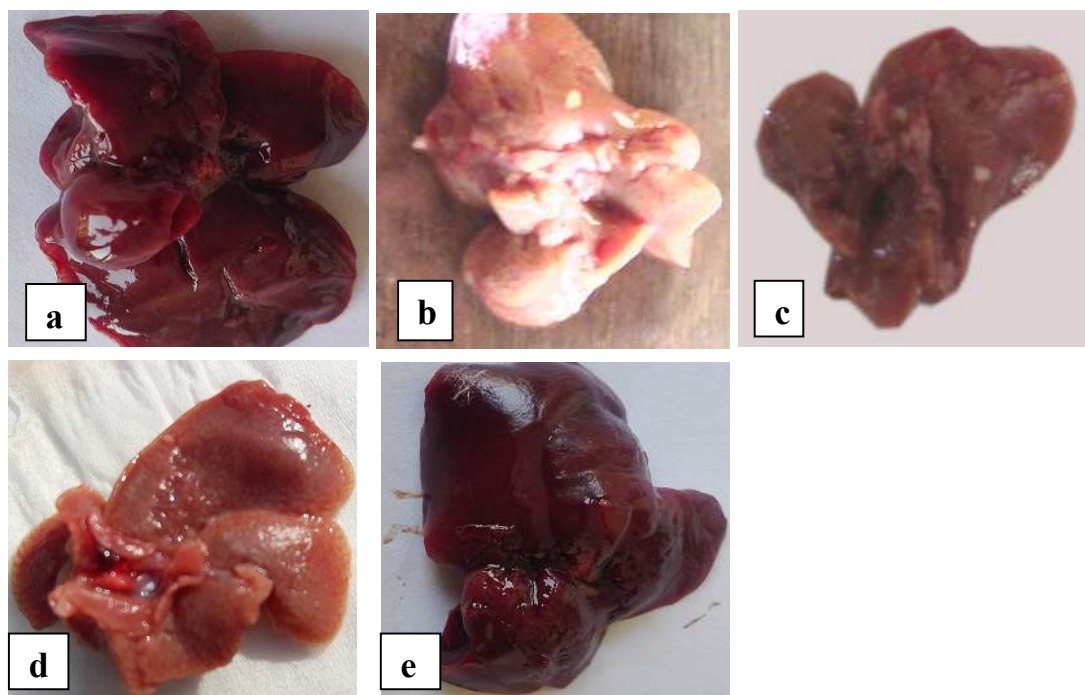


Figure 1: Comparative liver morphology of group v (e) showed high reduction on liver fibrosis in CCl₄ injured mice (a) due to strong therapeutic effect of Vit E and MSCs combintly as compared to group III (c), group IV (d).

Effect on liver weight

The result showed that the liver weight of CCl₄ treated mice (3.00gm) was significant increased as compared to normal liver. In group V mice the liver weight was significantly reduced (2.79gm, closely to normal), by the combined treatment of Vit E and MSCs, as compared to group III and IV (Table 2). Group IV mice liver weight (2.95gm) showed very slightly reduction due to less therapeutic potential of Vit E orally. Thus therapeutic power of Vit E and MSCs combintly was significantly high on CCl₄ injured mice, compared to Vit E and MSCs individually.

Tab 2: Effect of Vit E and MSCs on liver weight

Groups of mice	Group I	GroupII	GroupIII	GroupIV	Group V
weight in gm	2.71	3.00	2.86	2.95	2.79

Biochemical examination

To analyze the combined treatment effect of Vit E and MSCs on liver fibrosis, serum ALT and bilirubin level was studied in all experimental mice model. In CCl₄ injured mice ALT (290.52 units/L) and bilirubin (1.61 mg/dl) level was very high as compared to normal mice. Serum ALT level was significantly lowered by the combined treatment of Vit E and MSCs in group V mice (130.23 units/L) as compared to group III (170.42 units/L) and IV (260.52 units/L) (Fig. 2A). Likewise, serum bilirubin level in group V mice (0.85 mg/dl) was significantly lower than group III (1.36 mg/dl) and group IV mice (1.43 mg/dl), as shown in the figure (Fig. 2B). Collectively, these results clearly indicate that the combined therapeutic effect of Vit E and MSCs have high recovery on liver function in CCl₄ injured mice than either of Vit E or MSCs alone.

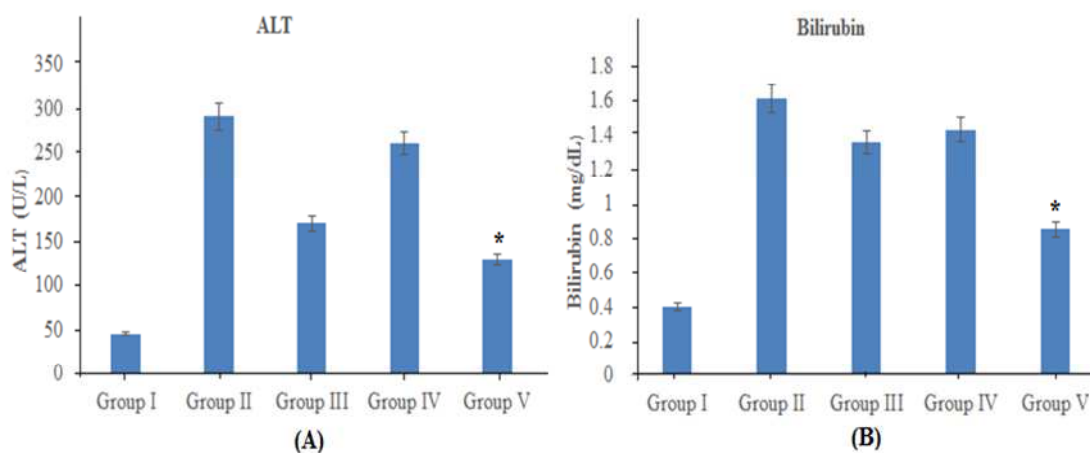


Figure 2: Biochemical examination of serum showed ALT and bilirubin level were significantly reduced in group V mice due to strong therapeutic effect of Vit E and MSCs compliantly as compare to group III and IV.

Gene expression analysis

The expression level of apoptotic and anti-apoptotic marker in all experimental groups were analyzed by using reverse transcriptase PCR. In this study β -actin is used as a standard and the expression level of other marker were compared with it. The expression of apoptotic marker (Bax) was very high in group II mice as compared to all other group. Bax expression was downregulated in Vit E and MSCs treated mice (Group V), compared to group III and IV (Fig. 3). In contrast to Bax marker, the expression level of antiapoptotic marker (Bcl-xl) in CCl₄ treated mice was downregulated. The Bcl-xl gene expression level in group V was significantly high as compared to group III and IV.

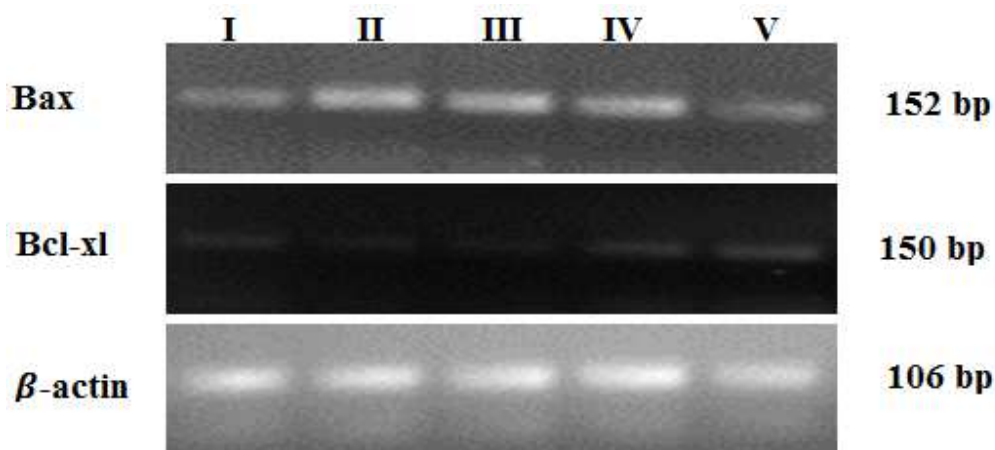


Figure 3: Expression of Bax, Bcl-xl and β -actin; line 1: group I mice, line 2: group II mice, line 3: group III mice, line 4: group IV mice, line 5: group V mice.

DISCUSSION

Liver fibrosis is one of the leading cause of death world-wise in which disruption occur in the architecture of hepatic tissue and extracellular matrix accumulate in response to chronic liver injury [17]. Chronic injury of the liver may be caused due to viral hepatitis, autoimmune, cholestatic, toxic compound, metabolic disorders such as nonalcoholic steatohepatitis[4]. CCl₄ is a toxic chemical which produces damage to the liver. When CCl₄ is given repetitively at low doses it causes liver fibrosis. The repetitive doses of CCl₄ produces rings of wound healing as a result hepatic stellate cells activation due to which imbalance occur between production and degradation of ECM

and liver fibrosis is developed [18]. Stem cell regenerative therapy is an alternative way for curing liver fibrosis. MSCs have a basic property to reduce different diseases such as fibrosis and also improve the function of lung, liver, heart, and brain when administered in these organs [19]. In the current study, BM-derived MSCs was isolated from femer and tebial bone of male albino mice and was cultured. These MSCs combined with Vit E were transplanted to CCl₄ injured mice tail vein.

In stem cell therapy, allogeneic or autologous stem cells were transplanted to the doner body (patient) through systemic infusion or by local delivery. For example in cancer such as leukemia HSCs transplantation has been used for many years [20]. Other most frequently used stem cells therapy, which have been recently reported, include marrow-derived MSCs. These MSCs have extensive spectrum of application therapy such as cardiovascular disease, lung fibrosis therapy, treatment of spinal cord injury and bone and cartilage therapy [3]. It has been investigated that locally migrated bone marrow cells can be useful in treating coronary artery disease, by generating de novo myocardium, and therefore have a dramatic improvement in worldwide heart activity [21].

Fibrotic scar produced during liver fibrosis, which is mostly made of type I and III collagen, proteoglycans etc[22]. It has been reported in our previous manuscript that the liver color of healthy mice is reddish black while that of CCl₄ induced liver injured mice was brownish- black colors [23]. In this study the liver morphology in regard to their color showed that group V mice have more similarity to group I due to strong therapeutic effect of Vit E and MSCs combined as compared to group III and IV mice (Fig. 1). This morphological result showed that the therapeutic effect of Vit E and MSCs combined was significantly high on CCl₄ injured mice as compared to Vit E and MSCs individually. Furthermore, mice treated with CCl₄ show a more increase in liver weight, compared to normal liver. MSCs and Vit E combined therapy reduces this increase in liver weight, caused by CCl₄ as shown in the table (Table 2).

The liver contains several important enzymes for drugs degradation and detoxification of dangerous materials. CCl₄ toxicity enhance the level of ALT and AST in blood and give rise to hepatocytes necrosis in rats [24]. In the current study, the effect of Vit E and MSCs were studied on the serum ALT and bilirubin level in mice. Vit E and MSCs transplantation individually restored the level of liver enzymes but the combined effect of Vit E and MSCs (Group V) have significant reduction on liver enzyme, closely to usual level as compared to group III and IV (Fig. 2).

PCR analysis showed that the expression level of *Bax*, which is an apoptotic markers is upregulated in CCl₄ injured mice while that of anti-apoptotic marker (*Bcl-xl*) was downregulated[23]. These apoptotic markers are the indicator that clearly demonstrates liver disease. These expression level of the apoptotic and anti-apoptotic markers were reversed after transplantation of Vit E and MSCs. Vit E with MSCs combined decreases the expression of Bax and increases the expression of Bcl-xl marker in group V mice as compared to group III and IV. Vit E combined with MSCs transplantation significantly restore the abnormal expression of Bax as compared to MSCs and Vit E administrated separately in CCl₄ treated mice (Fig. 3). Thus it is cleared that the combined therapy of Vit E and MSCs have high therapeutic effect on CCl₄ injured as compared to Vit E and MSCs individually. From all the results, it was concluded that the individual administration of Vit E and MSCs showed decrease effect on the reduction of liver fibrosis in CCl₄ injuredmice as compared to the combined therapy of Vit E and MSCs. Administration of Vit E combined with MSCs bring back the fibrotic liver to its normal state. Vit E acts as a strong antioxidant by increasing the antioxidant activity of liver enzyme, glutathione peroxidase and thioredoxinreductase, which neutralize free radicals and thus reduces the rate of liver hepatocyte damage. Therefore combined therapy of Vit E and MSCs revealed as the hopeful source of stem cells therapy for liver fibrosis.

Conclusion

Currently a very novel treatment option for fibrotic liver disease is the use of stem cell therapy. The present study suggested that Vit E and MSCs combined have strong therapeutic potential on the reduction of hepatic fibrosis in CCl₄ injured animal. VitE enhancing the antioxidant activity of liver enzyme such as glutathione peroxidase and thioredoxin reductase, which neutralize free radicals and thus reduces the rate of liver hepatocyte damage in CCl₄ injured mice. Similarly, MSCs transplantation enhancing liver function by stimulating hepatocyte regeneration in CCl₄ injured mice. Thus from morphological, biochemical and molecular studies, it was concluded that Vit E combined with MSCs transplantation have high therapeutic effect on reduction of liver fibrosis as compared to the individual effect of Vit E and MSCs. So it can be concluded that stem cells research has a valuable impact on human society as it would cures and provide treatment for every type of human disease i.e. from heart disease to cancer.

Competing Interest

All the authors declared that they have no competing interest.

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