The effect of HUCB Stem Cells Transnsplantation on Preservation of Liver Vasculature in Mice

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Abstract: Background and aim: Liver fibrosis is an accumulation of scar tissue in the liver caused by liver disease like hepatitis. However, numerous chemicals and drugs, like alcohol, can also cause fibrosis As a result, effective antifibrotic treatments are urgently needed. Recently, many studies demonstrated that stem-cell-based therapies might be developed for effective treatment of liver disease by ameliorate liver fibrosis and preserve vascular endothelial function by reducing the biochemical markers of inflammation (Cell adhesion molecules (CAMs)) and increase vascular endothelial growth factor (VEGF). Objective: The present work is designed to investigate the effect of HUCB stem cells transnsplantation on preservation of vasculature of liver and decrease inflammation and fibrosis of portal tract mice. Methods:. Induced hepatic fibrosis in mice with CCl_4, HUCB stem cells were infused systemically through the tail vein immediately after exposure to CCl_4 Then continues injection of CCl_4 for10 weeks, control mice received only saline infusion. After 10 weeks of the first dose of CCl_4 mice were killed under anesthesia, liver was taken for histopathological examination, Blood was collected for measuring sICAM- and vascular endothelial growth factor (VEGF). Results. Found that The serum level of sICAM-1 increased significantly in G2 (non treated) compared to G3(control group). Stem cells reduced the increase in sICAM-1 significantly ($P<0.05$). Induction of liver fibrosis increased significantly the release of sVEGF compared to the control group. Treatment with stem cells increased significantly the release and expression of sVEGF .Histological examination suggested that hepatic damage recovery was much better in the stem cells treated mice as the portal tract inflammation, fibrosis were statistical significantly lower in treated mice than in non treated. Conclusion. The results suggest that Human Umbilical Cord Blood Stem cells improve and preserve vasculature of liver and decrease inflammation and fibrosis of portal tract mice.

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Key words: Carbon tetrachloride CCl4, VEGF, Adhesion molecules (sICAM1) liver fibrosis.

1. Introduction

Liver fibrosis is an accumulation of scar tissue in the liver caused by liver disease like hepatitis. (1) However, numerous chemicals and drugs, like alcohol, can also cause fibrosis. Current treatments for cirrhosis are limited to remove the underlying injurious (2).

As a result, effective antifibrotic treatments are urgently needed. Recently, many studies demonstrated that stem-cell-based therapies might be developed for effective treatment of liver disease. (3)

Administration of carbon tetrachloride (CCl_4) to rodents is a widely used model to study mechanisms of hepatic injury. CCl_4 causes hepatocyte injury that is characterized by centrilobular necrosis and is followed by hepatic fibrosis (4)

VEGF (Vascular endothelial growth factor) is a potent and specific mitogen for vascular endothelial cells and it is related to the development of capillaries. Its expression is controlled by a transcriptional factor, namely hypoxia inducible factor-1α (HIF-1 α), which is known to be adaptive response during hypoxia (5).

Cell adhesion molecules (CAMs) are inflammatory markers of the endothelium.

They are a family of cell surface glycoproteins that are critical in endothelial/leukocyte and platelet/ leukocyte binding. Adhesion molecules, such as intercellular adhesion molecule 1 (sICAM-1), and vascular cell adhesion molecule 1 (sVCAM-1) present on endothelial cells (6).

Vascular endothelial growth factor (VEGF) is a principal regulator of blood vessel formation and haematopoiesis but the mechanisms by which VEGF differentially regulates these processes have been elusive. VEGF controls survival of haematopoietic stem cells (19).

VEGF receptor 1 stimulates stem-cell recruitment(20).Therefore, the present study was directed to examine the effect of the effect of HUCB stem cells transnsplantation on preservation of vasculature of liver and decrease inflammation and fibrosis of portal tract in mice through release and expression of VEGF, and by decreasing the expression of adhesion molecules (ICAM-1)

2. Methods

Experimental study: thirty Albino mice 6 weeks old 40 gm weight acclimatized for one week and kept with free access to standard pellet animal diet and tap

water under controlled conditions (temperature 23±3°C nd relative humidity 50±20 %0%).Animals were not received any immunosupressed agents. devided into: 3groups:

Group (1): n =10 mice, this group was subjected to: Induction of liver fibrosis with CCl_4 (2ml/kg body weight dissolved in 150µl of corn oil solution) in one subcutaneous injection each third day (twice weekly)was done until they were killed 10 weeks later with a total dosage of 40 ml/kg.

Transplantation of Human Umbilical Cord Blood (HUCB) stem cells of male fetus with a dose of 106 HUCB stem cells/mouse was injected through the tail vein immediately after exposure to CCl_4 Then continue injection of CCl_4 for10 weeks (liver fibrosis was completed before cirrhosis).The injection through the tail vein, is an easy and effective method for systemic administration of stem cells (7).

Group (2): n = 10 mice, this group was subjected to: Induction of liver fibrosis with CCl_4.This group did not receive HUCB stem cells and received the same volume of saline and served as one of the two control groups (+ve control).

Injection of CCl_4 for10 weeks

Group (3): n = 10 mice, this group received only corn oil (solvent of CCl_4). Each mouse received 2ml/kg of water dissolved in 150µl of corn oil over 20 subcutaneous injection once every 3 days and served as one of the two control groups (-ve control).

After 10 weeks of the first dose of CCl4, mice were killed under anesthesia, liver was taken from all animals and was fixed in 4% paraformaldehyde and paraffin embedded for sectioning and preparing for histopathological examination (Light microscopy examination).

Blood was withdrawn from retro-orbital sinus for assessment of sICAM-1 and sVEGF.

Biochemical analysis:

Two ml of blood was withdrawn from the retro-orbital sinus with heparinized microhematocrit capillary tubes (8). Sera were separated and frozen at –200C till the time of chemical analysis of Serum sICAM-1, and sVEGF (9). were estimated by ELIZA technique with specific kits for rat adhesion molecules.

Stem cell separation:

Cell sources: Umbilical cord blood (UCB) was obtained at the end of full term vaginal deliveries, after clamping and cutting of the cord (10).

The collections were made prior to the expulsion of the placenta; while the placenta was still in utero. Using strict aseptic techniques, the umbilical vein was cleansed with alcohol followed by Betadine®.

The umbilical vein was pierced and UCB was collected into sterile collection tubes containing citrate phosphate dextrose adenine-l (CPDA-l) anticoagulant (approximately 5 ml) since total collection was approximately 50ml. Samples were collected separately.

UCB units were stored at 4 °c & processed within 24 hrs. (10).

Steps of CD34 separation:

I-Isolation of low density Mononuclear Cells (MNC):

Isolation buffer used consists of: 100ml DW, 10ml phosphate buffered saline (PBS), and 1ml fetal calf serum (FCS).Then Dilution: 17.5ml blood and 17.5ml isolation buffer (1:1).

7ml diluted blood and 3ml lymphocyte separation medium (Ficoll).Then Centrifuge 20 min at 2000g and then the interface was collected, suspend cells in an equal volume of isolation buffer centrifuge again 20 min at 500g. Resuspending pellet in isolation buffer again and centrifuge 20 min at 300g.to a concentration in the range of 4 x 107 - 4 X 10 8 cells/ml in cold isolation buffer.The total volume should be at least 1 ml , Maintain at 2-8°C.And assessing the viability of cells by Trypan blue dye.

-Nearly 1 million CD34+ were separated from 100 million MNCs.

II- Separation and Purification of CD34+ cells:

Principle:-

Immunomagnetic cell selection using Dynabeads M-450 CD34 and DETACHaBEAD CD34 provides fast and reliable positive selection of (CD34+ hematopoietic progenitor cells. Dynabeads M-450 CD34 and DETACHaBEAD CD34 used to isolate CD34+ cells from a wide range of sample volumes.

Upon mixing and incubation. (CD34+ cells bind to Dynabeads M-450 CD34 and the rosetted cells were isolated from the suspension with a DYNAL Magnetic Particle Concentrator (DYNAL MPC).

A subsequent incubation with DETACHaBEAD CD34 gently detaches isolated cells from the beads.

A DYNAL MPC was used to separate the purified, positively selected CD34+ cells from the released Dynabeads M-450 CD34.

Materials supplied:-

Dynabeads M-450 CD34 were supplied as a suspension of 4 x 108 beads/ml in phosphate buffered saline (PBS), pH 7.4, containing 0.1% human serum albumin (HSA) and 0.02% (NaN_3).

DETACHaBEAD CD34 was affinity, purified polyclonal antibodies against the FAB portion of mAb 561. It was supplied sterile filtered in PBS, pH 7.4. No preservatives were added.

Additional material used: Magnetic separation device [Magnetic Particle Concentrator (DYNAL MPC-I)].

FLOW CHART ISOLATION OF CD34+ CELLS:

Isolate low density MNC, 2- incubate with Dynabeads M-450 CD34, 3- Wash rosette target cells, 4- Release Dynabeads from target cells with

DETACHaBEADS CD34, 5- Separate CD34+ cells from detached beads, 6- Collect and wash isolated CD34+cells

Histopathological examination:

Data of pathology was represented as the following items: portal tract inflammation, portal tract fibrosis and was not represented as grading or scoring because Knodell score is a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis and not used in CCl_4 induced liver fibrosis(11).

However, the degree of each pathological item (mild, moderate and marked) was according to Knodell score and was evaluated by two professors of pathology who were blind to the groups.

Statistical analysis:

SPSS statistical software was used applying appropriate statistical method. Data are presented as mean ± SD. Differences between two groups were assessed by the Student's paired t test, chi square test and Fisher's exact test. Comparison among the three groups was performed by one way analysis of variance. Chi square test and Fisher's exact tests were also used. *P* value <0.05 was considered significant (12)

3. Results:

1. The serum level of **sICAM-1** increased significantly ($p < 0.001$) in G2(non treated) compared to G3(control group). (360.55±129.78 ng/ml and 46.4±21ng/ml respectively). Stem cells reduced the increase in sICAM-1 significantly (263.18±92.7ng/ml).

Table (1): serum sICAM

tests / Groups	sICAM-1
Treated (G1)	263.18±92.7*
Non treated (G2)	360.55±129.78 *
Control group (G3)	46.4±21*
P value	0.001*

* Statistically significant

2.Effect of stem cells on **sVEGF** release in CCl_4 induced liver fibrosis

Table (2) showed that induction of liver fibrosis increased significantly the release of sVEGF compared to the control group. Treatment with stem cells increased significantly the release and expression of sVEGF (*P*=0.017).

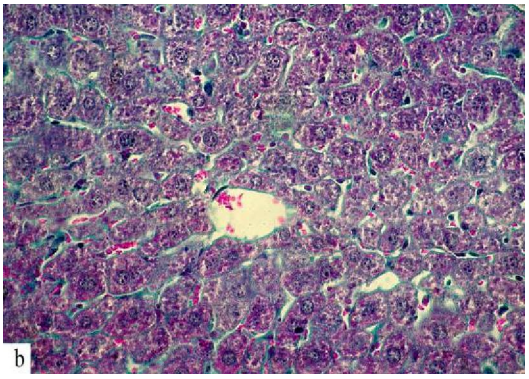
Table (2): sVEGF release

	Control G3	Non treated G2	Treated G1
VEGF Mean (pg/ml)	56.9*	210.6*	289.3*
±SD	±17.83	± 63	± 25.1

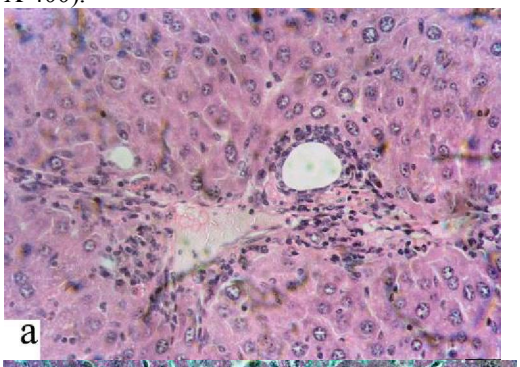
P<0.05 considered significant

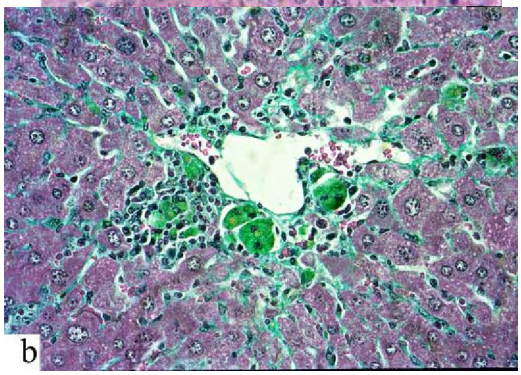
Results of Histopathology:

1-Section from normal liver tissue showing No portal tract fibrosis. Masson trichrome stain X 400.

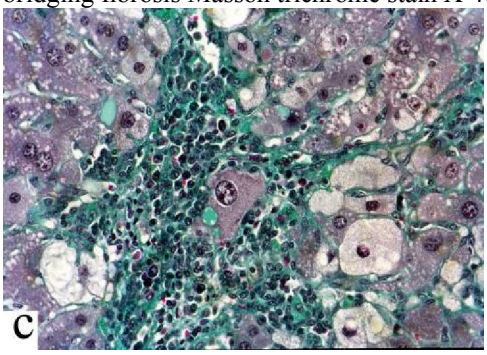


2-Section from liver tissue of G1 showing mild portal lymphocytic infiltrate (H&E & Masson Trichrome stain X 400).





3-Sections from liver tissue of G2 showing porto-portal bridging fibrosis Masson trichrome stain X 400.



1-Portal tract inflammation:

Table (3): Comparison between all groups according to portal tract inflammation,

Portal tract Inflammation / Groups	No inflammation	Mild inflammation	Moderate inflammation	Marked inflammation
Treated G1	20%	60%	10%	10%
Non treated G2	0%	20%	10%	70%
Control G3	100%	0%	0%	0%

Chi: 38.8 *P* value = 0.001

Portal tract inflammation was significantly different among the three groups.

In treated group there was no inflammation in 20%, on the other hand, there was 70% marked inflammation in non treated and no inflammation in 100% of the control

2- Portal tract fibrosis:

Table (4): Comparison between all groups according to portal tract fibrosis, the second item of pathology.

Portal tract fibrosis / Groups	No fibrosis	Mild fibrosis	Moderate fibrosis
treated G1	100 %	0%	0%
Non treated G2	.0%	100%	100%
Control G4	100%	0%	0%

Chi: 31.0 *P* value = 0.001

Portal tract fibrosis was significantly different among the three groups.

In treated group there was no fibrosis, the same results was found in the control group, while fibrosis in the non treated group was 100%,

4. Discussion:

Over the past decade umbilical cord blood (UCB) emerged as a new source of hematopoietic stem cells, with the potential to significantly alleviate the shortage of donors that has worried bone marrow transplantation. It appears to result in sustained engraftment of donor hematopoiesis similar to results achieved with marrow and peripheral blood hematopoietic stem cells, and is associated with a lower incidence and less severity of Graft Versus Host Disease (GVHD) than other sources of stem cells However, there are potential limitations to the widespread use of UCB as a source of hematopoietic progenitors for marrow replacement and gene therapy (13).

Administration of carbon tetrachloride (CCl_4) to rodents is a widely used model to study mechanisms of hepatic injury. CCl_4 causes hepatocyte injury that is characterized by centrilobular necrosis and is followed by hepatic fibrosis(14)

Baijun *et al.,* induced hepatic fibrosis in immunosupressed albino mice 6 weeks old by CCl_4 for 5 weeks but in this study induction of fibrosis by CCl_4 took 10 weeks and this may be due to the different mice generation, and different temperature and humidity in addition to the purity and efficacy of the drug (CCl_4).these results were according to pilot study which was done before research. (15)

This study was done on non immunosupressed mice and HUCB stem cells was not rejected this may be due to different and less advanced immune system in mice and may be due to affection of their immunity by the induction of liver fibrosis.

Turkdogan *et al.,* who evaluated the efficiency of systemically administered mesenchymal stem cells on CCl_4 – induced fibrosis in mice. In contrary to this study, we evaluated the efficiency of systemically administered human umbilical cord blood stem cells (HUCBSCs) on CCl_4 – induced fibrosis in mice. Human umbilical cord blood is a rich source of stem cells, which can be obtained without using invasive technique. (16).

In this study the serum level of **sICAM-1** The serum level of **sICAM-1** increased significantly in G2 (non treated) compared to G3 (control group). (360.55±129.78 ng/ml and 46.4±21ng/ml respectively) $p < 0.001$.

This result supported by Ara *et al.* (6) who found that sICAM-1(1720+/-1174 ng/ml) was significantly higher in patients with vasculitis than in healthy controls (*P*=0.001) .

In the current study The main findings was significantly reduced levels of circulating proinflammatory markers i.e. ICAM-1 by Stem cells treatment significantly (263.18±92.7ng/ml). As autologous bone marrow-derived stem cell therapy (ABMSCT) is an emerging modality to induce angiogenesis from endothelial progenitors (17).

In the current study induction of liver fibrosis increased significantly the release of sVEGF compared to the control group.

This is similar to Amarapurkar *et al.* (18) who found that Expression of VEGF was commonly found in early stages of fibrosis.

In this study the treatment with stem cells increased significantly the release and expression of sVEGF (*P*=0.017).which is found by Troy *et al.* as adult MSCs produced significantly more VEGF than embryonic MSCs and were noted to increase postischemic myocardial recovery(21)

More importantly, histological examination suggested that hepatic damage recovery was much better in the stem cells treated mice (22,23)

In this study, data of pathology was represented as the following items: portal tract inflammation, and portal tract fibrosis and was not represented as grading or scoring because Knodell score is a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis and not used in CCl_4 induced liver fibrosis(11).

Portal tract inflammation was significantly different among the three groups.

In treated group there was no inflammation in 20%, On the other hand, there was 70% marked inflammation in non treated and no inflammation in 100% of the control.

And Portal tract fibrosis was significantly different among the three groups.

In treated group there was no fibrosis, the same results was found in the control group, while fibrosis in the non treated group was 100%,

This is similar to Baijum *et al.,*who found that, areas of liver degeneration and necrosis in mice received CCl_4 were significant more than stem cells treated mice. (15), and Yoshiji *et al.,*found that the hepatic damage improvement in the histological examinations was much better in the stem cells treated mice.(22).

Conclusion:

There was no rejection of HUCB stem cells in non immunosupressed mice with CCl4 induced liver fibrosis.

Human Umbilical Cord Blood Stem cells improve and preserve vasculature of liver and decrease inflammation and fibrosis of portal tract mice.

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