CAN THE MESENCHYMAL STEM CELLS ATTENUATE APOPTOSIS IN ADULT MALE ALBINO RATS WITH ACETAMINOPHEN-INDUCED HEPATIC FAILURE

By

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ABSTRACT

Background: Liver failure is a worldwide health problem. Stem cells provide a great promise in regeneration of liver tissue. Objective: Assessment of the role of apoptosis in the pathophysiology of liver failure and bone marrow derived mesenchymal stem cells (BM MSCs) effect on it. Materials and Methods: Twenty four adult male albino rats of local strain were chosen as an animal model for this study. They were divided into control, liver failure (LF) rats, LF rats received culture media and LF rats received BM MSCs. Mesenchymal stem cells were separated from rat bone marrow, being identified by their morphology and immunophenotype (CD29, CD45 and CD90) by flow cytometry. BM MSCs were labeled with PKH26 dye before injection. LF was induced by oral administration of acetaminophen. At the end of the experiment (24 days), blood samples were obtained for estimation of serum alanine transferase (ALT) and aspartate transferase (AST). Animals were sacrificed and livers were obtained for measurement of BCL2 associated X protein (Bax), B-cell CLL/lymphoma 2 (BCL2) and transformation growth factor beta (TGFβ) gene expression in addition to histopathological examination of liver tissue. Results: BM MSCs were successfully separated from bone marrow, being identified as mesenchymal stem cells showing plastic adherence properties and fibroblastoid shape. The cells showed +ve expression of CD29 and CD90, -ve expression of CD45 proving that they were mesenchymal cells not hematopoietic cells. Acetaminophen showed significant increase in serum ALT and AST. There were significant increases of TGFβ and BAX gene expression in the liver tissue. There was significant decrease of BCL2 gene expression. BM MSCs showed significant decrease in serum ALT and AST, also there were significant decrease in TGFβ and BAX gene expression in the liver tissue in addition to significant increase in BCL2 gene expression. Histopathological examination revealed regeneration of the damaged liver tissue and restoration of normal architecture of liver tissue in BM MSCs-treated group in comparison with LF group. Conclusion: Apoptosis has important role in the pathophysiology of hepatic failure, and BM MSCs have significant role in its attenuation. Key word: BM MSCs, acetaminophen, apoptosis, liver failure.

INTRODUCTION

Prompt removal of unwanted cells, such as damaged, genetically mutated, or virus infected cells, is crucial for the maintenance of liver health. This process is naturally achieved through a highly regulated programmed form of cell death, i.e. apoptosis. In healthy organisms, the number of cells eliminated by apoptosis equals the number of cells generated by mitosis, ensuring the proper organ homeostasis. In addition, physiological apoptosis allows the removal of cells with virtually no release of proinflammatory
cytokines and minimal immune response. However, in pathophysiological situations, the balance between cell proliferation and cell death is often altered, with the consequent loss of tissue homeostasis and the onset of several liver diseases (Que et al., 1999). Excessive and/or sustained apoptosis can lead to acute injuries, such as fulminant hepatitis (Kohli et al., 1999), or even chronic sustained injuries, such as alcoholic liver disease, cholestatic liver disease, and viral hepatitis (Canbay et al., 2002). Therefore, therapeutic strategies to inhibit apoptosis in liver injury have the potential to provide a powerful tool for the treatment of liver disease (Guicciardi and Gores, 2005).

End-stage liver disease is a devastating condition with multiple etiologies. However, epidemiological data indicate an increasing worldwide prevalence of liver cirrhosis, related to chronic infection by hepatitis C or B viruses, alcohol consumption and non-alcoholic fatty liver disease (Poynard et al., 1997 and Parola et al., 2008). Currently, the only curative treatment is liver transplantation (Bernal & Wendon, 2004; Chen et al., 2005; Reding, 2005 and Fried- man, 2008). This method is limited by the critical shortage of donor organs, high cost, and the need for immunosuppression (Williams & Wendon, 1994 and Reding, 2005). Cell transplantation with hepatic stem cells could potentially replace liver transplantation in patients with end-stage liver diseases (Sanmartin et al., 2006 and Yang et al., 2007).

Cell-based therapies are quickly taking hold as a revolutionary new approach to treat many human diseases. MSCs are widely used because they are considered clinically safe, unique in their immune-capabilities, easily obtained from adult tissues, and quickly expanded as well as stored (Betancourt, 2013).

The pathophysiology of liver failure and the regenerative mechanism of MSCs are not fully understood. So, the aim of this research was to investigate the role of apoptosis in the pathophysiology of liver failure and, possible mechanism of MSCs on this model.

MATERIALS AND METHODS

Animals: Twenty four adult male albino rats of local strain were chosen as an animal model for this study. They were kept in suitable cages (20x32x20 cm for every three rats) at room temperature, with the natural light-dark cycle. They weighed 120 -140 g (average weight was 130 g). They were fed on a standard food in addition to green vegetables with free water supply. They were kept for 10 days for the adaptation to the new environments before the start of the experiment. The animals were divided into four equal groups as follows:

Group I (control group): received distilled water (0.5 ml/rat) by oral gavage for three days. Group II (LF group): LF was induced by oral administration of acetaminophen (500mg/kg) for three days (Gopi et al., 2010). Group III (LF + vehicle): Received culture media (Dulbecco’s modified Eagle’s medium DMEM), by single intravenous injection (1ml/rat) in the caudal vein in the fourth day after induction of LF. Group IV (LF + BM MSCs): received BM MSCs by intravenous injection in the caudal vein, (one million cells per rat) in the fourth day after induction of LF (Abdel Aziz et al., 2007). After twenty days from BM MSCs injection, blood samples were taken from the retro-orbital vein for measurement of
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serum ALT and AST. The animals were sacrificed to obtain liver tissue for histopathological examination and detection of TGFβ, BAX and BCL2 gene expression.

BM MSCs were prepared according to Abdel Aziz et al. (2007). Cells were identified as being MSCs by their morphology, plastic adherence (Dexter et al., 1981) and immunophenotyping using flowcytometry for CD29,CD45 and CD90 (Bieback et al., 2004). BM MSCs were labeled with PKH26 (Sigma, USA). Cells were centrifuged and washed twice in serum free medium. Cells were pelleted and suspended in dye solution. Cells were injected intravenously into rat tail vein. After twenty days, liver tissues were examined with a fluorescence microscope (Leica Microsystem, USA) to detect and trace the cells (Shao-Fang et al., 2011). Measurements were done for serum ALT (Reitman and Frankel, 1957), serum AST (Reitman and Frankel, 1957). Gene expression of BAX, BCL2 and TGFβ were done by real time polymerase chain reaction syber green I dye method (Aggarwal and Gupta, 1998).

Liver was excised for histopathological studies and detection of BM MSCs homing in the liver tissue. Different sections were obtained, stained with hematoxyline and eosin (Hx and E) and examined using a light microscope. Other slides were kept without staining to be examined by fluorescent microscope for detection of BM MSCs homing.

Statistical analysis: All the statistical analyses were processed using Statistical Program of Social Sciences (SPSS) for windows (version 17, SPSS Inc., Chicago, IL, USA). Values of the measured parameters were expressed as mean value ± standard deviation (SD), and the differences and significance were verified by one-way ANOVA followed by the Fisher’s least significant difference (LSD) post hoc test. P values less than 0.05 were considered statistically significant.

RESULTS

Twenty four hours from the primary culture (passage 0 = P0) of bone marrow derived mesenchymal stem cells, the cultured cells appeared crowded and suspended. They were variable in size and shape. Most of the cells appeared rounded (Figs.1a). Twelve days from the primary culture, the adherent cells reached 80-90% confluency (Fig.1b) and appeared triangular, star shaped and spindle shaped (Fig.1c). The cells were examined under fluorescent microscope after labeling with PKH26 dye (Fig.1d). The liver tissue was examined under fluorescent microscope for detection of homing of BM MSCs (Fig.1e) Immunophenotyping of BM MSCs using flowcytometry showed positive expression of CD29 & CD90, and negative expression of CD45 (Fig.1f,g & h).

Effects of BM MSCs on some liver functions Fig. (2):

In group I, the mean ± standard deviation of serum ALT and AST were 21.03±0.74 IU / L and 13.5±1.52 IU / L respectively. In group II, the mean ± standard deviation of serum ALT and AST were 106.53±4.8 IU / L and 86.01±1.41 IU / L respectively. There were significant increases in serum ALT and AST in group II when compared with group I. In group III, the mean ± standard deviation of serum ALT and AST were 104.38±3.13 IU/L and 87.16±2.04 IU/L respectively. There were no significant differences in group III when compared with group II. In group IV, the mean ±
standard deviation of serum ALT and AST were 40.28±1.34 IU/L and 31.17±2.32 IU/L respectively. There were significant decreases in serum ALT and AST in group IV when compared with group II.

**Figure (1):** phase contrast micrograph of BM MSCs under inverted microscope, the cell appeared rounded after 24hrs of separation (a), turned to fibroblastoid, star and triangular shape reaching 80 to 90% confluency (b,c). The cells have taken the colour of PKH26 dye under fluorescent microscope (d). Labeled cells were detected in liver tissue in the central canal and blood sinusoids taking (e). Histograms of flowcytometry showing +ve expression of CD29 and CD90 (f,g), and –ve expression of CD45 (h).
Effect of BM MSCs on TGFβ, Bax and Bcl2 gene expression in the liver tissue

Fig. (3):

In group I, the mean ± standard deviation of liver tissue levels of TGFβ, Bax and Bcl2 were 1.074±0.043, 1.091±0.062 and 1.087±0.079 respectively. In group II, the mean ± standard deviation of liver tissue levels of TGFβ, Bax and Bcl2 were 9.28±1.2, 7.93±0.33 and 0.39±0.028 respectively. There were significant increases in TGFβ and Bax in group II when compared with group I. Also, there was significant decrease in Bcl2 in group II when compared with group I. In group III, the mean ± standard deviation of liver tissue levels of TGFβ, Bax and Bcl2 were 10.13±0.88, 7.81±0.38 and 0.43±0.025 respectively. There was no significant difference in group III when compared with group II. In group IV, the mean ± standard deviation of liver tissue levels of TGFβ, Bax and Bcl2 were 3.45±0.28, 3.02±0.32 and 0.88±0.029 respectively. There were significant decreases in TGFβ and Bax in group IV when compared with group II. Also, there was significant increase in Bcl2 in group IV when compared with group II.

Histopathological examination:

In group I there was normal architecture of hepatic tissue, normal cords of hepatocytes radiating from normal central vein and normal blood sinusoids. Group II and III showed dilation, congestion of central vein, widening of blood sinusoid, congestion and dilation of portal tract with lymphocytic infiltration. Group IV showed more or less normal central vein, radiating regenerated cords of hepatocytes with normal blood sinusoids.
Figure (3): Effect of BM MSCs on TGFβ, Bax and Bcl2 gene expression in the liver tissue (Mean±SD).

Figure (4): Photomicrograph of the liver tissue by light microscope showed normal cords of hepatocytes radiating from normal central vein (a). Fig. b and c showed congestion and dilatation of the central vein (cv) and portal tract (pt) with lymphocytic infiltration of portal tract (li). There was showed restoration of normal cords of hepatocytes and central vein by regenerating cells (4d) (HX and E, X100).
DISCUSSION

MSCs can be separated from many tissues like adipose tissue, dental bulb, skeletal muscles, umbilical cord and even the brain tissue (Orbay et al., 2012). In the current work MSCs were separated from bone marrow showing characteristics which agreed with (Abd el Aziz et al., 2014). Only bone marrow-derived MSCs have documented evidence of stemness including the ability to form bone and bone marrow organ upon serial transplantation in vivo. In addition, they are multipotent cells (Sacchetti et al., 2007). Separation of MSCs from bone marrow can be done by several methods including plastic adherence (Bara et al., 2014), gradient density centrifugation (Insausti et al., 2012) and immuno-magnetic selection (Tillotson et al., 2016). In the current work MSCs were separated by plastic adherence method which depended on MSCs adherence to any plastic surface. In the current work mesenchymal stem cells were separated from bone marrow and labeled with PKH26 dye. Shao-Fang et al. (2011) found that labeling with PKH26 did not yield any differences in morphology, proliferation ability, apoptosis, and cell cycle of human umbilical mesenchymal stem cells which indicate that PKH26 did not change the physiological activity of cells.

The non-treated liver failure group showed significant increase in liver enzymes (ALT and AST) compared to control group. These results agreed with Zaher et al. (2008) and Gopi et al. (2010) who concluded abnormally higher activities of serum ALT and AST after paracetamol administration. When liver plasma membrane gets damaged, a variety of enzymes normally located in the cytosol are released into the circulation (Afroz et al., 2014).

Acetaminophen-treated group showed significant increase of TGFβ and BAX gene expression. Also, there was significant decrease in BCL2 gene expression which indicated occurrence of apoptosis in the liver tissue. This result agreed with Li et al. (2013). Accumulating evidence suggests that hepatocyte apoptosis plays a critical role in acetaminophen-induced hepatic injury (Hu & Colletti, 2010 and Havasi & Borkan, 2011). Acetaminophen-induced apoptosis is observed not only in primary hepatocytes (Sharma et al., 2011), but also in livers of mice treated with toxic doses of acetaminophen. Moreover, inhibiting apoptosis prevents the development of acute liver failure (Hu et al., 2010).

The transforming growth factor-β1 (TGF-β1) gene is located on chromosome 19. The TGF-β1 polypeptide is a member of the TGF-β superfamily of cytokines. It is a secreted protein that performs many cellular functions, including the control of cell growth, inflammation, extracellular matrix deposition, cell proliferation, cell differentiation, and apoptosis (Dobaczewski et al., 2011 and Wan et al., 2015). Increased transforming growth factor-β1 (TGF-b1) signaling is a highly potent inducer of collagen synthesis, and TGF-b1 pathway plays a vital role in the progression of hepatic fibrosis (Xiao et al., 2010).

BAX is a member of the Bcl-2 gene family. BCL2 family members act as anti- or pro-apoptotic regulators that are
involved in a wide variety of cellular activities. The encoded protein from BAX forms a heterodimer with BCL2, and functions as an apoptotic activator. This protein is reported to interact with, and increase the opening of the mitochondrial voltage-dependent anion channel, which leads to the loss in membrane potential and the release of cytochrome c (Kazi et al., 2011). Deng et al. (2015) reported that hepatitis C virus increases BAX gene expression which mediates apoptosis in the mitochondria. It was reported that TGFβ induces expression of BAX and this expression is a key factor of TGFβ induced apoptosis (Westphal et al., 2014).

BM MSCs-treated group showed significant decrease in TGFβ and BAX in addition to significant increase in BCL2 gene expression which indicates that BM MSCs significantly decreased occurrence of apoptosis in the liver tissue in this group. This agreed with Jin et al. (2013). BM-MSCs inhibit hepatocyte apoptosis by secreting cytokines, thus regulating cellular signal transduction pathways. In rats, BM-MSCs secrete vascular endothelial growth factor, which attenuates myocardial IR injury by activating the PI3K signaling pathway (Angoulvant et al., 2010), and the PI3K pathway can regulate the expression of BCL-2, an anti-apoptotic protein (Westphal et al., 2014). In rat neurons, MSCs secrete cytokines that reduce chronic ethanol-induced injury by modulating the extracellular-signal-regulated kinase (ERK)1/2 pathway (Liu et al., 2010). The ERK1/2 pathway regulates apoptosis by increasing the Bax/Bcl-2 ratio, Casp3 levels and TNF levels (Mohan et al., 2012).

REFERENCES


Can the Mesenchymal Stem Cells Attenuate Apoptosis in the Hepatic Lesion Induced by the Interleukin-1β and the Interleukin-2...