Role of Human Wharton's Jelly Derived Mesenchymal Stem Cells (WJ-MSCs) for Rescue of D-Galactosamine Induced Acute Liver Injury in Mice

Raghu Ramanathan*, Secunda Rupert*, Sakthivel Selvaraj*, Jeswanth Satyanesan*, Rosy Vennila*, Surendran Rajagopal†

*Centre for Advanced Research, Stem Cell Research Centre, Govt. Stanley Medical College and Hospital, Chennai, Tamilnadu, India and

†Director, Hepato-Pancreato-Biliary Centre for Surgery & Transplantation, MIOT International, Chennai, Tamilnadu, India

Background/Aim: Mesenchymal stem cells (MSCs) are multipotent precursor cells having self-renewal ability making them a candidate for use in regenerative medicine. Acute liver injury results in sudden loss of hepatic function leading to organ failure. Liver transplantation is often required to salvage patients with acute liver failure. Due to shortage of organs, identification of alternate method is the need of the hour. In view of this, an attempt has been made to check the regenerative ability of WJ-MSCs (Wharton's jelly derived MSC) in mice models for acute liver injury. **Methods:** Swiss albino mice weighing 25 ± 5 g were used in this study. The control mice (Group I), was given saline. Group II mice received D-Galactosamine (D-GalN—800 mg/kg; i.p). Group III mice similar with Group II, received WJ-MSCs (5×10^5 cells/0.5 ml DMEM) through tail vein, 24 h after D-GalN administration and Group IV mice received MSC alone. **Results:** Parameters, indicative of hepatotoxicity and oxidative stress were analyzed. A two-fold elevation in the marker enzymes of liver toxicity such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (SAP), and total serum bilirubin (TBIL) confirms hepatocellular injury, while a greater than four-fold increase in malondialdehyde (MDA) formation, along with around 40% fall in superoxide-dis-mutase (SOD) activity was indicative of oxidative stress and loss of hepatocellular membrane integrity induced by D-GalN. The above biochemical and pathological changes were significantly restored in mice that received WJ-MSCs indicating hepatoprotective and probable regenerative property. **Conclusion:** The present study showed that WJ-MSC treatment is able to rescue/ameliorate the hepatotoxicity induced by D-GalN in mice. (J CLIN EXP HEPATOL 2017;7:205–214)

The acute liver failure (ALF) is a clinical condition which results in both fulminant and subfulminant hepatic failure. There are many causes of ALF such as viral infections, drugs and chemicals-induced toxic injury, alcohol abuse, autoimmune disorders and genetic disorders leading to liver failure.¹ No specific medical treatment is presently available. Liver disease is an important cause of morbidity as well as mortality worldwide.² Liver transplantation is the only effective therapy available till date for all chronic end-stage liver diseases and many cases of ALF. Identifying an alternate remedy is the need of the hour, considering the shortage of available donor organs, high costs and requirement for lifelong immunosuppressive drugs following liver transplantation.

D-Galactosamine (D-GalN) is a well-known hepatotoxicant, which causes severe hepatocellular necrosis and other clinical features similar to that of human viral hepatitis in experimental animals. D-GalN is a specific hepatotoxic agent that inhibits macromolecule synthesis such as RNA and proteins resulting in organ dysfunction. Some studies have shown that the reactive oxygen species (ROS) generated by metabolism of D-GalN resulted in hepatotoxicity.³ A recent study by Pushpavalli et al. (2010),⁴ concludes that oxidative stress and inflammatory response are the two main factors for D-GalN-induced acute liver injury. Rodents administered with D-GalN show remarkable sensitization to lipopolysaccharide (LPS), and this D-GalN/LPS treatment in animals has been recognized as one of the promising model to study the clinical conditions of ALF in vivo.⁵ D-GalN is known to selectively block transcription and indirectly influence hepatic RNA and protein synthesis as a consequence of endotoxin toxicity.⁶ D-GalN mainly causes liver injury via the generation of free radicals and depletion of uridine triphosphate (UTP) nucleotides leading to progressive damage of cellular membranes and in higher doses, it is known to cause hepatic necrosis.^{7,8}

Stem cell based cell therapy has now gained interest as an alternative option for treating ALF.^{9,10} Specifically mesenchymal stem cells (MSCs) are being explored as an

Keywords: D-galactosamine, WJ-MSCs, oxidative stress, hepatotoxicity, cytogenetics

Received: 25.10.2016; **Accepted:** 14.03.2017; **Available online:** 1 April 2017

Address for correspondence: Rosy Vennila, Centre for Advanced Research, Stem Cell Research Centre, Govt. Stanley Medical College and Hospital, Chennai, Tamilnadu, India. Tel.: +91 044 25280032.

E-mail: stemcellstanley@gmail.com

Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; MSCs: mesenchymal stem cells

<http://dx.doi.org/10.1016/j.jceh.2017.03.010>

effective therapeutic tool in various types of experimental liver injuries in animals.^{10,11} MSCs can be isolated from various tissues, including bone marrow, muscle, tooth, amniotic fluid, placenta, adipose tissue.^{12,13} MSCs are also known to have regenerative and anti-inflammatory properties⁵ which make them suitable for use in regenerative medicine. Currently MSCs are being explored in inflammation-related diseases, such as acute lung injury, neuropathic pain, acute respiratory distress syndrome, and renal injury due to its immunomodulatory effects along with interleukins which play a major role against the above diseases.¹⁴ Recently it is found that WJ-MSCs has the ability to escape immune surveillance due to the absence of the antigen major histocompatibility complex II (MHC-II) and the co-stimulatory surface antigens CD40, CD80, and CD86.^{15–17} These findings suggest that WJ-MSCs has low immunogenicity and might be well tolerated in xenotransplantation without immunosuppression. Previous studies have also reported that transplantation of human MSCs without immunosuppression produces beneficial effect in animal models of Alzheimer's disease, Parkinson, traumatic brain injury, and stroke.^{18–22} So this interested us to use WJ-MSCs in our present study to analyze whether the MSCs derived from wharton's jelly is able to rescue the hepatotoxicity induced by D-GalN in acute liver injury in mice model.

MATERIALS AND METHODS

Isolation and Culture of WJ-MSC

Informed consent was obtained from mother prior to delivery for collecting the umbilical cord aseptically, sectioned and transported from Govt. Raja Sir Ramaswamy Mudaliar (RSRM) Lying-in Hospital, Chennai to the Stem cell research lab in Govt. Stanley Hospital, Chennai, Tamilnadu, India. The samples were transported within 15 min and processed immediately without further delay. MSCs were cultured by our previously standardized protocol.²³ Explants were removed from the culture flask on day 7 and the medium was renewed every 48 h and sub cultured on reaching 70–80% confluence.

Detection of Surface Markers of WJ-MSCs Using Flow Cytometry

Cell surface marker expressions were determined on all groups of MSCs, cultured in PL from P1 to P5. The primary antibodies used were fluorescent conjugated anti-human CD90-FITC, CD73-APC, CD105-PerCp-Cy5.5 (mesenchymal lineage), CD14-FITC, CD34-PE and CD45-APC-CY7 (hematopoietic lineage). Flow cytometry was performed using fluorescence activated cell sorter (BD FACS Aria II) and the results were analyzed with DIVA software.

G-banded Karyotyping

Standard cytogenetic procedures were followed for analysis of WJ samples. When the culture reached confluence of 80%, Colcemid (10 mg/ml) was added to each flask to a final dilution of 0.1 µg/ml and then incubated at 37 °C for 30 min. Changes in cell morphology were monitored using an inverted microscope. The MSCs were detached using 0.25% trypsin-EDTA. After 3 min of monitored detachment, few drops of FBS were added and the cells were transferred to a centrifuge tube containing medium. Samples were centrifuged at 400 × g for 10 min. For the hypotonic treatment, 10 ml of 0.075 M KCl was slowly and carefully added, followed by incubation at 37 °C for 30 min and fixation using methanol: acetic acid (3:1) solution. To improve the quality of metaphases, the samples were washed twice with methanol: acetic acid (3:1). Prior to slide preparation, slides were cleaned and kept in cold, immersed in water. Samples were dropped on to the surface of the slide from a distance to form a uniform blast. In order to obtain G-bands, the slides were baked at 80 °C for one hour and then aged at 37 °C for at least 16 h. Then, they were immersed in Trypsin solution (0.002 g/mL) for 5 s, washed in Sorenson's buffer and finally quickly rinsed in distilled water. The staining procedure was carried out using Giemsa (1: 20) producing Trypsin and Giemsa (GTG) bands. The band quality was evaluated under the microscope (magnification;100X) and the Trypsin and staining times were adjusted to produce clear well stained bands. Twenty five metaphases were analyzed per sample for karyotyping and the karyogram was performed using Applied Spectral Imaging (ASI) software.

Chemicals

Galactosamine (D-GalN), 1,1,3,3-tetramethoxypropane malondialdehyde (MDA), thiobarbituric acid, DMEM, Trypsin, antibiotics, Epinephrine, Bicinchoninic acid, Copper (II) Sulfate Pentahydrate, Bovine serum albumin (BSA), Glacial acetic acid and Giemsa were purchased from M/s Sigma-Aldrich Chemicals (St. Louis, Missouri, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS) and Karyomax–Colcemid were purchased from Gibco life technology, USA. The primary antibodies such as CD90-FITC, CD73-APC, CD105-PerCp-Cy5.5, CD14-FITC, CD34-PE and CD45-APC-CY7 for flow cytometer were purchased from M/s BD Biosciences, Franklin Lakes, NJ, USA. All the other chemicals used for various assay procedures were purchased locally and were analytical grade.

Animals

Swiss albino mice, weighing 25 ± 5 g procured from Institutional Animal House facility of Madras Medical College were used in this study. Animals were maintained

at controlled environmental conditions (temperature— $24 \pm 2^\circ\text{C}$; relative humidity—50–60%; 12 h dark–light cycle) in polypropylene cages over husk beddings and were provided standard pellet diet and water ad libitum. Institutional Animal Ethical Committee permission was obtained prior to experimentation (01/2015).

Experimental Design

In this study, the mice were divided randomly into four groups as Group I, II, III and IV respectively, each comprising six animals. The control mice (Group I), was given saline. Group II mice received D-GalN alone at 800 mg/kg b.w i.p. Group III mice were treated similar to that of Group II mice, along with WJ-MSCs (5×10^5 cells/0.5 ml DMEM) through tail vein 24 h after D-GalN injection and was considered as Day 0. The Group IV mice received MSC alone through tail vein injection. In Group II, three mice were sacrificed at 6 h and the remaining three at 24 h after the D-GalN treatment. Similarly in Group III, two mice were sacrificed at 24 h, two at 72 h and the remaining two at 120 h respectively. D-GalN was dissolved in saline and WJ-MSCs were suspended in DMEM. The dosage for D-GalN was selected based on the previous reports^{24,25} and for MSCs.²⁶

Sample Collection and Preparation

Under anaesthesia using isoflurane blood was collected from mice by retro-orbital-plexus, using capillary tubes at 6, 24, 72 and 120 h and serum was separated. The animals were then sacrificed by cervical dislocation. The abdominal cavity was opened and the liver was excised quickly, washed in ice cold saline to remove excess blood clot and other tissue debris. The livers were blotted to dryness and stored at -20°C (for up to 48 h) for further analysis.

Serum Assay

Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline Phosphatase (SAP), Total serum bilirubin (TBIL), albumin and protein levels were analyzed in serum according to the manufacture's protocol (Coral clinical system kit, India) using Biochemistry analyzer (Randox-RX-Daytona).

Lipid Peroxidation (LPO)

One ml of Liver tissue homogenates were mixed with 2 ml of trichloroacetic acid, thiobarbituric acid and HCl and heated in boiling water bath for 15 min, cooled and then centrifuged at 2000 rpm for 10 min. The MDA (the by product of LPO) concentrations in the supernatant was measured at 535 nm using spectrophotometer.²⁷ The MDA levels were evaluated by normalizing it with the total protein concentration of the same sample.

Superoxide Dismutase (SOD) Activity

Auto-oxidation of epinephrine in the presence of carbonate-bicarbonate buffer can be measured at an absorbance of 420 nm. The epinephrine auto-oxidation is inhibited by the SOD enzyme present in the sample. Quantification of SOD activity is evaluated as an increase in absorbance at 420 nm over time.²⁸ One unit of enzyme activity is defined as the quantity of enzyme required to produce 50% inhibition in epinephrine auto-oxidation.

Estimation of Protein by Bicinchoninic Acid (BCA)

To the liver tissue homogenate, bicinchoninic acid and copper sulphate pentahydrate was added and incubated. Spectrophotometric measurement of the blue colour that developed was measured at 562 nm, compared with blank and standard (BSA) as per kit protocol (Sigma, St. Louis, MO, USA).

Histopathology of Liver Using H&E

Phosphate buffered formalin (0.1 M; pH 7.4) fixed liver tissues were washed thoroughly and dehydrated subsequently in ethanol and embedded in paraffin wax. Thin sections (4 μm thickness) of liver tissues were stained using haematoxylin and eosin (H&E). The sections were mounted using DPX mount²⁹ and were studied.

Statistical Analysis

The results are presented as mean $\pm$ S.D. Statistical comparisons of data was performed by one-way analysis of variance (ANOVA). Post Hoc multiple comparison tests were employed to assess the degree of significance between means of various treatment groups employing Tukey's test using SPSS software (version 20.0). The P value < 0.05 was considered significant.

RESULTS

WJ-MSCs Morphology and Immunophenotype

The spindle shaped fibroblast like morphology was observed in MSCs which was densely packed with broad spindle shaped cells. Flow cytometric analysis WJ-MSCs were positive for MSC markers CD73, CD90 and CD 105 and negative for hematopoietic markers CD 14, CD34 and CD45 respectively (Figure 1).

Karyotype Analysis of WJ-MSCs

The passage 2 WJ-MSCs used in this study as well as passage 1 from which it was derived were arrested in the metaphase stage by colcemid. A normal diploid karyotype was observed i.e., 46 XY, no chromosomal abnormalities observed in these samples (Figure 2).

a) Culture of MSC

b) Flow cytometry

Figure 1 Mesenchymal stem cell (MSCs). (a) Spindle shaped MSC of passage 2 on day 5. (b) Flow cytometry analysis at 2nd passage shows 95.7%, 96.6% and 98.8% of the cells were positive for CD 73, CD 90 and CD105 and negative to CD14, CD 34 and CD45 respectively.

Liver Function Test

There were very highly significant increase ($P < 0.001$) in serum AST, ALT, SAP as well as bilirubin in D-GalN alone (Group II) treated group at 6 h which dipped to significant level ($P < 0.01$) at 24 h but no change was observed in protein and albumin levels. The mice in this group died within 48 h; hence data on 48, 72 and 120 h are not available. The Group III mice (that received MSCs 24 h after D-GalN) showed highly significant ($P < 0.01$) differences in the liver enzyme levels at 24 h compared to the saline control. These animals survived beyond 48 h and the liver enzyme levels of the samples taken at 72 and 120 h progressively dipped further significantly ($P < 0.001$; $P < 0.01$) as compared to levels at 24 h after D-GalN administration. At 120 h the enzyme levels in Group III had reached near normal values (of saline treated control). MSCs treated Group IV mice showed a highly significant increase ($P < 0.01$) in the levels of AST after 24 h treatment which gradually decreased at 72 and 120 h and to levels to that of saline treated control (Table 1).

Lipid Peroxidation (LPO)

Measurement of malondialdehyde (MDA), the byproduct of lipid peroxidation (LPO), is used to assess oxidative stress induced by drugs and chemicals. In the Group II treated mice there was a significant increase ($P < 0.001$) in/ the levels of MDA/lipid peroxidation at 6 h which dipped, though still at significant levels ($P < 0.05$) at 24 h post-treatment. Post-treatment with WJ-MSCs (Group III) significantly ($P < 0.001$) prevented/reversed the above changes at 120 h when compared to 72 and 24 h treatment. The MSCs alone treated Group IV was similar to that of control (Table 2).

Superoxide Dismutase (SOD)

SOD plays an important role in maintaining the oxidative homeostasis in cells. D-GalN alone treatment (Group II) showed around 40% fall ($P < 0.001$) in the activity of SOD at 6 and 24 h when compared to control (Group I) treatment. Post-treatment with MSCs (Group III) showed a

Figure 2 Karyogram of mesenchymal stem cell (MSCs). (a) Metaphase of WJ-MSCs with Giemsa Staining (magnification 400 $\times$); (b) karyotype analysis of MSCs at 2nd passage. Cytogenetic analysis showed no chromosomal abnormalities with normal male (46 XY) karyotype.

significant increase ($P < 0.001$) in levels of SOD at 120 h compared to 72 and 24 h. When MSCs were given alone (Group IV) no change was noted and it was similar to saline treated control (Table 2).

Histopathology of Liver

Histopathology of liver tissues is presented in Figure 3. The Livers of D-GalN alone treated mice (Group II) showed dilation of sinusoidal space with mild inflammatory cell infiltration at 6 and 24 h. Group III mice (that received MSCs 24 h after D-GalN) showed the above changes at 24 h, which was found reduced at 72 h, and near normal at 120 h. Livers of mice treated with MSCs alone (Group IV) showed a normal architecture similar to that of Group I control mice.

DISCUSSION

ALF is a sudden loss of hepatic cells/cellular function leading to organ failure for which liver transplantation is the only remedial measure available till date. Due to shortage of organs, alternate methods for liver regeneration are being explored. Stem cell therapy is now being (widely used) studied in many diseases/ailments. For stem cells to be effective in regenerative medicine, they should be

available in abundance and delivered via minimally invasive procedure that ensures donor safety. Bone marrow is the most common source for deriving MSCs. Bone marrow derived MSCs are capable of transdifferentiating into hepatocytes and bile duct cells in the repair process after severe liver injury.^{30,31} Harvesting Bone Marrow engages an invasive procedure. To overcome this, other perinatal tissues, such as umbilical cord blood (CB), wharton's jelly (WJ), amnion and chorion have been explored.^{12,13} WJ-MSCs has recently gained interest for its use in cellular therapy due to its efficiency and high yield without using invasive procedure. The WJ-MSCs also exhibit potent immunomodulatory and paracrine effects in comparison with BM-MSCs which makes it a better candidate for use in cellular therapy.³² The present study evaluates the in vivo hepatogenic potential of WJ derived MSCs in rescuing mice from acute liver injury induced by intra peritoneal galactosamine injection.

In the present study the mice treated with D-GalN started to die around 6 h after its administration, and the total mortality was observed within 24 h. Previous papers have used the combination of D-GalN and lipopolysaccharides (LPS) to induce acute injury.^{7,24} In our initial work, we had observed that the mice treated with both D-GalN and LPS succumbed within 4–6 h. Therefore in this study we have used only D-GalN at a dose of 800 mg/kg b.w to induce liver damage in mice (Group II), and sacrificed them 6 and 24 h and studied its effect on liver function. D-GalN treatment caused a significant increase in the levels of AST, ALT, SAP and bilirubin in serum of mice within 6 h and the toxicity was maintained till 24 h. The animals died between 26 and 48 h. In Group III mice, WJ-MSC treatment with D-GalN increased the survival rate and no death was observed in the group. This treatment significantly reduced the levels of the above liver function parameters at 120 h compared to 72 and 24 h respectively. Here the saline treated group as well as the group receiving MSCs alone served as control, D-GalN treatment as test control while MSCs following D-GalN was the intervention group that was studied.

The serum ALT, AST and SAP are the enzyme markers of liver toxicity.^{33,34} Our study confirms that D-GalN treatment induces liver injury in mice and the WJ-MSC were able to restore these injury. The increase in the levels of serum aminotransferase and alkaline phosphatase could be due to the leakage of these enzymes from liver cells as a result of membrane damage after D-GalN treatment.³⁵ Previous studies in rats have shown that D-GalN administration disrupts permeability of the plasma membrane, causing leakage of enzymes from the cell, leading to elevation in levels of serum enzymes and loss of liver cell membrane functional integrity.³⁶ Alkaline phosphatase is present in abundance in bile duct epithelium and canalicular membrane of the hepatocytes. Cholestasis is a characteristic of hepatocellular degenerative change in

Table 1 AST, ALT, SAP, Bilirubin, Protein and Albumin Levels in Serum of Mice Treated with D-GalN and MSCs.

Groups	Time interval	AST (IU/l)	ALT (IU/l)	SAP (IU/l)	Bilirubin (mg/dl)	Protein (mg/dL)	Albumin (mg/dL)
Group I	24 h	128.32 ± 29.72	76.16 ± 13.07	275.52 ± 29.47	0.24 ± 0.07	5.91 ± 0.14	2.96 ± 0.37
	6 h	260.48 ± 16.56***	152.20 ± 9.32***	576.58 ± 25.21***	0.77 ± 0.02***	6.60 ± 0.20	3.1 ± 0.11
Group II	24 h	233.38 ± 12.41***	145.31 ± 8.31***	547.58 ± 40.30***	0.50 ± 0.05**	5.92 ± 0.05	3.0 ± 0.09
	24 h	182.67 ± 10.37**	107.44 ± 6.55**	446.23 ± 39.40**	0.36 ± 0.04*	6.01 ± 0.09	3.1 ± 0.13
Group III	72 h	152.90 ± 2.21*	81.50 ± 7.32***	334.59 ± 33.30**	0.19 ± 0.03***	6.12 ± 0.12	3.0 ± 0.22
	120 h	136.23 ± 14.30*	75.21 ± 8.12***	307.45 ± 35.29**	0.15 ± 0.03***	5.80 ± 0.16	2.95 ± 0.30
Group IV	24 h	210.12 ± 15.72**	85.70 ± 2.55	311.40 ± 30.55*	0.24 ± 0.01	5.91 ± 0.23	2.7 ± 0.07
	72 h	188.56 ± 13.66**	81.63 ± 6.20	301.60 ± 23.50*	0.20 ± 0.20	5.93 ± 0.23	2.5 ± 0.03
	120 h	153.48 ± 18.30***	80.57 ± 7.12	285.45 ± 25.31	0.25 ± 0.04	6.02 ± 0.31	2.7 ± 0.28

Dose regimens and treatment protocols are described in Materials and methods section.

Group I is compared with Group II, III and IV.

**P* < 0.05 represents significant difference.

***P* < 0.01 represents highly significant difference.

****P* < 0.001 represents very high significant difference.

NS—not significant.

Values presented are mean ± S.D. of 6 nos. of mice in each treatment group. Post Hoc multiple comparison was performed by Tukey's test.

Table 2 Status of LPO and SOD in Liver Tissue of Mice Treated with D-GalN and MSCs.

Groups	Time interval	LPO (nM of MDA formed/min/mg protein)	SOD (units/mg protein)
Group I	24 h	1.08 ± 0.22	73.34 ± 3.55
	6 h	4.15 ± 0.39***	42.44 ± 4.02***
Group II	24 h	3.67 ± 0.46***	42.64 ± 3.79***
	24 h	3.02 ± 0.11***	42.39 ± 4.32***
Group III	72 h	2.47 ± 0.09***	51.35 ± 2.50***
	120 h	1.98 ± 0.55**	70.51 ± 2.98***
Group IV	24 h	1.25 ± 0.31***	73.20 ± 4.5 ^{NS}
	72 h	1.18 ± 0.22***	74.53 ± 3.6 ^{NS}
	120 h	0.98 ± 0.05***	75.93 ± 4.2 ^{NS}

Dose regimens and treatment protocols are described in Materials and methods section.

Group I is compared with Group II, III and IV.

**P* < 0.05 represents significant difference.

***P* < 0.01 represents highly significant difference.

****P* < 0.001 represents very high significant difference.

NS—not significant.

Values presented are mean ± S.D. of 6 nos. of mice in each treatment group. Post Hoc multiple comparison was performed by Tukey's test.

Figure 3 Histopathology of mice liver (H & E). Experimental protocol is described under materials and methods section. H—normal hepatocyte; CV—central vein; SP—sinusoidal space. Magnification 40 $\times$.

liver, and is indicated by increase in the levels of serum alkaline phosphatases, and serum bilirubin. It is proposed that the rupture of bile duct canaliculi leads to extravasation of bile into the sinusoidal space resulting in its accumulation and foamy degeneration of hepatocytes.³⁷ In the present study D-GalN treated mice showed a two-fold increase in serum alkaline phosphatases as well as in bilirubin levels indicating that the above chemicals precipitate cholestatic lesions in addition to hepatocellular damage.

In our present study, we found that WJ-MSC alone treated group showed a mild elevation of AST level at 24 h when compared to the saline treated control group. This could probably be due to the release of cytosolic AST from the red blood cells during infusion of MSC via the tail vein which caused a transient elevation AST enzyme in the Group IV mice.³⁸ However at 72 h and 120 h there was not much difference between Group I and Group IV mice.

The hepatocellular damage induced by D-GalN is also evident by histopathological changes, marked hepatocellular necrosis, neutrophilic infiltration around central vein and dilatation of sinusoidal space. These changes were observed in D-GalN treated mice (Group II) at 6 and 24 h respectively. Our results are in agreement with previous studies reporting leucocyte infiltration into the liver with in 24 h of D-GalN treatment.³⁹ Treatment of WJ-MSCs following D-GalN was able to mitigate the above changes to near normalcy compared to the D-GalN treatment alone.

The D-GalN (given intraperitoneally) is metabolized in liver and ROS such as OH^- , superoxide anion radicals, and hydrogen peroxide are liberated. ROS and free radicals such as hydroxyl ions (OH^-), peroxide (O_2^{2-}), superoxide anion ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2) etc., are liberated due to chemical reactivity in liver, leading to loss of oxidant-antioxidant homeostasis. The resultant oxidative

stress causes peroxidation of lipid bilayer of cellular membranes. Malondialdehyde (MDA) that is formed during this process can be measured to evaluate lipid peroxidation (LPO)/oxidative stress. This ROS formation results in the development of acute liver injury which could potentially cause lipid peroxidation and DNA damage of a cell or tissue.⁴⁰ Lipid peroxidation is a basic cellular deteriorating process in response to oxidative stress that occurs rapidly in tissues rich in highly oxidizable polyunsaturated fatty acids, and it is indicated by malondialdehyde (MDA) formation.⁴¹ Previous studies have suggested that D-GalN treatment in rats induces disruption of hepatic antioxidant defence system leading to liver injury.^{26,42} In the present study also the D-GalN treated mice exhibited oxidative stress by a four-fold increase in MDA levels.

ROS formation in tissues induces the oxidative defense mechanism. Superoxide dis-mutase (SOD) is a metalloprotein which catalyze the dismutation of superoxide radicals. Catalase (CAT) is a hemoprotein which causes the reduction of H_2O_2 to H_2O and O_2 . Both SOD and CAT act together to protect the liver from the effects of ROS. Oxidative stress is the imbalance between ROS and the antioxidant defence mechanisms whereby levels of SOD will be reduced and MDA will be increased. In the present study there was a two-fold decrease in the level of SOD in D-GalN treated mice suggesting that it was induced due to the oxidative stress caused in the liver which was similar to that of the previous report by Jia et al. (2014).⁴³ WJ-MS

treatment following D-GalN (Group III) showed a significant reduction in the levels of MDA and increased the SOD activity (Figure 4).

The MSCs exhibit plasticity and rapid expansion in vitro. While these properties render them a favourable choice for clinical cytotherapy in the future, they also confer a potential for acquiring gene disorders. Therefore, ensuring cytogenetic stability is important before considering them for transplantation. Karyotyping (G-banding) of the MSCs cultured from wharton's jelly in this study did not reveal any abnormality. Previous studies have reported that the protective effect of MSCs against the liver injury in animals could be due to the secretion of soluble factors by MSCs, rather than transdifferentiation into hepatocytes.⁴⁴ Some groups have stated that the hepatoprotective effect of MSCs is due to suppression of pro-inflammatory cytokines such as $TNF\alpha$ and IL-6 which arises during oxidative stress. These cytokines plays a major role in protecting the liver from necrosis and apoptosis. Hence, further studies are needed to understand the exact molecular mechanism by which WJ-MS could be explored for clinical transplantation.⁴⁵

CONCLUSION

The present study shows that WJ-MS was able to mitigate the liver injury in mice by decreasing the levels of liver enzymes, LPO and increasing the levels of SOD to an

Figure 4 Mechanism of WJ-MS in rescuing acute liver injury in mice.

appreciable level. The histopathology of the liver also confirms that WJ-MSc improved the liver structure in experimental mice with acute liver injury and significantly restored liver function. Hence, we derive a conclusion that WJ-MSCs may be suitable in cellular therapy for acute liver injury.

CONFLICTS OF INTEREST

The authors have none to declare.

ACKNOWLEDGEMENT

This study was funded by Indian Council of Medical Research, New Delhi, India.

REFERENCES

- Lee WM, Larson AM, Stravitz RT. AASLD position paper: the management of acute liver failure: update 2011. *Hepatology*. 2011;1–22. Available at: https://www.aasld.org/sites/default/files/guideline_documents/alfenhanced.pdf [consulted 07.11.16].
- Bernal W, Auzinger G, Dhawan A, Wendon J. Acute liver failure. *Lancet*. 2010;376:190–201.
- Lee WC, Kim JK, Kang JW, et al. Palmatine attenuates D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure in mice. *Food Chem Toxicol*. 2010;48:222–228.
- Pushpavalli G, Kalaiarasi P, Veeramani C, Pugalendi KV. Effect of chrysin on hepatoprotective and antioxidant status in D-galactosamine-induced hepatitis in rats. *Eur J Pharmacol*. 2010;631:36–41.
- Almeida-Porada G, Zanjani ED, Porada CD. Bone marrow stem cells and liver regeneration. *Exp Hematol*. 2010;38:574–580.
- Tang XH, Gao L, Gao J, et al. Mechanisms of hepatoprotection of *Terminalia catappa* L. extract on D-galactosamine-induced liver damage. *Am J Chin Med*. 2004;32:509–519.
- Ai Q, Jing Y, Jiang R, et al. Rotenone, a mitochondrial respiratory complex I inhibitor, ameliorates lipopolysaccharide/D-galactosamine-induced fulminant hepatitis in mice. *Int Immunopharmacol*. 2014;21:200–207.
- Wang XY, Zhang LN, Wei ZT, et al. The inhibitory action of PDCD4 in lipopolysaccharide/D-galactosamine-induced acute liver injury. *Lab Invest*. 2013;93:291–302.
- Stutchfield BM, Forbes SJ, Wigmore SJ. Prospects for stem cell transplantation in the treatment of hepatic disease. *Liver Transplant*. 2010;16:827–836.
- Yuan S, Jiang T, Sun L, Zheng R, Ahat N, Zhang Y. The role of bone marrow mesenchymal stem cells in the treatment of acute liver failure. *BioMed Res Int*. 2013;2013:251846.
- Zhang H, Fang J, Wu Y, Mai Y, Lai W, Su H. Mesenchymal stem cells protect against neonatal rat hyperoxic lung injury. *Expert Opin Biol Ther*. 2013;13:817–829.
- Wang X, Sha XJ, Li GH, et al. Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. *Arch Oral Biol*. 2012;57:1231–1240.
- Zagoura DS, Roubelakis MG, Bitsika V, et al. Therapeutic potential of a distinct population of human amniotic fluid mesenchymal stem cells and their secreted molecules in mice with acute hepatic failure. *Gut*. 2012;61:894–906.
- Ma H, Wu Y, Zhang W, et al. The effect of mesenchymal stromal cells on doxorubicin-induced nephropathy in rats. *Cytotherapy*. 2013;15:703–711.
- Weiss ML, Anderson C, Medicetty S, et al. Immune properties of human umbilical cord Wharton's Jelly-derived cells. *Stem Cells*. 2008;11:2865–2874.
- Bongso A, Fong CY. The therapeutic potential, challenges and future clinical directions of stem cells from the Wharton's Jelly of the human umbilical cord. *Stem Cell Rev*. 2013;9:226–240.
- Liu S, Yuan M, Hou K, et al. Immune characterization of mesenchymal stem cells in human umbilical cord Wharton's Jelly and derived cartilage cells. *Cell Immunol*. 2012;278:35–44.
- Yan M, Sun M, Zhou Y, et al. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopamine neurons mediated by the Lmx1a and neurturin in vitro: potential therapeutic application for Parkinson's disease in a rhesus monkey model. *PLoS ONE*. 2012;8:e64000.
- Hong SQ, Zhang HT, You J, et al. Comparison of transdifferentiated and untransdifferentiated human umbilical mesenchymal stem cells in rats after traumatic brain injury. *Neurochem Res*. 2011;36:2391–2400.
- Liu R, Zhang Z, Lu Z, et al. Human umbilical cord stem cells ameliorate experimental autoimmune encephalomyelitis by regulating immunoinflammation and remyelination. *Stem Cells Dev*. 2013;22:1053–1062.
- Lee HJ, Lee JK, Lee H, et al. Human umbilical cord blood derived mesenchymal stem cells improve neuropathology and cognitive impairment in an Alzheimer's disease mouse model through modulation of neuroinflammation. *Neurobiol Aging*. 2012;33:588–602.
- Mora-Lee S, Sirerol-Piquer MS, Gutierrez-Perez M, et al. Therapeutic effects of hMAPC and hMSC transplantation after stroke in mice. *PLoS ONE*. 2012;7:e43683.
- Secunda R, Vennila R, Mohanashankar AM, Rajasundari M, Jeswanth S, Surendran R. Isolation, expansion and characterisation of mesenchymal stem cells from human bone marrow, adipose tissue, umbilical cord blood and matrix: a comparative study. *Cytotherapy*. 2015;67:793–807.
- Huang H, Zhang X, Li J. Protective effect of oroxylin A against lipopolysaccharide and/or D-galactosamine-induced acute liver injury in mice. *J Surg Res*. 2015;195:522–528.
- Lu Y, Wang WJ, Song YZ, Liang ZQ. The protective mechanism of schisandrin A in D-galactosamine-induced acute liver injury through activation of autophagy. *Pharm Biol*. 2014;52:1302–1307.
- Liu Z, Meng F, Li C, et al. Human umbilical cord mesenchymal stromal cells rescue mice from acetaminophen-induced acute liver failure. *Cytotherapy*. 2014;16:1207–1219.
- Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol*. 1978;52:302–310.
- Misra HP, Ferdovich IC. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*. 1972;247:3170–3175.
- Bancroft JD, Cook BC. *Manual of Histological Techniques*. Edinburgh: Churchill Livingstone; 1984:49–51.
- Kisseleva T, Gigante E, Brenner DA. Recent advances in liver stem cell therapy. *Curr Opin Gastroenterol*. 2010;26:395–402.
- Sordi V, Piemonti L. Therapeutic plasticity of stem cells and allograft tolerance. *Cytotherapy*. 2011;13:647–660.
- Deuse T, Stubbendorff M, Tang-Quan K, et al. Immunogenicity and immunomodulatory properties of umbilical cord lining mesenchymal stem cells. *Cell Transplant*. 2011;20:655–667.
- Bjornsson E, Olsson R. Outcome and prognostic markers in severe drug-induced liver disease. *Hepatology*. 2005;42:481–489.
- Nyblom H, Berggren U, Balldin J, Olsson R. High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. *Alcohol Alcohol*. 2004;39:336–339.
- Sheth SG, Flamm SL, Gordon FD, Chopra S. AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. *Am J Gastroenterol*. 1998;93:44–48.

36. Mitra SK, Seshadri SJ, Venkataranganna MV, Gopumadhavan S, Udupa UV, Sarma DN. Effect of HD-03—a herbal formulation in galactosamine-induced hepatopathy in rats. *Indian J Physiol Pharmacol.* 2000;44:82–86.
37. Crawford JM. *The liver and biliary tract. Robbins & Cotran Pathologic Basis of Disease.* 7th ed. Kumar Abbas Fausto, Elsevier Publication; 2003:591–633.
38. Hayashi H, Ada HVV, Yoshimura T, Esaki N, Soda K. Recent topics in pyridoxal 5'-phosphate enzyme studies. *Annu Rev Biochem.* 1990;59:87–100.
39. Lin BR, Yu CJ, Chen WC, et al. Green tea extract supplement reduces D-galactosamine-induced acute liver injury by inhibition of apoptotic and proinflammatory signaling. *J Biomed Sci.* 2009;16–35.
40. Altan O, Pabuccuoglu A, Altan A, Konyalioglu S, Bayraktar H. Effect of heat stress on oxidative stress, lipid peroxidation and some stress parameters in broilers. *Br Poult Sci.* 2003;44:545–550.
41. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis.* 2005;15:316–328.
42. Gong X, Zhang L, Jiang R, Wang CD, Yin XR, Wan JY. Hepatoprotective effects of syringin on fulminant hepatic failure induced by D-galactosamine and lipopolysaccharide in mice. *J Appl Toxicol.* 2013;34:265–271.
43. Jia M, Jing Y, Ai Q, et al. Potential role of catalase in mice with lipopolysaccharide/D-galactosamine-induced fulminant liver injury. *Hepatol Res.* 2014;44:1151–1158.
44. Xagorari A, Siotou E, Yiangou M, et al. Protective effect of mesenchymal stem cell-conditioned medium on hepatic cell apoptosis after acute liver injury. *Int J Clin Exp Pathol.* 2013;6:831–840.
45. Liu nc, Meng ne, Li a, et al. Human umbilical cord mesenchymal stromal cells rescue mice from acetaminophen-induced acute liver failure. *Cytotherapy.* 2014;16:1207–1219.