Study on the therapeutic effect of hepatocytic stem cells in hepatic deficient canine patients

Rashmi Saini, NS Jadon, Priyanka Pandey, Manjul Kandpal, Ramanpreet Singh Sandhu and Arun Kumar

Abstract

The aim of this study was to determine the therapeutic efficacy of hepatocytic stem cells for hepatic regeneration in dogs. Twenty four canine patients suffering with hepatic dysfunction were divided into four groups i.e., A, B, C and D. The animals were subjected to different treatments viz group A (Control), group B (Inj. pepsid-C @ 1ml/10 kg b.wt. IM alternatively for 15 days), group C (hepatocytic stem cells @ 5 × 10^7 cells transplanted into peritoneal cavity of affected animal thrice at an interval of 5 days intraperitonially alternatively for 15 days) and group D (hepatocytic stem cells @ 5 × 10^7 cells transplanted into peritoneal cavity of affected animal thrice at an interval of 5 days + Inj. pepsid-C @ 1ml/10 kg b.wt. IM alternatively for 15 days IM). The efficacy of treatment was assessed by observing various haematological (Hb, TEC, TLC, DLC, PCV, BT and CT), biochemical (serum total protein, serum albumin, serum glucose, serum urea nitrogen, serum creatinine serum AST, serum ALT and serum GGT) changes. Pepsid-C and hepatocytic stem cells combination have better effect on liver regeneration, followed by hepatocytic stem cells (group C) alone and pepsid-C (group B) alone. Hence, hepatocytic stem cell+pepsid-C combination can be used safely by veterinarians for the treating various hepatic disorders in canine patients.

Keywords: Hepatic disorder, canine, pepsid-c, hepatocytic stem cell

1. Introduction

The liver is a vital organ of the body. It has a highly metabolic complex array of vasculature, endothelial and parenchymal cells that perform many functions in the body [1]. Hepatocytes constitute approximately 70% of the cellular population of the liver and perform major glucose homeostasis, urea synthesis and ketogenesis [2]. The liver is necessary for survival of the animal. There is no way to compensate for the absence of liver function. The liver performs multiple functions including lipid, carbohydrate, and protein metabolism; storage and metabolism of vitamins, storage of minerals, glycogen and triglycerides; extramedullary hematopoiesis; and coagulation homeostasis. The liver also has immunologic activity, contributes to digestion by producing bile acids and essential for detoxification of many endogenous and exogenous compounds. The liver has a large storage capacity, functional reserve and is capable of regeneration. These properties are somewhat protective against permanent damage. However, the liver is also predisposed to secondary injury because of its ability to metabolize, detoxify, and store various compounds.

The liver is important to maintain the metabolism of the body. It regulates the levels of substances such as fat, glucose, proteins, vitamins, iron and hormones in the blood. Also the foreign substances such as medicine are also processed. The substances in the blood which are derived from the vena porta become assimilated and then delivered to the blood in the vena hepatica. If it is a necessary substance then it will be secreted from the internal environment and out of the body through the gall bladder and the gastrointestinal system. Due to this regulation the concentration of blood substances will maintain steady. A adult liver has a remarkable regenerative capacity and can completely re-grow when upto 70% of its mass is removed [3–4]. However, its ability is impaired in numerous diseases such as advanced cirrhosis and hepatits resulting life threatening liver failure [5]. Liver is the major tissue in which ingested chemicals are largely metabolized. Hence, disease of the liver is one of the common medical problems [6]. Liver injury poises a serious problem in the metabolism and disposition of a large number of foreign chemicals to which vertebrates are continuously exposed.

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Stem cells are necessary for the creation of new cells for growth, development and regeneration. Embryonic stem cells are omnipotent; they are capable to differentiate into several cell types of the body [19]. These cells are needed for the development into a fully-grown adult and are eventually not necessary anymore. Another more restricted capability of differentiation can be found in adult stem cells. These cells are responsible for the development of new specific cells and also have potential to differentiate into different cell types but are mostly bipotent and are specific for the type of tissue they belong to. Therefore adult stem cells have less development potential than embryonic stem cells [7]. The adult stem cell for the liver is the liver specific progenitor cell (LPC) which can be classified as bipotent which means they can develop into two different cell types.

The liver has the capacity to regenerate to a certain degree. When liver tissue is damaged, through surgery or toxic substances, the liver cells start to proliferate. In the case of partial heptectomy (removal of a part of the liver) the proliferation stops when the original volume of liver tissue is reached. The growth is coupled with strong increase of mitosis during a couple of days, which will start 30 hours after a clinical intervention. The regenerated liver tissue is histologically comparable with the original liver.

Cell-based therapy is considered a new therapeutic tool that has shown great success in the recent years and is expected to replace whole-organ transplantation in the future [8]. The target of cell therapy is to substitute the defective cells in order to substitute organ function through transplantation of cells [9]. In case of larger tissue defects, cells alone cannot replace the function and tissue engineering is required to load cells on to 3-dimensional (3D) biodegradable scaffolding systems to support their growth and function. There are several sources of cells which can be used in prevention and treatment of permanent liver failure (PLF) [10]. The present study was designed to assess the efficacy of the allogenic hepatocytic stem cells and pepsid-C in the repair of hepatic damage with following objectives: To investigate the potential of allogenic hepatocytic stem cells for augmenting the healing of hepatic damage in canine and comparative evaluation of hepatocytic stem cells with and without pepsid-C in hepatic regeneration.

2. Materials and Methods
2.1 Ethical approval
The study was conducted in accordance with the guidelines laid down by the Institutional Animal Ethics Committee (IAEC) vide letter no. IAEC/CVASC/VS/359/dt. 21/12/2018.

2.2 Experimental animals
A total of 24 animals suffering with various hepatic disorders were included in this study. All animals were screened for hepatic disorders by performing liver function test.

2.3 Collection of liver sample for culture of hepatocytic stem cells
Collection was done as per procedure for partial liver resection in a dog, died recently due to accident. A midline laparotomy incision was made extending from the xiphoid cartilage caudally for approximately 20 cm. The incision continued through the fascia, muscle, and peritoneum. The liver was then exposed. The left lateral liver lobe was localized and the segment of the liver to be removed was selected. Thereafter, the segment was carefully dissected slipping the scalpel along the concave part of the clamp. The liver sample was collected in specimen bottles half full with culture medium (RPMI1640 with 10% FBS) and antibiotics (Plate 1, fig. a). The incision line was closed with a non-interrupted suture using 2-0 silk.

![Fig (a): Collection of the liver sample in specimen jar](image)

**Plate 1:** Collection of liver sample for culture of hepatocytic stem cells

2.4. Isolation of hepatocytic stem cells from liver biopsy and its propagation (Plate 2 and Plate 3)

2.4.1 Hepatocytic stem cell preparation
Hepatocytic stem cells were isolated from dog by in situ liver perfusion and enzymatic collagenase digestion as described by Berry and Friend (1969) and modified by Seglen (1976). Upon arrival in the laboratory, the liver sample was placed in the perfusion vessel and the edges were carefully examined in order to locate the various vein and artery entries that were cannulated and were used for perfusion. The liver was perfused with 1-2 L of HEPES buffer supplemented with antibiotics and fuzigone through the vein and artery entaries @ 1ml/min/g for 20 min. During this and further perfusion steps, the cannula was inserted successively in all veins or arteries present on the edge for approximately 30 s each (one vein or artery at a time). The liver was then circularly perfused with 500 ml of 0.5% collagenase I.V. solution at a constant flow of 100 ml/min. The duration of this step lasts for a maximum of 20 min.

![Fig (A): Centrifugation of filtrate for 5 min at 2500 rpm at room temperature](image)
Fig (b): Pellet containing hepatocyte at the base of centrifugation tube

Fig (C): Final pellet containing hepatocyte from tissue extract

Fig (d): Buffers and culture media used in hepatocyte propagation

Fig (E): Incubation of isolated hepatocytic stem cells for 24 hrs in T-25 flask and six well plates

Fig (F): Incubation of isolated hepatocytic stem cells at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in CO₂ incubator

Plate 2: Photographs showing isolation of hepatocytic stem cells from liver biopsy and its propagation

Fig (A): Hepatocytic stem cells were rounded at 0hrs

Fig. (b): Confluent monolayer of hepatocytic stem cells at 24hrs of culture

Fig (C): The hepatocytic stem cells have flattened out and show polygonal appearance at 48hrs of culture

Plate 3: Inverted microscopic photograph of hepatocytic stem cells at various time intervals

2.4.2 Non parenchymal epithelial cell isolation
At the end of the enzymatic digestion, the liver sample was transferred into a new stainless steel vessel, the Glisson’s capsule was opened in several places. The tissue was gently disrupted with scissors. The homogenate was complemented with 1-2L of BSA-HEPES buffer. The homogenate was filtered through a nylon filter (250μm mesh). The filter was washed twice with approximately 200 mL of BSA-HEPES
solution to collect the cells that were trapped in the undissociated tissue homogenate. Then, the filtrate was distributed into 50 mL centrifuge tubes (Plate 2, fig a). The tubes were centrifuged for 5 min at 2500 rpm at room temperature to pellet hepatocytic stem cells (Plate 2, fig b and c). This low speed centrifugation was repeated twice or thrice on the supernatant containing the non-parenchymal cells to remove residual hepatocytes. The supernatant was centrifuged at 400g for 10 min, at room temperature. The supernatant was filtered on a 40 µm cell strainer if the suspension contained large debris or cell aggregates. The pellet was resuspended in 5 to 10 mL of DMEM culture medium, cells were counted and the volume was adjusted so that the cells suspension was at 40 million/mL. This suspension was mixed (vol/vol) with the ficoll hypaque which was gently added at the top and centrifugation was performed for 15 min at 500g without break, at room temperature. Cells at the interface were collected and washed twice in Dulbecco’s modified Eagle’s medium (DMEM Himedia) (200g for 10 min at room temperature); resuspended in the complete DMEM culture medium and counted. Viability was checked with trypan blue dye exclusion test. If the viability was low but the total cell yield was sufficiently high, a Percoll density gradient centrifugation step was performed to improve the yield of viable cells.

2.4.3 Culture of non-parenchymal epithelial cells

The isolated hepatocytic stem cells were resuspended in the complete Dulbecco’s modified Eagle’s medium (DMEM Himedia) with 10% fetal bovine serum (FBS, Himedia RM10832), TBP (Tris buffer phosphate 1%) and antibiotic-antimycotic solution (Himedia A002A) (A mixture of 10,000 units/ml of Penicillin, 10 mg/ml Streptomycin and 25µg/ml Amphotericin B in 0.9 % normal saline). The isolated hepatocytic stem cells were then plated at desired volume in T-25 flask and incubated at 5 × 10^6/mL at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in CO₂ incubator (Plate 2, fig. e and f). After 24 h, nonadherent cells and cell debris were washed away with pre-warmed PBS and the expansion medium was added. After 48 hrs of incubation, the medium in T-25 culture flasks was changed every 2 days thereafter until the cultures became 80-90 % confluent. Once the cells (L-MSCs) attained 80-90% confluency, the cells (L-MSCs) was transplanted into the peritoneal cavity @5x10^7 cell of affected animal thrice at an interval of 5 days.

2.4.4 Morphology of isolated hepatocytes

The morphology of hepatocytes isolated from normal liver tissue at different time points after isolation (Plate 3). On initial plating cells appear round and bright, but during the next 24hrs cells gradually flattened out, forming a confluent monolayer and demonstrating a typical polygonal appearance

2.5 Experiment design

The experimental animals were divided randomly into four equal groups (A, B, C and D) of equal number of animals. The animals were subjected to the following treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-A</td>
<td>Control</td>
</tr>
<tr>
<td>Group-B</td>
<td>Inj. Pepsid-C IM 1ml/10kg on alternate days for fifteen days.</td>
</tr>
<tr>
<td>Group-C</td>
<td>Culture allogenic liver derived mesenchymal stem cell (hepatocyte stem cell) @5x10^7 cell transplanted into peritoneal cavity of affected animal thrice at an interval of 5 days</td>
</tr>
<tr>
<td>Group-D</td>
<td>Inj. pepsid IM 1ml/10kg on alternate days for fifteen days + Culture allogenic liver derived mesenchymal stem cell (hepatocyte stem cell)@5x10^7 cell transplanted into peritoneal cavity of affected animal thrice at an interval of 5 days</td>
</tr>
</tbody>
</table>

The regeneration of liver was evaluated on the basis of various clinical (Apetite, general body condition, skin coat, frequency of faeces and vomitus, activeness) and haematobiochemical parameters (Hb, TEC, TLC, DLC, bleeding time, clotting time, serum glucose, serum AST, serum ALT and serum GGT) at 0, 3rd, 6th, 10th, 15th and 20th day after treatment. About 5 ml venous blood was collected from the saphenous vein in sterilized disposable syringe, out of which, 2 ml was transferred into heparinized sterilize glass vials and rest 3 ml blood was used for serum.

2.5 Statistical analysis

Data was analyzed using analysis of variance [18]

3. Results and Discussion

Common clinical signs observed in hepatic deficient dogs were lethargy, depression, loss of appetite, vomiting, jaundice, diarrhoea, weight loss, dehydration, increased thirst and urination. The clinical condition of animals of different groups (A, B, C and D) improved following hepatic regeneration as evidenced by improvement in appetite and weight gain [9]. Haemoglobin level was comparatively lower in all animals suffering with hepatic disorders compared to normal values. A gradual increase in level of haemoglobin was observed after initiation of treatment in all four groups. An increase in haemoglobin level was observed in animals of group D (hepatocytic stem cells + pepsid-C), followed by group C (hepatocytic stem cells alone) and group B (pepsid-C) from 6th day of treatment onwards (Table 1). Total erythrocytic level increased non-significantly from their normal values throughout the study period in animals of group C and D as compared to group A and B. Major haematological finding was non-responsive anaemia which could have been the result of gastrointestinal bleeding [12]. Normocytic, normochromic, non-regenerative anaemia most likely occur due to chronic disease as the chronicity of many hepatic disease often lead to non-regenerative anaemia in which bone marrow is not able to respond to anaemic state [13].
Non-significant change in TLC level in animals of groups C and D indicated that there was no side effect of hepatocytic stem cells + pepsid-C (group D) and hepatocytic stem cells therapy alone (group C). However, a consistent and significant (p<0.05) decrease in TLC levels in animals of group A and B observed throughout the period of study, might be due to depression of reticuloendothelial system or as mild myelosuppression [20].

Non-significant decrease in the values of bleeding time and clotting time was observed (Table 1) in animals of all groups after initiation of treatment. A significant (p<0.01) increase in bleeding time and clotting time in dogs suffering with hepatic dysfunction may be related with low plasma prothrombin level due to liver damage, as damaged liver is unable to synthesize prothrombin in presence of adequate metabolites or in malabsorption of bile and fat soluble vitamin K, which is required for prothrombin synthesis. A non-significant increase in bleeding time in dogs suffering from hepatic disorders which is similar to above findings [14]. A significant (p<0.01) decrease in mean clotting time was observed in all the groups of animals suffering with hepatic disorders.

A hypoglycemic condition was observed at base level in all the animals of different groups suffering from hepatic disorders as compared to normal values. A gradual and significant (p<0.05) increase in the level of the glucose was observed after the initiation of treatment in animals of all the groups which reached to normal level on 20th, 15th, 10th and 6th day in animals of groups A, B, C and D, respectively (Table 1). Hypoglycemia in such cases may probably be due to excessive glucose utilization, secretion of insulin like growth peptide or some other hormonal induced alterations in the normal compensatory mechanism which prevents fasting hypoglycemia [15].

Serum AST was significantly (p<0.05) higher in all animals suffering with hepatitis compared to its normal value. A gradual decrease in the level of serum AST was observed in animals of all the groups after initiation of the treatment. Serum AST level was comparatively lower in animals of group D compared to animals of group C, at respective time interval. Significant (p<0.05) increase in level of serum ALT was observed in animals suffering with hepatic disorders as compared to its normal value. The level of serum ALT reached to near normal level on day 20th, 10th, 6th and 3rd after initiation of treatment in the animals of group A, B, C and D, respectively (Table 1). The liver is one of the main metabolic centers of the body and the cells contain a multiplicity of vital enzymes. When the liver damage occurs, the cell membrane may become more permeable or the cell wall may rupture so

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (mg/dl)</td>
<td>A</td>
<td>9.2±0.86</td>
<td>10.95±1.01*</td>
<td>12.01±0.57</td>
<td>12.4±0.06</td>
<td>12.3±0.45</td>
<td>12.67±0.41</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9.89±0.29</td>
<td>12.63±0.23</td>
<td>12.7±0.28</td>
<td>12.95±0.19</td>
<td>13.2±0.21</td>
<td>13.51±0.31</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10.2±0.43</td>
<td>12.38±0.46</td>
<td>13.34±0.39</td>
<td>13.46±0.39</td>
<td>13.57±0.41</td>
<td>13.88±0.38</td>
</tr>
<tr>
<td>TEC (10⁶/cumm)</td>
<td>A</td>
<td>10.4±0.41</td>
<td>13.8±0.46*</td>
<td>13.68±0.43</td>
<td>13.78±0.42</td>
<td>13.81±0.42</td>
<td>13.98±0.41</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1.95±0.03</td>
<td>1.91±0.02</td>
<td>1.91±0.01</td>
<td>2.22±0.04</td>
<td>1.98±0.04</td>
<td>1.99±0.04</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.93±0.01</td>
<td>1.93±0.01</td>
<td>1.93±0.00</td>
<td>1.98±0.01*</td>
<td>1.99±0.01</td>
<td>2.02±0.01</td>
</tr>
<tr>
<td>TEC (10⁶/cumm)</td>
<td>D</td>
<td>1.8±0.08</td>
<td>1.92±0.05</td>
<td>1.89±0.06</td>
<td>2.04±0.09</td>
<td>2.03±0.08</td>
<td>2.09±0.11</td>
</tr>
<tr>
<td>TLC (10⁶/cumm)</td>
<td>A</td>
<td>8.04±348.9</td>
<td>8.04±331.5</td>
<td>7.89±294.4</td>
<td>7.81±243.7</td>
<td>7.47±2.0</td>
<td>7.45±2.3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7.15±153.52</td>
<td>6.53±143.56</td>
<td>6.62±118.4</td>
<td>7.21±192.6</td>
<td>6.89±3.3</td>
<td>6.82±118.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>7.15±159.16</td>
<td>7.22±195.25</td>
<td>7.43±299.3</td>
<td>7.45±257.8</td>
<td>7.38±1.22</td>
<td>7.40±15.19</td>
</tr>
<tr>
<td>Glucose level (mg/dl)</td>
<td>D</td>
<td>6.82±25.06</td>
<td>6.91±24.75</td>
<td>7.03±290.5</td>
<td>7.11±198.2</td>
<td>7.11±184.5</td>
<td>7.16±256.0</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>A</td>
<td>55.4±2.86</td>
<td>55.9±4.66*</td>
<td>64.59±3.42*</td>
<td>76.54±1.82**</td>
<td>81.3±3.85*</td>
<td>91.65±2.86*</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>56.5±1.28</td>
<td>56.8±1.27</td>
<td>73.59±0.94</td>
<td>83.59±0.56*</td>
<td>91.43±0.98</td>
<td>100.15±1.12</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>57.5±1.94</td>
<td>67.7±2.09</td>
<td>82.59±1.78*</td>
<td>90.59±1.55**</td>
<td>101.03±1.9</td>
<td>108.65±1.94*</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>D</td>
<td>58.5±0.75</td>
<td>78.6±0.47</td>
<td>91.59±0.87</td>
<td>97.59±0.75*</td>
<td>110.63±1.05</td>
<td>117.15±0.75*</td>
</tr>
<tr>
<td>Bleeding time (min.)</td>
<td>A</td>
<td>190.3±2.1</td>
<td>189.9±2.16*</td>
<td>174.3±10.77</td>
<td>141.47±9.64</td>
<td>32.83±0.04</td>
<td>21.97±1.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>189.67±2.14</td>
<td>134.39±1.34</td>
<td>49.1±8.72</td>
<td>21.87±4.15</td>
<td>20.73±3.75</td>
<td>19.78±2.74</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>191±2.0</td>
<td>78.79±1.79</td>
<td>23.9±0.81</td>
<td>22.83±0.09</td>
<td>18.8±4.3</td>
<td>17.2±3.0</td>
</tr>
<tr>
<td>Clotting time (min.)</td>
<td>D</td>
<td>190.3±2.0</td>
<td>23.19±6.7</td>
<td>20.1±0.71</td>
<td>19.16±0.79</td>
<td>18.78±0.66</td>
<td>17.8±3.04</td>
</tr>
</tbody>
</table>
| * Significant at p<0.05 difference with 0 min time interval
** Significant at p<0.01 difference with 0 min time interval

~ 16 ~
that these enzymes diffuse into blood stream and increased levels are found in circulating blood. Thus the measurement of the serum activity of these enzymes can reflect the integrity of the walls of liver cells and can provide an important method of assessing liver damage. The cytoplasmic enzymes such as serum alanine amino transferase and serum aspartate amino transferase are affected by permeability of cell membrane whereas microsomal (Ribosomal and mitochondrial) enzymes like serum glutamate dehydrogenase need severe damage of liver cells before their elevation. Serum GGT level was significantly (p<0.05) higher in animals suffering with hepatic dysfunction compared to its normal values. A significant (p<0.05) decrease in serum GGT level was observed in animals of different groups after initiation of treatment. Liver enzymes ALT, AST and GGT were greatly elevated in dogs suffering with hepatitis due to hepatocytic necrosis and swelling after leakage from the cytosol.\(^{16,17}\)

### 4. Conclusion

Regenerative strategies in the liver seem important, as adult hepatocytes are widely known for their large regenerative capacity. However, advance research in the field of hepatology make clear that regeneration by hepatocyte replication is failing or absent in severe or chronic ongoing liver disease. To repopulate the liver, hepatic progenitor cells (HPCs) are a reserve compartment of progenitor and adult stem cells. Hepatic progenitor cells are a reserve compartment of adult stem or progenitor cells that reside within the liver and are found in rodents, humans, dogs and cats. Hepatic progenitor cells have potential to show self-renewal capacity and differentiate into cholangiocyte and hepatocytes. Cell-based therapy is considered a new therapeutic tool that has shown great success in the recent years and it is expected to replace whole organ transplantation in the future.

On the basis of parameters observed in this study it was concluded that combination of hepatocytic stem cells and pepsid-C gave an early and better recovery in hepatic deficient canine patients as compared to the hepatocytic stem cells and pepsid-C alone. The therapeutic efficacy of the different combinations used in this study can be grouped in order of hepatocytic stem cells + pepsid-C (Group D), hepatocytic stem cells (Group C) and pepsid-C (Group B).

### 5. References