Long Term Evaluation of Human Umbilical Cord Blood Mesenchymal Stem Cells in the Management of Total Coronary Occlusion (Experimental Study in Dogs)

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ABSTRACT
Cardiomyocytes in the border zone of an old infarct are condemned to die by necrosis and apoptosis caused by a persistent impairment of the coronary vasodilatory reserve even after successful recanalization of coronary artery total occlusion. Therefore, Chronic Total Occlusion (CTO) remains one of the more challenges for coronary interventions with uncertainty regarding procedural success and long term benefits. Twenty dogs were subjected experimentally to total coronary occlusion; half were treated with human Umbilical Cord Blood derived Mesenchymal Stem Cells (hUCB-MSCs). Clinical, electrocardiographic, echocardiographic, histopathological, biochemical and immunohistochemistry assessments were performed for 6 months at different time intervals. Demonstrated improved systolic function after one month following MSCs injection that was manifested by gradual increase of Fractional Shortening (FS%) and Ejection Fraction (EF). Electrocardiography exhibited improvement of ECG pattern with resolving of abnormal changes (elevated ST segment and inverted T) till retaining approximately normal ECG. After hUCB-MSCs myocardial infiltrations, specific genes in cardiac tissue were highly expressed indicating the differentiation of hUCB-MSCs into endothelial cells. The markedly increased vWF-positive cells in the damaged zone, suggested that angiogenesis is induced by promoting VEGFR2 expression at cardiac injury sites. This improved ischemia, thereby promoting body repair. Improved cardiac efficiency was expressed differently through various assessment parameters.

Key words: Coronary, occlusion, myocardial, infarction, stem cells

INTRODUCTION
Chronic total coronary artery occlusion (CTO) remains one of the more challenges for coronary interventions (Han et al., 2006). It is one of the commonest reasons for referral for coronary artery bypass surgery and many are left untreated because of uncertainty regarding procedural success and long term benefits (Delacretaz and Meier, 1997). Even after successful recanalization of CTO by angioplasty with stent implantation, the myocardium partially remains hibernating (Wijns et al., 1998). Cardiomyocytes in the border zone of an old infarct are condemned to die by necrosis and apoptosis caused by a persistent impairment of the coronary vasodilatory reserve (Prasad et al., 2007).
Mesenchymal Stem Cells (MSCs) as a multipotent adult progenitor cells appear to have multilineage differentiation capacity in vitro including osteoblasts, adipocytes, chondrocytes, cardiomyocytes, fibroblasts and endothelial cells (Friedenstein et al., 1976; Pittenger et al., 1999; Jiang et al., 2002). The MSCs are thought to be excellent tools for the field of regenerative medicine, because of their differentiation potentials. In addition to inhibit proliferation, differentiation and activation of immune cells including T cells, B cells, NK cells and dendritic cells (Uccelli et al., 2006; Kang et al., 2008). Preclinical models have shown the potential ability of undifferentiated hUCB-MSCs to undergo site-specific differentiation into a functional cardiac muscle phenotype after injection into sheep. Thus, they seem to avoid detection by the host immune system (Liechty et al., 2000; Di Nicola et al., 2002; Hua et al., 2011). The isolation of MSCs have been described in several species and from different tissues, including Bone Marrow (BM), adult fat, Umbilical Cord Blood (UCB) and skeletal muscle (Laitinen and Laine, 2007). Stem cells from UCB have many advantages because of the immature nature of newborn cells compared to adult cells. UCB express a lower level of Human Leukocyte Antigen (HLA) class I than BMMSCs, suggesting the lower immunogenicity and the superiority for clinical use in Hematopoietic Stem Cell Transplantation (HSCT) (Lee et al., 2004; Kern et al., 2006; Anzalone et al., 2013). The UCB appear to have greater immunosuppressive effect, indicating their better role in the management of Graft Versus Host Disease (GVHD), do not induce teratomas and have anticancer properties (Pham et al., 2014). These advantages are important considerations for their use in cell based therapies and treatment of cancers (Huang et al., 2011; Pham et al., 2014). Therefore, UCB represents a good alternative source of MSCs and should not be discarded as medical waste (Bajpai and Andreadis, 2012).

The aim of this study was to surgically establishing experimental Acute Myocardial Infarctions (AMI) model via ligation of the distal 1/3 of Left Anterior Descending artery (LAD) and to investigate the long term potential therapeutic effects of hUCB-MSCs and their differentiation.

MATERIALS AND METHODS

The study comprised 20 male domestic mongrel dogs, aging between 2-3 years and weighing between 15-20 kg. Animals were obtained, housed, supervised and taken care-off at the kennels of the Department of Surgery, Anaesthesiology and Radiology, Faculty of veterinary medicine, Cairo University. An approval was obtained from the ethical committee of the same institute. The design of this study was divided into in vivo and in vitro procedures. The in vitro procedures included the followings.

Stem cells isolation and characterization: Twenty samples of human CB were harvested and hUCB-MSCs were isolated by high density concentration gradient. The separated hUCB-MSCs were subcultured at a concentration of 1×10^6/100 mm dish in mesencult basal medium (Stem Cell Technologies, Vancouver, Canada) and used for in vivo experiments. The cells were incubated at 37°C in 5% CO₂ in a humidified atmosphere. When the cells reached 80% confluency, they were detached with 0.25% trypsin and replated. The hUCB-MSCs at P3 were assessed for multilineage differentiation. The adipogenic, osteogenic and chondrogenic differentiation ability of hUCB-MSCs was determined as briefly described. For chondrogenic, osteogenic and adipogenic differentiation, 5×10⁵ hUCB-MSCs were cultured with adipogenic, osteogenic and chondrogenic differentiation kit (Invitrogen, Germany), respectively for 3 weeks. To evaluate the chondrogenic, osteogenic and adipogenic differentiation, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and stained with 0.5% Alcian blue, Alzarin red and Oil Red (Sigma-Aldrich, Milan, Italy) in methanol (Sigma-Aldrich, Milan, Italy) for 20 min at room temperature.
Intracardiac infiltration of the cell product was administrated. Aliquots of the cell product for infiltration were taken to assess cell viability (trypan blue exclusion test).

**Animal groups:** The study was performed on 20 experimental animals that were divided equally into 2 groups (n = 10). In both groups; all dogs were subjected to open surgery LAD artery ligation. On day 7 after the ligation procedures: Group I: (control group), received a single intramyocardial infiltration of 2 mL Phosphate Buffer Saline (PBS) in the ischemic area below the site of ligation and group II: (cell-treated group), received a single intramyocardial MSCs-infiltration of a total of 6×10^6 cells in 2 mL PBS. Half the animals of each group (n = 5) were sacrificed after 3 months and the other half was sacrificed at 6 months post-operatively.

**Surgical procedures:** Anaesthesia was induced with intravenous injection of xylazine (1 mg kg\(^{-1}\)), diazepam (0.5 mg kg\(^{-1}\)) and ketamine (10 mg kg\(^{-1}\)). After intubation; dogs were maintained with isoflurane 1% and fentanyl (0.25 mg kg\(^{-1}\), i.v.). In all dogs, AMI was induced through left thoracotomy (Fig. 1) followed by ligation of the LAD distal to the first diagonal branch. All animals were given a systemic course of antibiotic for 5 days. All animals were exercised once daily. Animals were left for 1 week before commencing treatment. At the end of the experiment and according to the sacrifice timetable, dogs were put to sleep through intravenous injection of thiopental sodium and samples were collected for evaluation.

**In vivo assessment:** Assessment methods/techniques used in this study included the clinical, radiological, electrocardiography, echocardiography, laboratory/biochemical analysis and histological/immunohistochemistry assessments.

**Clinical assessment:** The vital parameters were recorded. The respiratory and cardiovascular signs following the operation were monitored.

**Electrocardiography:** A standard 12-lead ECG was used as non-invasive cardiac monitoring using ECG-E22A (Shenzhen Oriental Science and Tech. Co., Ltd). Electrocardiography was carried out before and after operation. Electrocardiographic monitoring was carried at various time intervals following operation that included (2, 24 h, 7 day, 1, 3 and 6 months). The ECG was then analysed for abnormalities and compared with the previous ones.

**Echocardiography:** Two-dimensional and M-mode echocardiography was performed before and after operation for detection of any abnormalities. Echocardiographic monitoring was carried out at various time intervals (24 h, 7 days, 1, 3 and 6 months following operation). A 2-4 MHz microconvex transducer attached to an ultrasonographic machine (Samsung Madison, SONOACE-R3-Korea) was used. Echocardiography was performed through right parasternal approach from the third to the fifth intercostal spaces where the posterior wall of left ventricle could be visualized properly according to Thomas *et al.* (1993).

The following measurements were recorded, RVIDd: Right Ventricular Internal Dimension at end-diastole, LVIDd: Left Ventricular Internal Dimension at end-diastole, LVIDs: Left Ventricular Internal Dimension at end-systole, LVWTd: Left Ventricular Wall Thickness at end-diastole, LVWTs: Left Ventricular Wall Thickness at end-systole, LA/AO: Left Atrium to Aortic Root ratio, EPSS: Mitral valve E-point to the ventricular septal separation, FS%: Left ventricular Fractional Shortening and EF: Left ventricular Ejection Fraction.
Echocardiographic measurements and indices were analysed and compared with the previous measurements.

**Laboratory and biochemical analysis:** This included colorimetric assessment of troponin I and creatinin kinase-MB fraction to evaluate myocardial damage induced immediately before (baseline) and at 24 h, 7 days, 3 and 6 months after cell/placebo infusion.

**Animal scarification for the followings**

**Histological examination:** The hearts of all groups were exposed by median sternotomy and quickly removed. After gross examination, the hearts were divided into two parts: One part was fixed in 10% formaldehyde for 48 h and embedded in paraffin. Sections (5 μm) were stained with Haematoxylin and Eosin (HE) and Masson Trichome (MT) for qualitative histopathological analysis for scar tissue, new blood vessels development, inflammation, infarction lesions, fibrosis and collagen deposition. Other frozen sections were unstained for immunofluorescence assessment of injected hUCB-MSCs differentiation into endothelial cells. Slices were dried at room temperature, PBS-washed for 5 min 4 times, incubated with 0.4% Triton X-100 (PBS preparation) at room temperature for 20 min, PBS washed for 5 min 4 times, incubated with 10% bovine serum albumin antigen at room temperature for 20 min, incubated with human anti-rat vWF multi-clonal antibody (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), incubated at 4°C overnight, PBS washed for 5 min 4 times, incubated with TRITC-labeled goat anti-mouse secondary antibody (1:100; Beijing Zhongshan Golden Bridge Biotechnology Company) and incubated at 37°C without light for 3 h, PBS washed for 5 min 4 times. Images were acquired using inverted fluorescence microscopy (Leica, Germany).

**Real time PCR:** Human VEGFR2 and eNOS mRNA expression level of heart tissue for all groups were quantified by qRT-PCR to assess hMSCs homing and angiogenesis. Thousand nanogram of the total RNA from each sample were used for cDNA synthesis by reverse transcription using high capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 min at 95°C for enzyme activation followed by 40 cycles of 15 sec at 95°C, 20 sec at 55°C and 30 sec at 72°C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean Critical Threshold (CT) values of GAPDH housekeeping gene by the ΔΔCt method. One micromolar of both primers specific for each target gene was used as demonstrated (Table 1).

**Statistical assessment:** The data was presented as Mean±SD. Data was analyzed using SPSS 15.0 software (SPSS Inc.). The ANOVA was used to determine significant differences between different groups. Significant differences were considered when p<0.05.

**Table 1: Primers sequence for each target gene:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-R2</td>
<td>GATGTGGTTCTGAGTGCCGCTCT</td>
<td>CATGGGCTCTGCTCTCCTTTTG</td>
<td>NT-022853.15</td>
</tr>
<tr>
<td>eNOS</td>
<td>ATTATATTCTACACAAAGCTCCAG</td>
<td>TCTTCAAGTGCCCAGTTTAC</td>
<td>NT-007914.15</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCTCTACTGGCGCTGCAAGGCT</td>
<td>GTCCACCACGACGTTGG</td>
<td>NT-009759.16</td>
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RESULTS

Clinical assessment: The vital parameters of both controlled and treated groups were within normal. No specific respiratory or cardiovascular signs (congested blood vessels, prolonged capillary refill time or lower limbs edema) were reported following operation. At 3 months interval, all dogs within the controlled group showed lethargy, weakness, decreased activities and deep inspiratory pattern. The above mentioned signs continued until six-month interval. No obvious clinical signs were noticed in treated dogs.

ECG assessment: In controlled group, the ECG changes appeared more prominent and progressed by time. The ST segment elevation started within 2 h after ligation and after 24 h, more ST elevation was noticed. Old MI revealed depressed ST segment with inverted T was noticed (Fig. 1).

In treated group, the ECG changes improved one week following injection. One month post injection, the resolving of changes becomes more pronounced. At 3 month interval, ECG appeared normal that’s continued until the end of the study (Fig. 2).

Echocardiogram: The infarcted myocardium was thinner and appeared echodense than normal as the affected areas became fibrotic. The decrease in thickening occurred suddenly. The reduction of wall motion toward the centre of the ventricular chamber during systole on transverse or long-axis real-time images revealed the infarcted areas of myocardium. By comparing the echocardiographic measurements for the 2 groups: In group I (controlled group), the FS% showed significant decrease as the ratio between diastole and systole increased due to disturbance in

Fig. 1(a-c): Control group, (a) After 2 h, the ST segment elevation is noticeable, (b) At 24 h, the elevation of ST segment become more pronounced and (c) One week following ligation. The ST segment appeared depressed with inverted T

Fig. 2(a-c): hUCB-MSCs treated group, (a) After 1 month showed improved ST segment elevation, (b) After 3 months showed progressed improvement of ST segment and (c) After 6 months showed approximately normal electrocardiogram
Table 2: Echocardiographic measurements for control group. The measurements are presented as Mean±SD

<table>
<thead>
<tr>
<th>Variables</th>
<th>0 P.O</th>
<th>7 days</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVIDd</td>
<td>1.01±0.25</td>
<td>1.05±0.22</td>
<td>1.03±0.23</td>
<td>1.02±0.71</td>
<td>1.02±0.55</td>
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<tr>
<td>LVIDd</td>
<td>4.33±0.70</td>
<td>4.32±0.40</td>
<td>4.33±0.30</td>
<td>4.33±0.50</td>
<td>4.35±0.60</td>
</tr>
<tr>
<td>LVIDs</td>
<td>3.01±0.50</td>
<td>3.00±0.40</td>
<td>2.80±0.40</td>
<td>2.80±0.60</td>
<td>2.60±0.40*</td>
</tr>
<tr>
<td>LVWTd</td>
<td>0.91±0.11</td>
<td>0.91±0.11</td>
<td>0.83±0.21</td>
<td>0.81±0.33</td>
<td>0.81±0.25*</td>
</tr>
<tr>
<td>LVWTS</td>
<td>1.41±0.11</td>
<td>1.43±0.12</td>
<td>1.36±0.12</td>
<td>1.31±0.21</td>
<td>1.30±0.21*</td>
</tr>
<tr>
<td>LA/Ao</td>
<td>0.99±0.02</td>
<td>1.03±0.11</td>
<td>1.02±0.22</td>
<td>1.01±0.21</td>
<td>0.99±0.36</td>
</tr>
<tr>
<td>EPSS</td>
<td>0.73±0.03</td>
<td>0.7±0.02</td>
<td>0.7±0.03</td>
<td>0.70±0.07</td>
<td>0.70±0.08</td>
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<tr>
<td>FS (%)</td>
<td>37.48</td>
<td>37.56</td>
<td>33.36</td>
<td>30.06</td>
<td>30.83*</td>
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<tr>
<td>EF</td>
<td>58.21</td>
<td>58.32</td>
<td>49.53*</td>
<td>44.33*</td>
<td>41.11*</td>
</tr>
</tbody>
</table>

*p<0.05 significant difference when compared with 0 time

Table 3: Echocardiographic measurements for hUCB-MSCs group. The measurements are presented as Mean±SD

<table>
<thead>
<tr>
<th>Variables</th>
<th>0 P.O</th>
<th>7 days</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVIDd</td>
<td>1.09±0.06</td>
<td>1.0±0.02</td>
<td>1.02±0.30</td>
<td>1.01±0.04</td>
<td>1.07±0.60</td>
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<tr>
<td>LVIDd</td>
<td>4.52±0.63</td>
<td>4.53±0.65</td>
<td>4.69±0.62</td>
<td>4.60±0.03</td>
<td>4.61±0.61</td>
</tr>
<tr>
<td>LVIDs</td>
<td>3.16±0.61</td>
<td>3.22±0.52</td>
<td>3.44±0.41*</td>
<td>3.33±0.25</td>
<td>3.30±0.25</td>
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<tr>
<td>LVWTd</td>
<td>0.88±0.05</td>
<td>0.86±0.01</td>
<td>0.90±0.02</td>
<td>0.92±0.07</td>
<td>0.95±0.06</td>
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<tr>
<td>LVWTS</td>
<td>1.55±0.22</td>
<td>1.51±0.32</td>
<td>1.59±0.18</td>
<td>1.52±0.21</td>
<td>1.45±0.61</td>
</tr>
<tr>
<td>LA/Ao</td>
<td>1.06±0.02</td>
<td>1.01±0.03</td>
<td>1.0±0.03</td>
<td>0.98±0.04</td>
<td>0.98±0.10</td>
</tr>
<tr>
<td>EPSS</td>
<td>0.70±0.06</td>
<td>0.70±0.06</td>
<td>0.69±0.01</td>
<td>0.69±0.02</td>
<td>0.70±0.02</td>
</tr>
<tr>
<td>FS (%)</td>
<td>41.09</td>
<td>36.20</td>
<td>32.51</td>
<td>30.61</td>
<td>36.15</td>
</tr>
<tr>
<td>EF</td>
<td>57.47</td>
<td>55.70</td>
<td>43.47*</td>
<td>51.66</td>
<td>53.15</td>
</tr>
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</table>

*p<0.05 significant difference when compared with 0 time

Table 4: CK-MB and troponin-I levels in ischemic and ischemic/ hUCB-MSCs treated canines

<table>
<thead>
<tr>
<th>Time</th>
<th>CK-MB U L⁻¹</th>
<th>Troponin-I U L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic/PBS</td>
<td>63.30±6.103*</td>
<td>61.51±5.906*</td>
</tr>
<tr>
<td>Ischemic/MSCs</td>
<td>40.21±2.034*</td>
<td>20.44±0.434</td>
</tr>
<tr>
<td>Ischemic/PBS</td>
<td>21.17±0.626</td>
<td>20.11±0.629</td>
</tr>
<tr>
<td>Ischemic/MSCs</td>
<td>21.31±0.519</td>
<td>20.44±0.434</td>
</tr>
</tbody>
</table>

*p<0.05 significant difference when compared with 0 time

systolic function. The ejection fraction which measures the efficiency of left ventricle showed disturbance due to impaired systolic motion of the left ventricle. The features of acute myocardial ischemia on echocardiograms include reduced wall or septal systolic thickening, dyskinetic wall motion and systolic thinning (Table 2).

In hUCB-MSCs treated group, marked improvement in fractional shortening and ejection fraction started almost one month post operatively. The improvement of both FS% and EF continued until 6 months after operation where it is become within normal indices in dogs (Table 3).

**Laboratory and biochemical analysis:** About 24 h after LAD ligation, CK-MB and troponin I levels showed marked significant elevation compared to control/placebo group indicating occurrence of myocardial infarction (p<0.05). When CK-MB and troponin I levels were measured at one week after ligation and after intramyocardial hUCB-MSCs infiltration at 3 and 6 months, showed suppression of their levels demonstrating cardiac enzymes improvement (Table 4).
PM findings: Gross examination revealed clearly distinguished white area under the site of ligation in controlled group. The same area in treated group was difficult to be distinguished from surrounding cardiac tissue with presence of reddish patchy areas spread all over this area.

Histological assessment: In ischemic/PBS group, signs of Infarction were clearly noticed. At 3 months scarification, myocytic necrosis in the form of empty cells (sarcolysis) was minimal but necrotic cells were mostly of the eosinophilic granular necrosis type with loss of central nuclei. Granulation tissue rich in fibroblasts and inflammatory cells (macrophages and lymphocytes). Congestion of blood vessels and hemorrhages in the infarction area and nearby the intramuscular zones. Atrophy of myocytes surrounding necrotic zones was seen and the fibrous tissue was extensive and edematous (Fig. 3).

After 6 months from the operation, myocytic necrosis was prominent in the form of empty cells (sarcolysis) and eosinophilic granular necrosis with loss of central nuclei (viability poor compared to 3 months. Atrophy of myocytes surrounding necrotic zones. Fibrous tissue rich in fibroblasts and very dense mostly collagen-I was noticed. Fibrosis was denser and band like with less vacuolization (Fig. 4).

Fig. 3(a-c): Ischemic/PBS cardiac musculature at 3 months scarification, (a) Eosinophilic necrosis, myocytes with pink granular cytoplasm and lost nuclei HE-400x, (b) Necrotic myocytes are surrounded by edematous interstitial tissue (arrow) MT-400x and (c) Rich in fibroblasts and some inflammatory cells MT-400x

Fig. 4(a-c): Signs of necrosis and fibrosis of cardiac muscles at 6 months following operation (a) Dense fibrosis, Fibrofatty Infiltration (FI) and necrosis as well as atrophic myocytes (arrow) HE-x400, (b) Sarcolysis of myocytes (arrow) HE-x200 and (c) Necrotic myocytes (arrows) and dense band like fibrosis (FT), MT-x200
In cell treated group, after 3 months from treatment, the viability of myocytes was better with minimal necrosis. Neovascularization was pronounced with MSCs collections. Very minimal myocytic necrosis was evident in the form of empty cells (sarcoclysis) and eosinophilic granular necrosis with loss of central nuclei. Atrophic myocytes surrounding necrotic zones was noticed. Mild fibrosis and fatty infiltration in addition to inflammation (macrophages, lymphocytes and few polymorph nuclear lymphocytes) were also detected. Mild congestion of blood vessels and many capillaries and large blood vessels with budding and branching were visible indicating neovascularization of the area. Collections of dark oval small cells slightly larger than lymphocytes were visualized in a perivascular and intravascular localization that probably indicates collection of injected MSCs that differentiated into new blood vessels (Fig. 5).

In the cell treated group, after 6 months from treatment, mild Congestion of BV was seen. Many capillaries and thick walled blood vessels with evidence of budding and branching indicating neovascularization of the area in addition to angiomatoid formation were detected. Fatty infiltration of muscle was prominent (fat replaces lost muscle fibres) (Fig. 6).

Fig. 5(a-c): Signs of muscular tissue healing and new blood vessels formation at 3 months following MSCs treatment, (a) Branching of Congested Blood Vessel (BV) in infarct area, HE-x400, (b) Dilated congested large vessel surrounded by fibrosis (FT) and Fatty Infiltration (FI) MT-x400 and (c) MSCs collections: Dark blue intravascular collections (arrow) of stem cells, HE-x400

Fig. 6(a-c): Six months following treatment showed increased neovascularization, (a) Branching of congested blood vessel in infarct area (angiomatoids) and thick walled medium sized artery, HE-x200, (b) Long, branching and interconnected blood vessels, HE-100x and (c) Neovascularization in septal zone, HE-x200
Angiogenesis immunostaining assessment: Hearts of ischemic/PBS and ischemic/hUCB-MSCs treated dogs were examined as regarding immunostaining of new blood vessels formation at 3 and 6 months after scarification. The results of vWF immunofluorescence staining to assess angiogenesis (Fig. 7) showed that the number of positive vessels in the ischemic/PBS group at 3 and 6 months were 9.31±2.518 and 10.5±2.521, respectively and 14.01±3.78 and 20.74±5.44 for the ischemic/MSCs group, respectively. The number of vWF-positive vessels in the injured groups at 6 months was significantly higher than at 3 months (p<0.05, Table 5).

Quantitative PCR assessment: The QRT-PCR gene expression of human VEGFR2 and eNOS was assessed to evaluate homing and functional angiogenesis in heart tissues of all groups. There is high significant difference between VEGFR2 and eNOS genes expression in different times scheduled (3 and 6 months) in ischemic/MSCs group (Table 6). This indicates homing, localization and differentiation of transplanted MSCs into endothelial cells. Human VEGFR2 and eNOS were not detected in heart tissues of ischemic/PBS group.

Table 5: vWF-positive blood vessels in hearts of the two groups at different times scheduled

<table>
<thead>
<tr>
<th>Group</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic/PBS</td>
<td>9.31±2.518</td>
<td>10.5±2.521</td>
</tr>
<tr>
<td>Ischemic/MSCs</td>
<td>14.01±3.78</td>
<td>20.74±5.44</td>
</tr>
<tr>
<td>p-value</td>
<td>0.000</td>
<td>0.000</td>
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</tbody>
</table>

Table 6: Quantitative genes expression in ischemic/hUCB-MSCs at different times

<table>
<thead>
<tr>
<th>Group</th>
<th>VEGFR2 3 months</th>
<th>VEGFR2 6 months</th>
<th>eNOS 3 months</th>
<th>eNOS 6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic/MSCs</td>
<td>1.251±0.416</td>
<td>2.041±0.581</td>
<td>0.839±0.135</td>
<td>1.932±0.509</td>
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<tr>
<td>p-value</td>
<td>0.000</td>
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</table>

Fig. 7(a-d): Number of vWF-positive blood vessels (a-b) Ischemic/PBS and (c-d) Ischemic/hUCB-MSCs groups. Scale bar = 100 μm
DISCUSSION

Reports on immunogenicity of MSCs have stated that allogenic MSCs are non-immunogenic in vitro but can provoke immune responses after in vivo injection (Poncelet et al., 2007). Nevertheless, it was also reported that long term evaluation of allogeneic MSCs isolated from young healthy donors have excellent regenerative potential and immediate availability for clinical application (Dhingra et al., 2013).

Early-phase trials of intravenous administration of allogeneic MSCs demonstrated a trend toward improved clinical outcomes. Preserving prostaglandin E2 level prevents rejection of implanted allogeneic mesenchymal stem cells and restores post-infarction ventricular function. Reports have demonstrated that differentiation of allogeneic stem cells after implantation in the infarcted myocardium was associated with loss of their immune-privilege, cell rejection and progressive ventricular dysfunction (Huang et al., 2010).

Human Umbilical Cord Blood-derived Mesenchymal Stem Cells (hUCB-MSCs) have recently emerged as a promising solution for allogeneic cell therapy (Chang et al., 2008). Several studies have reported that hUCB-MSCs can be successfully isolated, expanded and differentiated into multi-lineages (Kogler et al., 2004; Zhang et al., 2013). Cells used previously in autologous stem cell therapy are acquired from patients with multiple cardiovascular risk factors that are known to suppress the function of stem cells, whereas, hUCB-MSCs are extracted from relatively young and healthy donors who have low cardiovascular risk factors.

Although, myocardial infarction is not common in dogs but its experimental induction resulted in reduced systolic function with dyskinetic wall motion. Dogs of both groups showed no immediate or early clinical signs following the LAD-ligation. At 3 months following ligation, all dogs within the control group started to showed lethargy, weakness, decreased activities and deep inspiratory pattern. These signs continued until the end of the experiment (Buchanan and Bucheler, 1995; Boon, 2011). No obvious clinical signs were noticed in treated group. Radiographically, the heart appeared within normal size according to the Vertebral Heart Score (VHS) in both control or treated groups (James, 2000; Boon, 2011) and normal cardiothoracic ratio (Torad and Hassan, 2014). This may be attributed to the localized nature of ischemic area following ligation of LAD.

Various ECG criteria have been proposed in the past as indicators for myocardial infarction during ventricular pacing (Barold et al., 1987). Three ECG criteria were found to have independent value in the diagnosis of acute myocardial infarction: ST-segment elevation of ≥1 mm in the presence of a positive QRS complex; ST-segment depression of ≥1 mm in lead V1, V2 or V3 and ST-segment elevation of ≥5 mm in the presence of a negative QRS complex (Sgarbossa et al., 1996).

An anterior wall myocardial infarction occurs when anterior myocardial tissue usually supplied by the left anterior Descending Coronary Artery (LAD) suffers injury due to lack of blood supply. In the control group, the ECG changes appeared more prominent and progressed by time. The ST-segment elevation started 2 h after ligation and increased after 24 h. Old MI exhibited depressed ST-segment with inverted-T that was noticed one month following induction. The elevation of ST-segment above the baseline indicated injury pattern (more than 1 mm in limb leads and more than 2 mm in chest leads) followed by deep symmetrically inverted T wave ischemia. Hyperacute T wave may be an early sign of AMI. The repolarization changes usually include ST-T changes while necrosis mostly included depolarization changes (QRS changes).

In the treated group, the ECG changes started resolving one week following hUCB-MSCs injection. One month post injection, the improvement became more pronounced and at 3 months, the ECG appeared normal and until the end of the study.
On echocardiographic examination, both control and treated groups clearly showed localized reduction of the ventricular wall thickness at one month post-injection. The area of infarction appeared hyperechoic than neighboring normal part of the ventricular wall. This may attributed to the fibrotic changes occurring following the infarction (Pandian et al., 1982; Sabia et al., 1991). The decrease in systolic thickening did not extend to the adjacent area in control group, while in treated group, the thickness of infarcted area increased gradually until reaching approximately the original thickness of the wall.

The fractional shortening percentage (an indicative of systolic function) showed gradual decrease in both control and treated groups at one month. This may be a result of decreased systolic function. The decreased fractional shortening continued until the end of experiment in control group whereas, in treated group, the fractional shortening increased gradually until re-claiming its normal values at 6 months interval which can be attributed to the improved systolic function in treated group manifested by improved ventricular contractility (Guth et al., 1984; Sabia et al., 1991).

The ejection fraction in the control group gradually decreased all over the study period, on the contrary, it gradually increased until obtaining nearly normal range in the treated group. This may be a result of improved ventricular contractility in treated group (Torry et al., 1991).

Gross examination of the heart after euthanasia revealed localized white area in control group below the site of ligation that was easily distinguished from the surrounding tissue. In treated group, the area below the site of ligation appeared reddish in color with the presence of patchy reddish areas on the surface at 3 months interval. At 6 months interval, the infarcted area could not be differentiated from the surrounding structure.

On histopathological examination, study demonstrated that intramyocardial transplantation of MSCs in canine model with AMI was associated with neovascularization and improvement of cardiac function. The localization and homing of transplanted DILDL-UEA-1 human MSCs in canine cardiac tissues was identified by detection of these labeled MSCs as fluorescent collected cells between striated cardiac muscles.

This study confirms the potential adherence growth rate of mesenchymal stem cells derived from human umbilical cord blood on plastic culture plates and their differentiation capacity in vitro towards reference lineages. Umbilical cord blood is proposed as the most valuable, non-invasive source for MSCs on the basis of the following observations. Firstly, an isolation success rate of 100%, which might indicates that the isolation methods and culture conditions for hUCB-MSCs from these sources have been optimized. Secondly, regarding the osteogenic and adipogenic differentiation, hUCB-MSCs produced a mineralized matrix and adipocytes respectively. Other studies using microarray data (Wagner et al., 2005) reported 478 genes changed when compared human MSCs from three tissue origins (bone marrow, adipose tissue and umbilical cord blood) to differentiated fibroblasts.

This study also demonstrated that after MSCs infiltration, VEGFR2 and eNOS genes expression in cardiac tissue were highly expressed indicating differentiation of human MSCs into endothelial cells. Results of vWF-positive cells in the damaged zone were markedly increased, suggesting that angiogenesis in the damaged zone is induced by promoting VEGFR2 expression at cardiac injury sites after MSCs transplantation. This improved ischemia, thereby promoting body repair. Previous study showed that the ideal time of angiogenic therapy intervention based on VEGF and neuronal stem cell was presumed to be 2 days to 2 weeks after the acute spinal cord injury (Oudega, 2012). The results showed that vWF-positive cells at injury sites increased after MSCs transplantation and lasted for 60 days.
CONCLUSION

It is concluded that, hUCB-MSCs improved the cardiac efficiency and different assessment techniques form an integral parts in evaluating AMI management with hUCB-MSCs injection. Improved cardiac efficiency was expressed differently through various assessment parameters. Consequently, further immunological studies.

REFERENCES


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