Role of Endothelial Progenitor Cells in Management of Myocardial Infarction Following Total Coronary Occlusion in Dogs.

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ABSTRACT

Myocardial infarction caused by ischemic heart disease is a major health problem being the most prevalent cause of premature death in human. Chronic total occlusion (CTO) remains one of the most difficult challenges for coronary interventions. The currently available therapeutics only delay disease progression and mainly aim to enable patients to survive with a heart that is working at a fraction of its original capacity. Recent approaches suggest that the primary goal of cells therapy is to induce neovascularization rather than to replace myocardial tissue. In this paper, twenty dogs were subjected experimentally to CTO; only half were treated with endothelial progenitor cells (EPCs). Clinical, electrocardiographic, echocardiographic, histopathological, biochemical, and immunohistochemistry assessments were performed for 6-months at different time intervals. Results demonstrated improved systolic function. The fractional shortening (FS %) and the ejection fraction (EF) gradually decreased in both groups. In the treated group, the FS% and EF increased gradually until retaining their normal values at 6-months. ECG pattern improved with resolving of abnormal changes. Quantitative gene expression showed high significant difference between expressions on infarcted tissues versus non-infarct tissues. The intracardiac administration of EPCs is shown to be a useful cell therapy as potentially promoting both neovascularization and cardiac regeneration in ischemic heart disease. Large-scale studies should be induced to examine the potential effects of this novel approach on the risk of death and complications in patients with large acute myocardial infarctions and depressed left ventricular contractile function.

Keywords: Coronary, occlusion, endothelial progenitor cells, EPCs, myocardial infarction.

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INTRODUCTION

Heart failure due to ischemic heart disease is a major health problem, being the most prevalent cause of premature death in human (1). Chronic total occlusion (CTO) remains one of the more difficult challenges for coronary interventions (2) and its presence often exclude patients from treatment by percutaneous coronary intervention (3). Currently available therapeutics only delay disease progression and mainly aim to enabling patient to survive with a heart that is working at a fraction of its original capacity. Heart transplantation is the only surgical procedure that is performed on patients with end stage heart failure or severe coronary artery disease, (4). Recent new insights into cardiac repair suggest that the primary goal of stem cells therapy would be to induce neovascularization in heart rather than to replace myocardial tissue (5). Bone marrow-derived cells can be mobilized from the bone marrow into the peripheral circulation and can subsequently home to sites of ischemia. A variety of experimental studies investigating the function of endothelial progenitor cells (EPCs) in vivo, especially in vessel endothelium formation (6,7). EPCs have been recognized to be pivotal in endothelial maintenance and their number is of great significance to the regulation of arterial regeneration (8). Experiments in animals show that systemic application or mobilization of stem cells and progenitor cells beneficially influences the repair of endothelial cells after injury and the progression of atherosclerosis (9).

The aim of the present work was to test the ability of EPCs to form new blood vessels and to differentiate into cardiomyocytes in an experimental model (dog) with acute myocardial infarction (AMI).

MATERIALS AND METHODS

The present work included in vitro and in vivo studies. The in vitro procedures included the acquisition, culturing and analysis of the EPCs while the in vivo study included surgical procedures in dogs (20 male domestic mongrel dogs, aging between 2-3 years and weighing between 15-20 kg) and follow up procedures. The in vitro study was carried out at department of Biochemistry, Faculty of Medicine, Cairo University, while the in vivo study was performed at the department of Surgery, Anaesthesiology and Radiology, Faculty of Veterinary medicine, Cairo University. The study was approved by ethical committee of Faculty of Veterinary medicine, Cairo University, Egypt.

In vitro study:

EPCs acquisition:

The blood (50 ml) was collected from human umbilical cord after obtaining informed consent. The mononuclear cell fraction (MNCs) was isolated from the buffy coats through density-gradient centrifugation with Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY). Anticoagulated blood sample was carefully layered on 20 ml Ficoll, and then centrifuged for 35 min. at 400xg rpm.

The upper layer was aspirated leaving the MNC layer undisturbed at the interphase. The interphase (MNC layer) was carefully aspirated and washed twice in PBS containing 2 mMEDTA and centrifuged for 10 minutes at 200xg rpm at 20°C. The cell pellet was resuspended in a final volume of 300 μl of buffer. (10, 11)

EPCs culture:

EPCs were identified in culture by formation of a Colony Forming Unit (CFU) (12). After MNCs culturing for 7 days, CFUs were formed. For the EPCs counting assay,6×10⁵ MNCs were layered onto fibronectin coated 96-well plates and cultured in M199 medium supplemented with 20% fetal calf serum (FCS), 0.1% human vascular endothelial growth factor-1 (VEGF-1) and 0.1% insulin-like growth factor (IGF-1) at 37°C for 48 hours after which the supernatant was removed. After 7 days, cells were stained and labelled with 1, 1′-dioctadecyl-3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated LDL (DiLDL) and FITC-labelled Ulexeuropaeus agglutinin I (UAEC-1, Sigma Chemical Company); this double staining is specific for EPCs identification. (10-13)

Cells were counterstained with 4’, 6-diamidino-phenylindole (DAPI); 0.2 μg/ml in 10 mmol/l Tris-HCl, pH 7.0, 10 mmol/l EDTA, and 100mmol/l NaCl) for 10 min and cells visualized with a distinct blue cytoplasm under inverted fluorescent microscope; DAPI staining was used to ensure cells viability.
Only double stained cells (DILDL-FITC labeled UAE-1) with a distinctly blue cytoplasm (DAPI positive cells) were counted in a five random fields under fluorescent inverted microscope (Lieca, Germany) (10).

**EPCs analysis:**

Analysis of EPCs was based on cell surface marker expression. Flow Cytometry Analysis (FACS analysis) for CD34+ of cultured EPCs was done as an identification surface marker of EPCs.

Mononuclear fractions (6×10⁶ cells) were incubated with FcR blocking reagent (MiltenyiBiotec) for 10 min, and then incubated with CD34-FITC (MiltenyiBiotec). Dead and dying cells were excluded with 7-AADD (1 μg/10⁶ cells, Molecular Probes). Isotype control antibodies were used to set baseline fluorescence levels.

Intra cardiac infusion of the cell product was the route of administration in dogs. Aliquots of the cell product for infusion were taken to assess cell viability (trypan blue exclusion test).

**Animals and groups:**

The 20 dogs were divided equally (n=10) into 2 groups; **Group I**: Control group (intramyocardiac infiltration with 2 mL saline). **Group II**: Cell-treated canines (intramyocardiac infiltration with EPCs a total of 6×10⁶ cells in 2 mL solution). Half the animals of each group (n=5) were euthanized after 3-months, and the other half was euthanized at 6-months post-operatively.

**Surgical procedures:**

Dogs were premedicated with xylazine (1 mg/kg IV), diazepam (0.5 mg/kg IV) and ketamine (10 mg/kg IV) was used for induction of anaesthesia. After intubation, dogs were maintained with isoflurane 1% and fentanyl (0.25 mg · kg⁻¹ · min⁻¹ IV). AMI was induced by ligation of the left anterior descending artery (LAD) distal to the first diagonal branch through left thoracotomy approach (14). All dogs received intravenous antibiotic (Ceftixone HCl 25mg/kg) daily for 5 days. After one week, all dogs were re-operated and subjected to intramyocardiac infusion of either 2 ml saline (control group) or EPCs in 2ml solution (cell treated group). At the end of the experiment, and according to the sacrifice timetable, dogs were put to sleep through intravenous injection of thiopental sodium (30mg/kg.bwt, i.v).

**Follow up procedures:**

The follow up procedures included the clinical, radiological, electrocardiographic, echocardiographic, laboratory/biochemical analysis, and histological/immunohistochemistry assessments were done. Animals were evaluated at several time intervals that are varied according to the method of assessment.

**Clinical assessment:**

Complete physical evaluation was performed daily during the first month after surgery then each week till the end of experiment (14).

**Radiological assessment:**

Sequential lateral thoracic radiography was performed at the following time; before and after operation (PO), 24 hours following operation, 7 days, 1, 3 and 6 months post operatively. Radiographs were then evaluated and compared with the previous ones (15, 16).

**Electrocardiography:**

Electrocardiography was carried out before and after operation using standard 12- lead ECG. Electrocardiographic monitoring was carried at various time interval following operation that included (2h, 24h, 7day, 1, 3, and 6 months). ECG was then analysed for abnormalities and compared with the previous ones.
Echocardiography:

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 similar to (17 and 18). All dogs were examined by two-dimensional and M-mode echocardiography before and after operation for detection of any abnormalities. Echocardiographic monitoring was carried out at various time intervals (24 hours, 7 days, 1, 3, and 6 months following operation). A 2-4 MHz microconvex transducer attached to an ultrasonographic machine (Samsung Medison, SONOACE-R3) was used. The following measurements were recorded; 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; EPSS: Mitral valve E-point to the ventricular septal separation; FS%: Left ventricular fractional shortening and EF:left ventricular Ejection Fraction. Measurements were expressed as mean±SD. Echocardiographic measurements and indices were analysed and compared with the previous measurements.

Laboratory and Biochemical analysis:

This included blood count to assess inflammatory response, troponin I and creatin kinase-MB fraction to assess myocardial damage obtained immediately before (baseline) and at one, seven and end days after cell/saline infusion.

Post-mortem examination:

Euthanasia was carried out at the previously determined intervals (3 and 6 months postoperatively) and the hearts were collected for gross examination. The area under ligation was thoroughly inspected before histological evaluation.

Histological and Immunohistochemistry assessments:

The target area was divided into 2 parts; one part was fixed in 10% formaldehyde for 48h and embedded in paraffin. Sections (5 μm) then stained with haematoxylin and eosin for qualitative histopathological analysis specifically targeted to assess for scar tissue, new blood vessels development, inflammation, infarction lesions, fibrosis and collagen deposition.

Molecular genes expression:

Total RNA was isolated from collected cultured human EPCs using Qiagene cells/tissue extraction kit (Qiagene, USA) according to instructions of manufacture. The purity (A260/A280 ratio) and the concentration of RNA were obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA). The extracted and purified RNA samples were subjected to RNase inhibitor at 37°C for 20 min and stored at –80°C for further use. Two μg RNA was reversed into cDNA using high capacity cDNA reverse transcription kit (#K1621, Fermentas, USA). The cDNA 25 μl master mix was prepared; first strand buffer (10x) 5µl, 10 mM dNTP’s, RNase inhibitor (40 U/µl), MMLV-RT enzyme (50 U/µl), and DEPC-treated water. RT mix was incubated for one hour at 37°C followed by inactivation of enzymes at 95°C for 10 min, and cooled at 4°C. qPCR was performed using an Applied Biosystem with software version 3.1 (StepOne, USA). cDNA including previously prepared samples (for VEGFR-2 and eNOS genes expression), internal control (for GAPDH gene expression as housekeeping gene), and non-template control (to assure absence of DNA contamination), were in duplicate. Each 25 μL of reaction mix contained 12.5 μL of SYBR Green (Fermentas), 1 μL of each primers (10 μmol/L), and cDNA (1 μg/mL) for sample determination. The thermal reaction was initiated by activation of Taqpolymerase at 95°C for 5 min, followed by 40 amplification cycles: 10 s denaturizing at 95°C, 50 s annealing at 59°C (VEGF-R2) or 61.2°C (eNOS). After the RT-PCR run the data were expressed in Cycle threshold (Ct) of assessed genes (VEGFR-2 and eNOS) and the house keeping gene (GAPDH). Therefore, Relative quantitation (RQ) of target genes expression was assessed and related to housekeeping gene by previously published method RQ= 2_ΔΔCt Sequence of primers was designed as shown (Table 1).
Table (1): Shows the primers sequence for the VEGF-R2 & eNOS genes:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Accession number</th>
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<tbody>
<tr>
<td>VEGF-R2</td>
<td>GATGTGGTTCTGAGTCCGTCT</td>
<td>CATGGCTCTGCTTCTTTTG</td>
<td>NT_022853.15</td>
</tr>
<tr>
<td>eNOS</td>
<td>ATTATATCTACACACACTCCAG</td>
<td>TCTTCAAGTTGCCCCATGTAC</td>
<td>NT_007914.15</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCTCTACTGGCCTGCAAAGCCT</td>
<td>GTCCACCACTGACACGTTGG</td>
<td>NT_009759.16</td>
</tr>
</tbody>
</table>

Statistical assessment:

The data were presented as mean±SD. Data were analyzed using SPSS 15.0 software (SPSS Inc.). ANOVA was used to determine significant differences between different groups. Significant differences were considered when P value <0.05.

RESULTS

Clinical evaluation:

The respiratory and heart rates 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-month interval, all dogs within the controlled group showed lethargy, weakness, decreased activities, deep inspiratory pattern. The above mentioned signs continued until six-month interval. No obvious clinical signs were noticed in treated dogs.

Radiology:

Sequential radiography revealed that one week following operation, hypervascularization; increase size and number of blood vessels with marked dilatation and tortuosity of vessels; was noticed. This radiographic signs disappeared in the following radiographs. Mild pleural effusion was noticed in 4 dogs (2 controlled and 2 treated). The plural fluid (transudation) disappeared within one month post operatively in all cases.

ECG assessment:

Two hours after ligation, a characteristic and prominent ECG change was reported which is an elevated ST segment. This elevation increased within the first 24 hours following induction of myocardial infarction (MI). In old MI, depressed ST segment with inverted T were noticed (Fig. 1).

Figure (1): Electrocardiographic (ECG) assessment of animals with AMI at different intervals (2 hours, 24 hours & 1 week) post induction.

In treated group, the ECG changes approximately started resolving one week following injection. One month post injection, the resolving of changes becomes more pronounced. Finally and by 3-month interval, ECG appeared normal that’s continued until the end of the study (Fig. 2).
Figure (2): Electrocardiographic (ECG) assessment of animals with AMI at different intervals (1, 3 & 6 months) post induction.

One month post injection, improvement of ST segment elevation
3-month post injection, the improvement became more pronounced
6-month post injection, the ECG appeared approximately normal

Echocardiographic assessment:

The infarcted part of the myocardium appeared thinner with marked decrease in its thickness when compared with the neighbouring part and appeared echodense than normal. Marked reduction in left ventricular wall motion toward the centre of the ventricular chamber during systole was noticed in both groups but marked improvement of systolic wall motion was noticed in treated group, starting at 1-month and continued till the end of experiment. Both FS% and EF showed gradual decrease that continued till the end of experiment. In treated group, marked improvement in fractional shortening and ejection fraction started almost one month post operatively. The improvement of both FS% and EF continued till 6-month where they became within normal values in dogs (Table 2 and 3).

Table (2): Mean±SD of echocardiographic measurements (cm) and indices for control group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Time</th>
<th>0</th>
<th>P.O</th>
<th>7 days</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd</td>
<td></td>
<td>4.3±0.7</td>
<td>4.32±0.4</td>
<td>4.33±0.3</td>
<td>4.33±0.5</td>
<td>4.35±0.6</td>
<td>4.38±0.6</td>
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<tr>
<td>LVIDs</td>
<td></td>
<td>3.01±0.5</td>
<td>3.00±0.4</td>
<td>3.30±0.4</td>
<td>3.80±0.6</td>
<td>3.82±0.4</td>
<td>3.98±0.4</td>
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<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>
<td>0.75±0.32</td>
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</tr>
<tr>
<td>LVWTs</td>
<td>1.41±0.11</td>
<td>1.43±0.12</td>
<td>1.36±0.12</td>
<td>1.38±0.21</td>
<td>1.37±0.21</td>
<td>1.33±0.31</td>
<td></td>
</tr>
<tr>
<td>EPSS</td>
<td>0.73±0.03</td>
<td>0.73±0.02</td>
<td>0.76±0.03</td>
<td>0.83±0.07</td>
<td>0.84±0.08</td>
<td>0.83±0.04</td>
<td></td>
</tr>
<tr>
<td>FS%</td>
<td>30</td>
<td>31</td>
<td>24</td>
<td>12</td>
<td>12</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>EF</td>
<td>58.21</td>
<td>58.32</td>
<td>47.73</td>
<td>26.63</td>
<td>26.51</td>
<td>20.27</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 significant difference when compared with normal (0 time)

Table (3): Mean±SD of echocardiographic measurements (cm) and indices for EPCs treated group.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Time</th>
<th>0</th>
<th>P.O</th>
<th>7 days</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd</td>
<td></td>
<td>4.3±0.55</td>
<td>4.3±0.62</td>
<td>4.5±0.22</td>
<td>4.4±0.48</td>
<td>4.4±0.77</td>
<td>4.3±0.61</td>
</tr>
<tr>
<td>LVIDs</td>
<td></td>
<td>3.34±0.51</td>
<td>3.32±0.62</td>
<td>3.92±0.33</td>
<td>3.60±0.33</td>
<td>3.54±0.26</td>
<td>3.42±0.51</td>
</tr>
<tr>
<td>LVWTd</td>
<td>0.80±0.06</td>
<td>0.82±0.04</td>
<td>0.83±0.02</td>
<td>0.84±0.06</td>
<td>0.82±0.21</td>
<td>0.85±0.03</td>
<td></td>
</tr>
<tr>
<td>LVWTs</td>
<td>1.44±0.19</td>
<td>1.41±0.06</td>
<td>1.39±0.31</td>
<td>1.38±0.32</td>
<td>1.42±0.21</td>
<td>1.43±0.09</td>
<td></td>
</tr>
<tr>
<td>EPSS</td>
<td>0.69±0.02</td>
<td>0.69±0.03</td>
<td>0.71±0.12</td>
<td>0.72±0.06</td>
<td>0.72±0.09</td>
<td>0.71±0.03</td>
<td></td>
</tr>
<tr>
<td>FS%</td>
<td>22</td>
<td>23</td>
<td>13</td>
<td>18</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>EF</td>
<td>45.30</td>
<td>46.09</td>
<td>27.83</td>
<td>37.93</td>
<td>40.38</td>
<td>42.08</td>
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</table>

*p<0.05 significant difference when compared with normal (0 time).

One week postoperatively, small amount of pericardial fluid was noticed in all cases where it appeared sonographically as accumulated anechoic fluid between the pericardium and cardiac wall. One month postoperatively, the accumulated pericardial fluid subsided in all cases except 2 dogs in both groups. At 3-
months postoperatively the pericardial fluid was within normal. The features of acute myocardial ischemia on echocardiograms include reduced wall or septal systolic thickening, dyskinetic wall motion, and systolic thinning.

**Laboratory and Biochemical analysis:**

24 hours after LAD ligation, CK-MB and Troponin I levels showed marked elevation indicating occurrence of myocardial infarction. One week after intramyocardial EPCs transplantation, CK-MB and Troponin I levels showed gradual decrease till obtaining their approximate normal values at 6-month post injection, (Table.4).

**Table (4): CK-MB and Troponin I levels in EPCs treated canines .Data were expressed as Mean ± SD**

<table>
<thead>
<tr>
<th></th>
<th>CK-MB U/L</th>
<th>Troponin I ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours after ligation.</td>
<td>58.50 ± 5.126</td>
<td>0.6117 ± 0.04071</td>
</tr>
<tr>
<td>1 week after EPCs infiltration</td>
<td>36.16 ± 2.021</td>
<td>0.1631 ± 0.01756</td>
</tr>
<tr>
<td>One month after EPCs infiltration</td>
<td>31.61 ± 1.266</td>
<td>0.1300 ± 0.01075</td>
</tr>
<tr>
<td>3 months after EPCs infiltration</td>
<td>26.73 ± 1.35</td>
<td>0.1243 ± 0.01721</td>
</tr>
<tr>
<td>6 months after EPCs infiltration</td>
<td>21.63 ± 1.29</td>
<td>0.1114 ± 0.01421</td>
</tr>
</tbody>
</table>

**PM findings:**

The hearts of dogs within the controlled group showed white area under the site of ligation. This white area was easily distinguished from neighboring areas. In EPCs treated dogs, it was difficult to distinguish the area below the site of ligation from surrounding cardiac tissue with presence of reddish patchy areas spread all over this area (Fig. 3).

**Figure (3): Gross examination of the hearts of both EPCs treated (a) and controlled (b) groups at 6-month following injection. White area under the site of ligation in controlled group while the same area in EPCs treated group appears approximately similar to neighbouring area.**

**Histological Assessment:**

In controlled group, signs of infarction were noticed as myocytic necrosis, in the form of empty cells (sarcologysis) were seen. Necrotic cells were mostly of the eosinophilic granular necrosis type with loss of central nuclei. Atrophy of myocytes surrounding necrotic zones was seen. Granulation tissue rich in fibroblasts and inflammatory cells (macrophages, lymphocytes and few PNLs). Congestion of blood vessels and haemorrhages were observed in the infarction area and nearby the intramuscular zones(Fig. 4). A necrotic muscle with wide zone of fibro-inflammatory tissue was also seen.
Figure (4): (a)-Eosinophilic necrosis (EN): myocytes with pink granular cytoplasm and lost nuclei H&E-400x; (b)-Necrotic myocytes are surrounded by edematous interstitial tissue (arrow) MT-400x, (c&d)- Rich in fibroblasts (FT) and some inflammatory cells.

In cell treated group, after 3-months from treatment, fewer amounts of fibrous tissue forming streaks were seen with increased number of vessels and prominent neovascularization. Atrophy of myocytes surrounding necrotic zones. Smaller amounts of fibrous tissue forming streaks of denser fibrosis between the muscle fibres compared to control. There was increased number of vessels, congestion and tissue haemorrhages. Many congested capillaries and congested large BVs with budding and branching indicating neovascularization of area and angiomatoids (interconnecting vessels) between the muscle bundles. Collections of dark oval small cells slightly larger than lymphocytes in a perivascular localization (EPC collections) were also seen (Fig.5 and 6).

Figure (5):(a)-Chords of endothelial cells forming new vessels between muscle fibers (arrows), H&E-x200; (b)-Collection of large blood vessels in a fibrotic zone (FT), MT-x200.

Figure (6):(a)-Congestion, hemorrhages and increased intramuscular vessels MT-x100; (b)-Many small new vessels of the capillary type surrounded by edematous fibrous tissue MT X200
In the control group, after 6-months from the operation, poor myocytic viability and prominent fat infiltration was seen as well as dense band like fibrosis. 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 (Fig.7 and 8).

Figure (7):(a)-Dense fibrosis, fibrofatty infiltration (FI) and necrotic (N) as well as atrophic myocytes (arrow) H&E-x400; (b)-Sarcolysis of myocytes (arrow) H&E-x200.

Figure (8): (a) Necrotic myocytes (arrows) and dense band like fibrosis (FT), MT-x200, and (b) 400.

Whereas, in the cell treated group, after 6-months from treatment, all tissues were viable and looked nearly normal except some atrophic areas. Increased vascularity was observed with no fibrosis, inflammation or
necrosis. Heart looks normal in appearance just mild atrophy of some myocytes particularly in the oedematous area around blood vessels which were probably ischemic. Increased number of vessels in the cardiac muscle and fibrosis was minimal similar to the control group (Fig. 9 and 10).

**Figure (9):** (a) Normal appearance of cardiac muscle No fibrosis, MT-x200; (b) Increased number of small intramuscular vessels surrounded by atrophic myocytes, H&E-x400

**Figure (10):** (a) Atrophic cells (arrows); (b) No necrotic non-viable muscle fibres around a vessel surrounded by oedematous tissue (H&E- x400)

**DISCUSSION**

Total coronary occlusion is not common in dogs and the absence of clinical signs in both controlled and treated group following the induction and may be attributed to the small area of infarction which resulted following ligation of LAD. At 3-month following induction, all dogs within the controlled group showed lethargy, weakness, decreased activities and deep inspiratory signs that continued until six-month following induction. Although myocardial infarction is not common in dogs but its experimental induction resulted in reduced systolic function with dyskinetic wall motion. This explained the signs appeared in controlled dogs (18,19).

No radiographic changes in cardiac size were noticed in both controlled or EPCs treated groups, as the heart appeared within normal size according to the vertebral heart score (VHS) (15) and cardiothoracic ratio (16). This may be attributed to localized nature of ischemic area following ligation of LAD. The other radiographic changes; vascular pattern or pleural effusion, may be related to the surgical interference but not related to the induced coronary occlusion as this signs appeared approximately with all thoracic interventions.

The ECG is the most important source for the early diagnosis of an acute myocardial infarction. Various ECG criteria have been proposed in the past as indicators for myocardial infarction during ventricular pacing (20). 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. (21).

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 controlled group, the ECG changes appeared more prominent and progressed by time. The ST segment elevation started within 2 hours after ligation and after 24 hours, more ST elevation was noticed. Old MI revealed depressed ST segment with inverted T- that noticed one month following induction. The elevation of ST segment above the baseline indicating 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. Hyper acute 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(19-21).

In treated group, the ECG changes approximately started resolving one week following injection. One month post injection, the resolving of changes becomes more pronounced. Finally and by 3-month interval, ECG appeared normal that’s continued until the end of the study.

On echocardiographic examination of both controlled and treated groups, at one month interval, the localized reduction of ventricular wall thickness could be clearly identified. This may be attributed to localized fibrotic changes at the area supplied by LAD. The area of infarction appeared echodense than normal (hyperechoic than neighboring part of the ventricular wall. This may attributed to the fibrotic changes occurred following infarction(17, 18).

The decrease in systolic thickening did not extend to the adjacent area in controlled group, while in treated group, the thickness of infarcted area increased gradually until obtaining approximately original thickness of the wall.

The FS % which an indicative of systolic function showed gradual decrease in both controlled and treated groups at one month interval. This may be a result of decreased systolic function. The decreased fractional shortening continued until the end of experiment in controlled group. In treated group, the fractional shortening increased gradually until retaining its normal values at 6-month interval. This may be attributed to improved systolic function in treated group manifested by improved ventricular contractility (22,23).

The ejection fraction for controlled dogs gradually decreased all over the study period but it gradually increased until obtaining its approximate normal range in treated group. This may be a result of improved ventricular contractility in treated group (18,24).

Gross examination of the heart after euthanasia revealed that, in controlled group a localized white area below the site of ligation which could be easily differentiated from the surrounding tissue was noticed. In treated group, the area below the site of ligation appeared reddish in color at 3-month interval. At 6-month interval, the infarcted area could not be differentiated from the surrounding structure. The color of area below ligation appeared nearly homogeneous with the surrounding tissue (25).

On histopathological examination, study demonstrated that intramyocardial transplantation of EPCs in canine model with AMI was associated with neovascularization and improvement of cardiac function. Our in vivo results suggested that injected EPCs into ischemic myocardium, improved myocardial function and increased vascularity. Histopathological cardiac tissue analysis of infarcted cardiac muscle transplanted with human EPCs showed many congested capillaries, congested large blood vessels with budding and branching indicating neovascularization in the area of infarction (11, 25)

In our study we detected transplanted EPCs were differentiated into cardiomyocyte-like cells within the fibrotic area of the infarction and identified by staining with a specific cardiac marker as TroponinI. Our results agreed with Badorff who stated that EPCs from healthy volunteers and CAD patients can transdifferentiate in vitro into functionally active cardiomyocytes when co-cultivated with rat cardiomyocytes. According to cell-to-cell contact but not cellular fusion mediates EPC trans-differentiation (26,27).
Autologous EPCs transplantation may help cardiomyocyte regeneration in patients with ischemic heart disease. A novel type of cell-to-cell communication between EPCs and cardiomyocytes in vitro was proved by further studies (28,29). This communication might enable EPCs through autocrine action to acquire a cardiomyogenic phenotype without permanent cellular or nuclear fusion (30, 31). EPCs can contribute not only to vasculogenesis but also to myogenesis in ischemic myocardium in vivo (31).

To detect homing, localization of transplanted EPCs and their role of in cardiac repair, gene expression of human VEGFR-2 gene and human eNOS gene were measured by Real-time qPCR on infarcted tissues & on adjacent infarct tissues in group1: EPCs-AMI treated canines. Quantitative gene expression of both human VEGFR-2 and human eNOS genes showed high significant difference between expressions on infarcted tissues compared to expressions on adjacent infarct tissues. Human VEGFR-2 and eNOS genes expression 373 was not expressed by QRT-PCR in group 2 canine heart tissues as they were not injected with human EPCs (11,25).

In conclusion, the intracardiac administration of EPCs enhances neovascularization after induced experimental AMI. Therefore, EPCs might be useful for cell therapy as potentially promote both neovascularization and cardiac muscle regeneration in ischemic heart disease. EPCs can be isolated from the human umbilical cord blood and trans differentiated into cardiomyocyte like cells in AMI experimental model. Large-scale studies should be induced to examine the potential effects of this novel approach on the risk of death and complications in patients with large acute myocardial infarctions and depressed left ventricular contractile function.

REFERENCES


