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Abstract

**Background:** This study evaluates the effects of *Ailanthus excelsa* Roxb methanolic extract (AER-ME) in rats induced with Myocardial Infarction (MI) followed by transplantation of MSCs.

**Material and Methods:** Rats were induced with MI by ligation technique of left coronary artery. The sham-operated the control and AER-ME treated group of rats received transplantation of PKH-26 and marked MSCs followed by normal saline and AER-ME treatment (200mg/kg/day of AER-ME extract) respectively for 30 days. Parameters such as cardiac function, inflammation, oxidative stress, apoptosis and differentiation of MSCs (angiogenesis) were evaluated. Histological studies of infracted myocardium revealed anti-inflammatory activity of AER-ME treatment.

**Result and Discussion:** Oxidative stress parameters revealed decrease in levels of malondialdehyde (MDA) and increase in superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHpx) activity significantly indicating antioxidant activity of the extract. There was a reduction in cell death rate of treated rats due to the decrease in apoptotic index with prolongation of MI when compared to both control and sham-operated groups. The expression of Fas protein was parallel to apoptotic index. The vascular density increased significantly in extract treated group. The treatment showed improved cardiac activity with decreased left ventricular end diastolic (LVEDP) and arterial pressure while the left ventricular end systolic pressure (LVEP) and dp/dtmax increased significantly when compared to both control and sham-operated groups respectively showing the protective effect of the extract as necessitated by the transplantation of MSCs. The study marked the protective outcomes of AER-ME treatment for MSCs in microenvironment of infracted myocardium by improving their viability and increasing differentiation into cardiomyocytes.

**Key words:** Mesenchymal stem cells, Myocardial Infarction, *Ailanthus excelsa* Roxb

Introduction

Ischemic heart diseases top the list of the growing cause of deaths in developing countries. Post infraction condition results in heart muscles being reduced to diminished capacity for self-renewal; hence restructuring itself with low left-ventricular activity Pfeffer et al. (1990). Myocardial infarction (MI) is associated with a loss of valuable cardiomyocytes and eventual gain of fibroblasts which contributes a major reason of heart failure. Studies have reported presence of precursor cells in heart, destruction of valuable cardiomyocytes in major leads to heart failure Oh et al. (2004). Treatment lines for MI include traditional drug therapies and surgical procedures to improve the blood perfusion to infracted cardiac muscles, restoring myocardium would be a choice of treatment. Recently treatments involving the use of growth factors for angiogenesis are being adopted VEGF and found to be a key responsible growth factor by increasing the differentiation of cells. (Heba et al. 2001; Cohen et al. 1994; Xie et al. 1997).

Mesenchymal stem cells (MSCs) originate from non-hematopoietic system. These cells are found in all tissues Da Silva et al. (2006) namely bone marrow, fat tissue, synovial membrane, muscles, placenta, amniotic fluid and umbilical cord Orbay et al. (2008). Chiefly found in bone marrow, MSCs have the ability to differentiate into variety of cells including nerve and vascular endothelial cells and reports also suggest their differentiation into skeletal muscles [Jiang et al. 2002]. MSCs have hence demonstrated promising role recently in Cell based therapies. Several studies are reported for differentiation of MSCs in-vitro and in-vivo. Reports indicating differentiation of MSCs in vitro in dexamethasone enriched media have been concluded Differentiation into osteo-cell lines and hence participation in bone formation (Bruder et al. 1997, 1998; Siddappa et al. 2007). Studies suggesting possibilities of MSCs playing pivotal role in regeneration of vascular tissues are said to play a key role in associated therapies such as impotence, disseminated sclerosis and conditions associated with spinal cord injury (Albersen et al. 2011; Steffenhagen et al. 2011). Regenerative cell therapies are making use of MSCs as cell sources for repairing damaged tissues, heart muscles and bone Chatterjea et al. 2010. Literatures suggested clinical trials directed towards the improvement of cardiac functions followed by myocardial infarction (Abdel-Latif et al. 2007; Wollert et al. 2004).

Although with MSCs playing a key role in cell mediated therapies of MI, the clinical application of MSCs still remain under concerns due to their poor viability into the destructed myocardium post-implantation. Studies suggested MSCs died within 1 week after transplantation into infracted myocardium environment in human heart Toma et al. 2002. This report concludes the unsuitability of infracted myocardium for survival of MSCs and there further differentiation into cardiomyocytes which either could encourage cardiac function in the acute phase of infraction (Dong et al. 2006; Piao et al. 2005; Kamihata et al. 2001). Hence for a MI therapy followed by MSCs transplantation, the success relies on increasing the survival rate and there further differentiation.

*Ailanthus excelsa* Roxb is tall and leafy tree widely distributed in world with prevalence in countries like Japan, China and regions of Australia. The bark AER is found to contain sitosterol, Quassinoids and Atlantic acid Kumar et al. (2010). The bark of AER finds place in ancient Chinese medicine for treating alignments such as diarrhea and dysentery associated blood in stools Chopra et al. 2006; Chopra et al. 1997; Chopra et al. 1996.
As far as the use of traditional drug therapies associated with chronic myocardial infarction are concerned, studies have shown synergistic role of Angiotensin Converting Enzyme inhibitors (ACEi) in expression of VEGF in process of angiogenesis in myocardial infarcted rats treated with MSCs cell lines. Studies have concluded role of angiotensin system in angiogenesis and growth factors. There are few reports correlating link between role of ACEi and expression of VEGF in process of angiogenesis Dan-Yang et al. (2004).

The goal of present study was to evaluate possible working mechanisms for AER-ME on transplanted MSCs in rats subjected to infarction. The study would also establish experimental evidences for the use of this flavonoids enriched methanolic extract as a supplement during cellular therapy of MI.

Materials and Methods

Chemicals

For the experiments, reagents and diagnostic kits involved in the study include Fetal bovine serum (FBS), penicillin and streptomycin, Low-Dulbecco’s modified Eagle’s medium (L-DMEM), trypsin-EDTA, were procured from Gibco-BRL (USA); oxidative stress measuring kits for assay of MDA, SOD, CAT and GSHpx were obtained from Nanjing Jiancheng Bio. Ins. (China). The methanolic extract of Ailanthus excels (Roxb) was produced in own lab from leaves of the same using a soxhlet assembly. The Cell Death Detection kit (In situ) was procured from Nanjiang Bio. Ins. China; Antibody used in the study Fas was obtained from San Diego, CA, USA (Antibody Design Labs) remaining chemicals obtained from Sigma (USA).

Animals

For the study, male Sprague-Dawley rats weighing between 250 ± 20 g were supplied by Shanghai Medical College from the Department of Experimental Animal Center (Shanghai, China). Animals were kept in polyethylene cages supplied with adequate food and water and maintained as per the guidelines given by institutional ethical committee.

Isolation and Culture of MSCs

The process started with the collection of bone marrows from tibias and femurs of male Sprague-Dawley rats. The MSCs were separated by method described by Yan-Li Liu et al [27] in which femurs were injected by a needle to collect the marrow under strict aseptic conditions. After collecting the marrow the cells were subjected to repeated washing by L-DMEM. After washing cells underwent centrifugation in a cooling centrifuge at 1500 x g for 20 min followed by resuspension in DMEM medium enriched by 10% FBS, 100 U/ml penicillin, 2 mM/L L-glutamine followed by 100g/L streptomycin. The whole mixture was subjected to seeding into flasks. After 24 hr the non-adherent free cells were removed by switching the culture medium after 48 and 72 hr.

After MSCs had grown to 80% obtained cells were plated again in two separate new flasks. Preparation of subculture was done by re-suspending the cells in mixture of 0.25% trypsin + 0.02% EDTA and tagged with 4′6-diamino-2′-phenylindole (DAPI, Roche Diagnostics) before implanting them in the experiment.

Model for Myocardial Infarction and Transplantation of MSCs

The MI model was created by ligation technique of left coronary artery in Sprague-Dawley rats according to the process described by Pouzet et al. (2000). Pentobarbital sodium (45 mg/kg Intrapertioneally (IP)) was used to anesthetize rats which were ventilated tracheally by normal environmental air using a ventilator (CIV 101, Colombus Instruments, Colombus, OH). The heart was operated for left thoracotomy, the procedure for Ligation of coronary artery was carried out 1-2 mm directed away from the line between the left and the right border of the pulmonary conus and left atrial appendage respectively. Echocardiography (ADI Instruments, Houston, USA) was done to confirm MI by monitoring the ejection fraction of the ligation operated rats. The Ligated rats were transplanted with 200 μl of suspension bearing a total sum-up of 1x10^7 MSCs which were tagged with a cell tracker dye PKH-26 red fluorescent cell (Red Fluorescent Cell Linker Kit; Sigma) injected in four different sites surrounding infarcted myocardium using a tuberculin injection. The animals underwent surgery for closure of chest cavity followed by transplantation of MSCs. Grafted rats underwent recovery in a chamber under controlled temperature conditions until were found reasonably alert and mobile. Animals were then shifted to cages.

Rats were divided into three groups as sham-operated, control and AER-ME treated groups (n=18). The sham-operated group rats were subjected to MI infarction by ligation but were not transplanted with MSCs cell cultures. The control group received MSCs transplantation followed by 3 ml normal saline solution (0.9% Sodium chloride IP) and AER-ME treated post MSCs transplanted group received 200 mg/kg/day of methanolic extract IP. The treatment regimens were split into two IP injections/day. After 1, 7 and 30 days of MSCs transplantation and respective treatments hearts of six rats from each group were surgically removed and fixed using 4% neutral formaldehyde for further studies.

Assessment of Cardiac Activity

After MSCs transplantation cardiac function was measured as per the protocol described by Yan-Li Liu et al (2014). After 30 days sham-operated, control and AER-ME treated group rats (6 from each group); cardiac activity of animals was monitored using a physiological recorder (J. Tongyong Indu. Co., Ltd., China). Rats were anesthetized using Ketamine 100mg/kg and Xulazine 1.5 mg/kg after recording the weights of each subject carefully followed by application of transducer (Jinjiang T. Ind. Ltd. China) on the thorax.
region (Left). The visualization of heart (left ventricle) was done by viewing axially opting a 2-dimensional mode.

A cannula was inserted inside the left ventricle. Parameters such as LVEDP, LVESP, left ventricular ± dp/dt (maximum rate of pressure rise) and arterial pressure were calculated using EC Toolbox software (Emory University, USA).

Histopathological Examination

All the animals treated for 1 and 30 days of treatments of normal saline and AER-ME were sacrificed by a high amount of anesthesia. The sacrificed rats were operated and cardiac tissues of the left ventricle were recovered and subjected to fixation process in 4% p-formaldehyde (for in situ perfusion fixation) by inserting a needle in the left ventricle via left atrium. The recovered tissues of myocardium were fixed in wax paraffin and were sliced into sections of 5 μm for histological studies. Staining of sections was done by hematoxylin and eosin dies (H&E) as per the instructions given by the manufacturers (Beyotime Bio. Inst.China). Stained sections were subjected to microscopic analysis at 100x and 400x.

Determination of Oxidative Stress Markers

For the study the rat models for MI divided into three group treatments were used. Sham-operated, control and AER-ME group rats were sacrificed after 1, 7 and 30 days of treatments using high dose anesthesia. Animals were operated along the greater curvature for removal of heart and washed with phosphate buffer saline (PBS). The heart was homogenized using a tissue homogenizer and then the homogenates were subjected to centrifugation (10,000 rpm for 15 min), the supernatant of heart tissue homogenates were subjected to oxidative stress assay for antioxidant enzymes using kits for measuring activity of GSH-Px, CAT and SOD. Marker for lipid per oxidation MDA content assays were done using kits, all the protocols were done according to the procedure given by the manufacturer.

In Situ Cell Death Detection and Histochemical Analysis

The tissue sections from animals were subjected to Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay using cell death detection kit (Nanjing KeyGen Biochemical Institute, Nanjing, China). The procedure followed was as per the manufacturer instructions. The stained tissue sections were observed by conofocal microscope (Leica, Germany). AI was computed by dividing count of TUNEL-positive cells with that of transplanted PKH-26 pre-labeled cells.

Vascular density studies were carried in 6 animals from each group; animals were sacrificed on day 30 after transplantation of MSCs followed by respective treatments. The sections were subjected to hematoxylin and eosin staining. Counting of capillary vessels was done in zone of tissues subjected to infraction. Angiogenic effect followed by MSCs transplantation was assessed by making capillary vessels count within the infacted zone under light microscopy at 400 x magnification for five different regions in the infacted area in both the groups and results were represented as the average of capillary vessels/0.2 mm²area.

Study for Expression of VEGF Protein

Western blot analysis was done using polyclonal antibodies of rabbit against VEGF (Zymed lab.Co.). The myocardial tissues of rats were homogenized in mixture of 0.1 % Tween-20 buffer and protease inhibitor. Protein of volume 50 μg was transferred to tissue containing buffer and was bundled on a 7.5 % sodium dodecyl sulfate-polyacrylamide gel followed by blotting using a polyvinylidene fluoride membrane. After 2 hr waiting time the membrane was transferred with primary antibody (1:200) and incubated. The membrane was then incubated with horseradish peroxidase labeled with a secondary antibody diluted at a ratio of 1:1000. Densitometric analysis revealed formation of positive protein bands obtained on exposure to di-aminobenzidine. Quantitative analysis of myocardial tissue for VEGF was calculated by performing enzyme immunoassay. The VEGF levels were quantified similarly 7 days after the culture of transplanted MSCs Xu et al. (2005).

Statistical Treatments

All results are described as average SEM and were calculated using statistical software (GraphPad Prism 5). P values were calculated using the data.

Results

Characterization of Mscs for Presence of Surface Markers

The cultured MSCs were found positive for mesenchymal markers including CD44, CD105, and CD90 and did not express surface markers for hematopoietic stem cells i.e. CD45, macrophages CD14 and markers for lymphocytes CD34.

Assessment of Cardiac Function Followed by AER-ME Treatment

The sham-operated, control and AER-ME group rats (n=6) were assessed for variety of parameters of echocardiography followed by analysis of hemodynamics. Both the AER-ME and control groups demonstrated decreased left ventricular end diastolic pressure (LVEDP) and Arterial pressure (systolic and diastolic) while the LVESP and dp/dtmax increased showing protective effect of
MSCs treatments. Results when compared to control group the AER-ME group showed significant improvement in cardiac function parameters proving ameliorating effect in MI rats. The results are presented in Table 1.

### Effect on MI-Induced Heart Inflammation

Histopathology of myocardial tissues subjected to H&E staining, the images showed MI induced changes in the myocardium makeable by the presence of mononuclear cells, eosinophils, macrophagocytes, polymorph nuclear neutrophils and fragments of necrotic myocardial fibers. When compared to group supplemented with AER-ME followed by MSCs transplantation after 30 days, reduction in number of inflammatory cells was observed in the stained sections (Fig. 1).

Table 1: Results of cardiac function, data are produced as the mean ± SEM for n=6. *P<0.005 compared to sham-operated day 1, †P<0.005 compared to day 1 rats, ‡P<0.05 compared to sham-operated and control groups.

| Groups                  | Day-1          | Day-30        |
|-------------------------|----------------|---------------|
|                         | Sham-operated | Control       | AER-ME Treated | Sham-Operated | Control | AER-ME Treated |
| LVEDP (mmHg)            | 11.2±0.46      | 9.1±0.33      | 9.3±0.40      | 12.9±0.68      | 8.3±0.45      | 7.9±0.23      |
| LVESP (mmHg)            | 101.3±2.93     | 88.9±2.33     | 90.1±2.98     | 92.3±2.2       | 102±2.44     | 110±3.16      |
| dp/dtmax (mmHg/sec)     | 5960±75.3      | 6167±93.4     | 6250±69.52    | 5530±57.8      | 6650±81.5    | 8430±57.74    |
| Arterial pressure (systolic) (mmHg) | 126±5.77      | 102±3.01      | 107±2.92      | 155±6.45       | 110±2.89     | 116±2.78      |
| Arterial pressure (diastolic) (mmHg) | 102±2.89      | 88±3.15       | 95±2.7        | 112±2.92       | 93±3.01      | 89±3.01       |

Figure 1: Anti-inflammatory effect using H&E staining images of cardiac muscles (A) The sham operated group showing necrosis of myocardial tissues, signs of infiltration of neutrophils. (B) Control group rats showing healing of necrosis of myocardial tissues (C) AER-ME treated group shows decreased tissue necrosis and infiltration of neutrophils.

### Anti-Oxidant Effects of AER-ME on the Heart Tissue Homogenates

For the study AER-ME, control and sham-operated group rats were sacrificed (day 1,7 and 30) and cardiac tissue homogenates were evaluated for levels of SOD, CAT and GSH-Px and MDA. Results demonstrated significant antioxidant potential of MSCs treatments in both the treated groups showing an increase in activity levels of SOD, CAT and GSH-Px and decrease in levels of Malondialdehyde a lipid per-oxidation product as compared to sham-operated rats. The results demonstrated a synergistic effect of AER-ME treatment on oxidative stress markers, the results were significant (p<0.05) and are presented in Figure 2.
Effect of AER-ME Extract on MI-Induced Mscs Apoptosis

The MI induced rats were transplanted with PKH-26 labeled MSCs cells into the infarcted region. The apoptosis of cells was identified by TUNEL assay staining positive with green. The images by confocal microscope showing PKH-26 (red color) and TUNEL (green color) which suggested programmed cell death post implantation of MSCs cells (figure 3). Results demonstrated apoptotic index (AI) significantly decreased in AER-ME treated rats (P<0.001) against control and sham-operated group of rats. After transplantation of MSCs the AI index increased in all the three groups when analysed on day-1 and day-30. The results are presented in figure 4.

Evaluation of Angiogenic Effect (Vascular Density Studies)

Vascular density studies were done to mark the angiogenic effects. Microscopic evaluation was done by light microscope (magnification, 400×) for sections stained with hematoxylin and eosin. Counting of capillaries in infarcted zones revealed differentiation of MSCs into new capillaries in the ASE-ME and control groups which were subjected to infraction followed by MSCs transplantation, while the sham-operated group showed no evidence of angiogenesis and also showed a higher death rate. The group of rats supplemented with AER-ME treatment (for 30 days) presented results having vascular density significantly higher (P<0.001) versus the control group rats, suggesting a synergistic role of the AER-ME supplementation. Examination of peri-infracted area do not exhibited a variation in vascular density in both the groups. The results of vascular density are shown in figure 5.
Figure 4: Results of AER-ME treatment on AI in sham-operated, control and AER-ME treated groups. *P<0.005 against respective sham-operated group, $P<0.005$ against respective control, $P<0.005$ against respective sham-operated group, @P<0.005 against respective control.

Analysis of VEGF (Western Blot Analysis)

Results of Western blot analysis (figure 6) showed increase in the VEGF protein levels 7 days after MSCs transplantation in both the control and AER-ME treated groups. It was found that levels of VEGF decreased in sham-operated rats. The enzyme immunoassay (ELISA) suggested levels of VEGF increased significantly in AER-ME treated rats as compared to control group rats. Results are presented in figure 6.

Figure 5: Results of Vascular density analysis in sham-operated group, control group and AER-ME treated group of rats. *P<0.001 against sham-operated, $P<0.001$ against control.

Figure 6: (A) Results of Western blot for expression of Fas protein (B) Quantitative study of myocardial tissue contents of VEGF. AER-ME treated group *P<0.001 compared to sham-operated and @P<0.001 against control.
Discussion

Therapy involved with grafting of MSCs have found to improve heart functions in infarcted animal models, the property is contributed by differentiation of MSC into myocardial cells. Differentiation is however required further for improved cardiac functions specifically in infarcted zones of myocardia. The present research examined role of methanolic extract of Ailanthus excelsa Roxb (AER-ME) which was reported to have flavonoids. The study indicated MSCs transplantation in myocardial infarcted rats followed by treatment of AER-ME ameliorates the cardiac functions in MI induced rats. This also confirms partial protective effect of AER-ME for the transplanted MSCs against the MI caused apoptosis and also the treatment of extract promotes the differentiation of grafted MSCs into cells of myocardia.

Literatures confirm presence of six flavonoids in methanolic extracts exhibiting ACE inhibitory and anti-inflammatory activity, the extract is documented to treat asthma and ulcers also Kumar et al. (2010). Literatures also support role of ACE inhibitors in expression of VEGF proteins in process of angiogenesis in myocardial infarcted rats gifted with MSC Izuagie et al. 2015. Present study confirmed that AER-ME increases the expression of VEGF proteins and thereby enhancing survival and differentiation of transplanted MSCs into myocardial cells in infarcted rats. The study concluded that supplementation improved cardiac functions as compared to untreated group of rats.

Induction of MI in rat models is often associated with cardiac inflammation; studies have confirmed that inflammation remains one of the main causes for death of grafted MSC in treatments associated with MI. Present study confirmed treatment of AER-ME significantly brought down the infiltration of inflammatory cells into the infarcted zone of myocardium confirming its established anti-inflammatory activity.

The treatment of extract confirmed the antioxidant property which was consistent with other studies performed in vivo and in vitro Kumar et al. (2010). The extract treated groups established significant antioxidant effects by showing enhanced activity levels of enzyme SOD, CAT and GSHpx and reduced levels for MDA. Study responded well with time and showed significant results after 30 days of treatments.

The study further confirmed protective effects of AER-ME against infract induced apoptosis of MSCs in the process of implantation in rats. The TUNEL-positive rate of the MSCs decreased significantly in rats subjected to AER-ME treatments and was inversely proportional with treatment time of AER-ME in rats.

The Fas protein expression studies were carried to determine the possible pathway of cell apoptosis. The studies concluded that treatment of AER-ME significantly down regulated the manifestation of Fas protein and was found to be inversely proportional to treatment regime of the AER-ME. Hence the studies partially confirm role of Fas signaling pathway as possible mechanism behind cell apoptosis, however further studies are required for a clear mechanism.

Evaluation of cardiac functions in infarcted rats treated with AER-ME extract reveled improved cardiac functions. The vascular density increased significantly in both control and AER-ME treated rats gifted with MSC as compared to sham-operated rats, however the extent of angiogenesis was significantly high in AER-ME treated rats confirming a synergistic role of extract in angiogenesis.

The results of present work revealed protective role of AER-ME on MI which was followed by transplantation of MSCs. The study also established protective role of AER-ME extract for transplanted MSCs against the MI caused apoptosis. The study also confirmed Fas arbitrated pathway for cell apoptosis. Study established role of AER-ME against inflammation in infarcted zones of myocardium. The results suggested that treatment reduced oxidative stress and also increased expression of VEGF protein and hence increased vascular density. The study proves promising role of AER-ME extract in MSC grafted MI therapy. However presence of six flavonoids in methanolic extract of Ailanthus excelsa Roxb increases need of further investigation with detailed mechanisms of specific flavonoids involved.

The present study concludes and evidences that AER-ME is able to ameliorate the cardiac function in myocardial infarcted rats transplanted with MSCs. Study also established that treatment of AER-ME increased the endurance and differentiation of transplanted MSCs; the treatment also attenuated inflammation in tissues and reduced oxidative stress in the myocardial tissues of MI induced rats.

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