

Original Article

Establishment of a rat model of myocardial infarction with a high survival rate: A suitable model for evaluation of efficacy of stem cell therapy

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Abstract

The most common rat model of myocardial infarction (MI) is by ligation of left anterior descending (LAD) coronary artery but it is associated with high mortality and large variations in the infarct size. We evolved certain innovations/modifications in the existing technique including immobilization of the heart without exteriorization, identification of the LAD by pressing it proximal to the site of ligation by an ear-bud, and subsequently its ligation 8 mm from its origin, no touch technique of the lungs during surgery, removal of air from the chest cavity prior to its closure using an in-house tubing, and deflation of the lungs before extubation. We induced MI in 24 Sprague- Dawley (SD) rats using these modifications and carried out post-MI evaluation of hemodynamic parameters, serum cardiac enzymes and histological studies upto 90 days using 13 sham operated and 3 healthy SD rats as controls. Three of the 24 rats (13%) died <24 hours of MI, but thereafter no mortality was observed till the follow-up period of 90 days. The infarct size was consistent in all the rats (21±4% of left ventricular area). This model with low early and no long-term mortality may be suitable for studying efficacy of stem cell therapy in MI, where a follow-up of at least 13 weeks is required to assess myocardial regeneration.

Key words; Rat Model, Myocardial Infarction, Innovations in LAD Ligation, High Survival Rate, Stem Cell Therapy

Introduction

Regeneration of myocardium by local or systemic injection of stem cells is an emerging promising therapy for myocardial infarction (MI) and a number of pre-clinical studies have been conducted in this area ^[1,2]. However, a standard protocol for stem cell therapy of MI is yet to be established because the data obtained is variable and several issues like the most appropriate stem cell type, the safety of this cell type, optimal dose, route of delivery and timing after MI for injection, still remain to be addressed for which a suitable animal model is needed.

We have previously successfully induced MI in cats and rabbits ^[3,4] and other groups in rodents, dogs, and primates ^[5], but the rat model of MI has dominated because of the convenience in handling, housing and the lower cost involved in their experimentation ^[6]. The most common procedure used for induction of MI in these animals is ligation of left anterior descending (LAD) coronary artery. However, the mortality is high, ranging from 27.67% and the success rate of MI is also low (49%) with large variations in infarct size (4-59%) ^[7,8,9,10,11]. In the present study, we have developed a rat model of MI, with high survival rate and a consistent infarct size by modifications and innovations in existing surgical techniques for LAD coronary ligation. Post-MI, we have studied hemodynamic, biochemical and histopathological parameters at different time points for up to 90 days, to use this model for evaluation of myocardial regeneration after stem cell therapy.

MATERIALS AND METHODS

Induction of MI

Twenty four male SpragueDawley (SD) rats ranging from 6 to 8 weeks in age and weighing 180-250 g were selected for inducing MI by LAD ligation. All procedures involved were approved by the

Institutional Ethics Committee for Use and Care of Laboratory Animals. Anesthesia was induced and maintained in animals with an intra-peritoneal injection of 80mg/kg ketamine and 10mg/kg xylazine. After anesthesia, the animals were intubated and ventilated with a 16 gauge intravenous catheter and were placed in a supine position on a temperature control pad. Left-sided thoracotomy was performed by a small incision between the third and fourth intercostal spaces. The incision was expanded by a blunt ended retractor in such a manner that the lungs are avoided in the area of retraction. The pericardial sac surrounding the heart was cut open to access the heart. The heart was not exteriorized; once the site of ligation of left anterior descending coronary artery (LAD) had been determined 8 mm away from the origin, a cotton ear bud was used to gently press on the artery a little below the site of ligation, immobilizing the heart and also making the artery prominent and easy to identify. Using a tapered atraumatic needle a 6-0 silk ligature was passed underneath the LAD and tied with three knots. Visible blanching and cyanosis of the anterior wall of the left ventricle and swelling of the left atrium were indicative of successful ligation. Ribs and muscles were closed using 6.0 vicryl dissolvable sutures leaving a small gap to aspirate air left in the chest cavity. The air was aspirated by an in-house tube (2 mm diameter), commonly used for endotracheal intubation of rabbits, in a manner that the lungs are not touched. At the time of closure neomycin powder and betadine were applied onto the muscle and skin stitch sites, respectively. Before extubation, the lungs were deflated by placing the outlet tube connected to the endotracheal tube in an under-water seal. The surgical site was dressed daily to avoid any infection and to monitor for any dehiscence of the suture site. In 7 to 10 days the sutures were removed.

The entire procedure was performed with in 20 min after induction of anesthesia. Thirteen sham operated rats underwent the same procedure but with out any ligation.

Post- MI evaluation

The evaluation of hemodynamic and biochemical parameters, and of histopathological changes were done in the MI and sham operated rats, as given below, on days 1,7,14,21,32,64 and 90, in groups of 3 rats at each time point.

Hemodynamic parameters

The blood pressure, heart rate and ECG were evaluated essentially according to the method of described ⁽¹²⁾. Rats were anaesthetized with ketamine and xylazine as above and their carotid artery was exteriorized. A catheter filled with 10 IU/ml of heparinized saline was inserted into the artery and then connected to pressure transducer lined with a data acquisition system (Biopac system, USA) to record the blood pressure and heart rate of the animals at different time points of the follow-up. ECG was also recorded under anesthesia using standard lead II (Biopac system, USA).

Biochemical tests

The animals were sacrificed at different time points by administering an overdose of ether. The creatinine kinase MB fraction (CK-MB) and lactate dehydrogenase (LDH) levels were measured in the serum obtained from the heart blood, by spectrophotometer using commercially available kits (Merck Diagnostics, India).

Assessment of Infarct Size

To evaluate the infarct size, triphenyltetrazolium chloride staining (TTC, SISCO Research Laboratories, India) staining was performed in control rats and in those with induced MI. The hearts of the animal were removed and after rapid washing in cold water, they were frozen at -20°C for 60 minutes. The frozen hearts were cut transversely into 10 mm thick sections.

The sections were stained with TTC by placing them in pre warmed 1% TTC solution in phosphate buffered saline (pH 7.2) and incubating at 37°C for 30 min in dark. The TTC stained sections were fixed with 10% formaldehyde and infarct size was measured by custom free Image-J analysis software of NIH ^[9].

Histopathological Changes

Infarcted hearts tissues obtained at different time-points were fixed in 10% formalin and serial sections of 5 mm were stained with hematoxylin and eosin (Glaxo Smith Kline, India) to evaluate sequential histopathological changes.

Statistical Analysis

The results were calculated as mean \pm SD. The difference between healthy and experimental animals (MI & sham operated rats) was evaluated by One-Way ANOVA test. P value of <0.5 was considered to be significant.

Result

The induction of MI was successful in all the 24 rats and the procedure was completed within 20 minutes. Three rats (13%) died within 24 hrs of the procedure but there was no mortality in remaining 21 rats till their sacrifice at different time points up to 90 days, leading to an overall survival rate of 87% by this procedure. In the sham operated control group 1 out of 13 (8%) rats died within 24 hrs of the procedure and thereafter there was no mortality. There was no significant difference in mortality between the MI induced and sham operated group of rats ($p < 0.001$).

The hemodynamic parameters showed a significant decrease in blood pressure from day 1 and of heart rate from day 7 onwards after induction of MI up to 90 days (Table 1). ECG recordings immediately after induction of MI showed significant ST segment elevation.

Table 1

Hemodynamic parameters in healthy and of MI induced rats at different time points.

Groups	Time points	Heart rate (BPM)	Blood Pressure (mmHg)
MI group	Day 1	370±12	89.34±1.29***
MI group	Day 7	361±11*	82.31±1.23***
MI group	Day 14	353±11**	79.16±1.26***
MI group	Day 21	349±05**	75.00±1.38***
MI group	Day 32	345±10**	72.09±1.50***
MI group	Day 64	335±15***	71.09±1.10***
MI group	Day 90	321±13***	70.82±1.21***
Healthy Control	Day 0	400±20	120.00 ±2.57

Table 1: Heart rate (beats per minute: BPM) and blood pressure (mmHg) in healthy and of MI induced rats at different time points. Values are mean ± SD of 3 rats. *p<0.05, **p< 0.01, ***p< 0.001 (MI induced rats vs. healthy rats).

Serum levels of CK-MB and LDH began to rise on day 1, reached their peak levels at day 7 after MI, and then declined gradually and normalized by day 21. In sham operated rats serum levels of CK-MB remained normal for the entire period of follow-up, whereas LDH in sham operated rats significantly increased on day 1 but normalized from day 7 onwards (Figs 1 and 2).

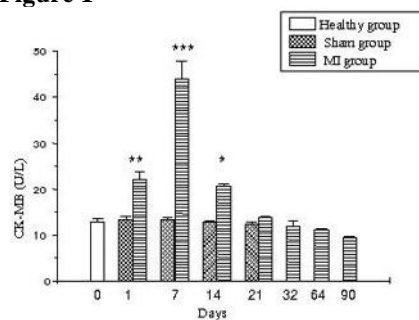
Figure 1

Fig1. Serum levels of Creatinine Kinase-MB (CK-MB) in healthy rats, sham operated rats and MI induced rats at different time points of follow-up. Values are mean ± SD of 3 rats *p<0.05, **p< 0.01, ***p< 0.001 (MI induced or sham operated rats vs. healthy rats).

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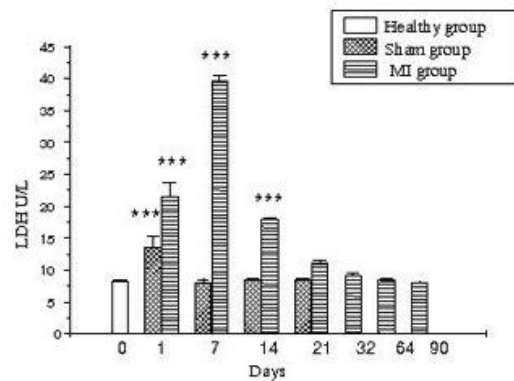
Figure 2

Fig 2. Serum levels of Lactate Dehydrogenase (LDH) in healthy rats, sham operated rats and MI induced rats at different time points of follow-up. Values are mean ± SD of 3 rats *p<0.05, **p< 0.01, ***p< 0.001 (MI induced or sham operated rats vs. healthy rats).

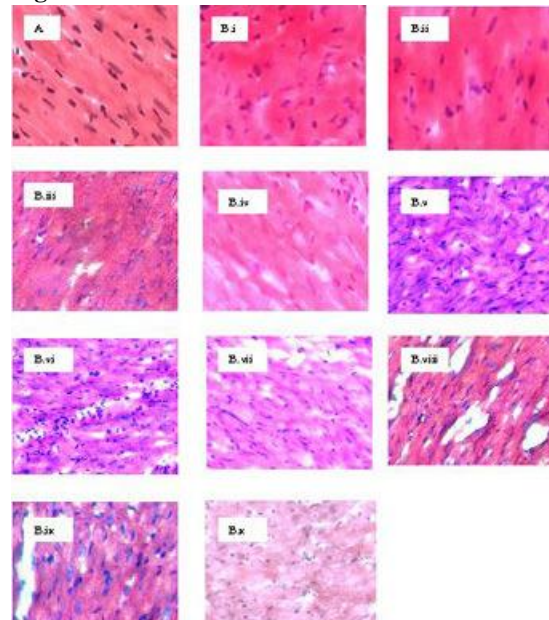
Figure 3

Fig 3. Representative heart sections stained with H&E (X400) of (A) healthy rats showing centrally located nuclei in intact myocytes and (B) of MI induced rats showing the following sequential histopathological changes after MI: (B.i) nucleomegaly and (B.ii) loss of cross striations in myocytes at 3-6 hours; (B.iii) loss of cardiomyocyte boundary after 24 hours; (B.iv) infarct zone after 24 hours; (B.v) wavy fibers and (B.vi) neutrophilic infiltration at 3 days; (B.vii) degeneration of myocytes and cellular swelling and (B.viii) early granulation tissue with formation of fibroblasts, fibrocytes and capillaries at 1 week; (B.ix) granulation tissue with early scar formation at 2 weeks; (B.x) granulation tissue with dense scar formation at 3 weeks.

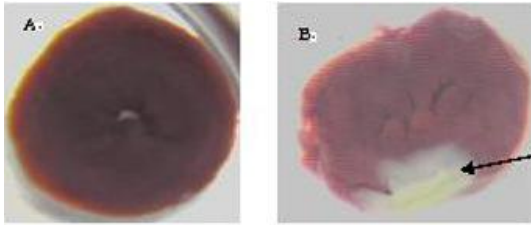
Figure 4

Fig 4. Triphenyl Tetrazolium Chloride stained cross-section of the heart of (A) Healthy rat showing uniform staining pattern (B) MI induced rat, with the arrow showing a pale, TTC negative area of infarct.

The histopathological examination of the heart of rats with MI at 3-6 hours showed nucleomegaly and loss of cross striations of cardiomyocytes and at 24 hrs a loss of cardiomyocyte boundary with formation of an infarct zone. At 3 days there was infiltration by acute inflammatory cells including neutrophils and wavy fibers. Degeneration of cardiomyocytes, early granulation tissue with capillary formation and infiltration by some fibroblasts was seen at 1 week and by 2 weeks early scar formation was evident. Dense scar formation was observed at 21 days (Fig. 3).

The TTC staining in MI animals showed a mean infarct size (TTC negative myocardial area) of $21 \pm 4\%$ (range 17% to 25%), while healthy and sham operated animals had uniform deep red colored TTC staining throughout the myocardium (Fig 4).

Discussion

We have developed a rat model of MI with successful induction of MI in all rats and a consistent infarct size measuring $21 \pm 4\%$ of left ventricular area and a survival rate of 87%. The early mortality (<24 hrs) in MI induced rats (13%) was similar to that observed in the sham operated rats (8%; $p > 0.05$), showing that the mortality in the model was not per se related to the induction of MI.

The ligation of LAD coronary artery is the most common method for induction of MI in rats and other small animals. It is being widely used since 1954, when Johns and Olson et al^[7], described it for the first time. However, a high mortality ranging from 27.67% remains a limiting factor of this method^[7, 8, 9, 10]. The standard operating protocol of St. Vincent's Health Center, Australia, for establishment of rat models of MI (www.svhm.org.au) has also shown a mortality rate of 15 to 30%. We observed during standardization that the mortality associated with this method could be dramatically reduced by certain modifications in the existing technique.

Firstly, the site of ligation the LAD is very critical. The LAD is usually ligated close to its origin because of its clear visibility at this site and surgical ease. However, this results in large sized infarcts and hence high early mortality. Further, it has been shown that rats with infarct sizes greater than 46% of left ventricular area developed elevated filling pressures, reduced cardiac output, minimal response to pre- and post-load stress and finally died due to congestive heart failure after 21 days^[10]. We ligated the LAD 8 mm away from its origin by gently pressing the LAD by an ear bud near the site of ligation to make it prominent proximal to the point of pressure. Ligation at this site resulted in infarct sizes ranging from 17 to 25% and low early mortality of 13% and no long-term mortality. An important feature of our model was a minimal variation in the infarct size. It has been reported that large variations in infarct size (4-59%) occur mainly due to improper ligation^[10]. Thus in our model the method and site of ligation was consistent in all the animals.

Secondly, exteriorization of the heart and its manual handling to immobilize it, affects the mortality. This causes compression of the heart leading to severe hemodynamic disturbance, mechanical trauma to the heart/lung and ischemic injury to both the heart and brain. Moreover, by this method,

the safe time for immobilization of the heart is very limited and thus the LAD suture has to be placed very quickly, which is likely to lead to misplacement in site of the suture, and may result in inconsistency in infarct size.

Thirdly, we reduced the mortality by judiciously preventing any trauma to the lungs by avoiding any manual touching of the lungs, removal of air from the chest cavity before closing the chest, and by deflation of the lungs before extubation, it also shown a significant reduction in mortality by removing air from the chest cavity after surgery and by deflation of the lungs at the end of the procedure^[12].

In addition, we observed during our initial standardization process that post-operative infections are another important cause of late mortality and it is a misconception that rodents have an innate resistance to bacterial infections. Such infections may not be apparent on casual observation, but will cause loss of vessel cannulations and several physiological changes in the animals^[13, 14]. We provided proper post-operative care to avoid any infection related mortality.

We have carried out a detailed time course study of hemodynamic parameters, cardiac injury enzymes, and histopathological changes up to 90 days after induction of MI. There is only one study in which a time course evaluation has been done after induction of MI, but for a period of only 32 days^[12]. In this study a significant decrease in heart rate and blood pressure was observed from day 8 to day 32. We observed a significant decrease in the heart rate and of blood pressure from day 1 onwards, up to the period of observation of 90 days. After induction of MI, serum levels of CK-MB and LDH were elevated on day 1, reached their peak at day 7, and declined thereafter. In sham operated rats except for increase in serum levels of LDH on day 1, perhaps due to muscle injury, there were no other changes in cardiac injury enzymes. The post

MI histopathological changes observed in our model, including nucleomegaly, loss of cross striations in myocytes and cardiomyocyte degeneration and timing of emergence of infarct zone were similar as previously reported in mouse^[15] and rat^[12]. The time sequence of formation of granulation tissue and scar tissue observed in our model was similar to that observed in human MI^[16].

In conclusion, we have established a rat model of MI with a consistent infarct size, low early and no long-term mortality. This model may be especially useful in evaluating efficacy of therapies where a follow-up of at least 12 weeks is required like regeneration of infarcted myocardium after MI by stem cell therapy.

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