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Therapeutic Efficacy of Single Dose of Amniotic Fluid Derived Stem Cells on Global Myocardial Ischemia in Rat

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Abstract

Stem cell therapy is gaining attention in the repair of damaged myocardium. The current study was planned to see the therapeutic effects of stem cells derived from amniotic fluid (AFSCs) on global myocardial ischemia (MI) induced by injecting Isoproterenol in rats to see physiological, biochemical, and functional changes as well as histopathological changes in heart tissue. Functional changes in heart were recorded by small animal ECHO -Ultrasound. Stem cells from the amniotic fluid through amniocentesis, were isolated, cultured and cells were harvested for experimental design. One week after induction of Myocardial Infarction, $2x10^6$ AFSCs or normal saline was given intravenously through the tail vein of MI rats. After 4 weeks of hAFSCs administration in the MI group, rats showed weight gain, and enhanced ejection fraction of myocardium. AFSCs-treated MI rats had a significant decrease in other functional cardiac parameters, along with enzyme biomarkers (LDH and CK-MB) in comparison to MI group. Haematoxylin-Eosin and Masson Trichome staining in heart tissue showed less necrosed heart tissue and lesser expression of collagen and fibrosis in AFSC-treated MI rats. AFSCs limit infarction, reduce fibrosis in heart tissue, and improve left ventricular systolic function in MI rats. Our results suggest that AFSC has potential to treat MI and the use of small animal ECHO-ultrasound to record functional changes could be standard in preclinical settings.

Keywords

ECHO-Ultrasound, AFSC, Myocardial Infarction, Rat, Stem Cell therapy.

INTRODUCTION

Heart disease is a major cause of death globally despite medical advances. Fibrosis and loss of myocardium due to necrosis is the major cause of heart pathology in myocardial infarction.Though multifactorial pathophysiology of myocardial infarction is treated with coronary artery intervention, coronary artery bypass grafting strategies along with use of conventional medicines such as antihypertensive, and antiplatelet agents, the rise in heart failure continues [1]. A heart transplant is not a choice of treatment modality due to a shortage of heart donors [2]. Adult heart tissue loses its ability to regenerate after injury, making it difficult for patient survival.

Search for other treatment modalities leads utilization of stem cells for the regeneration of damaged cardiac tissue. Various multipotent stem cells from different sources are implemented for therapy against myocardial infarction (MI) and coronary artery disease (CAD) [3] [4] [5] [6] [7] [8]. The partial protection offered by these multipotent cells on cardiac diseases is caused by various multiple pathways. Mesenchymal stem cells (MSCs) have limited capacities of differentiation and proliferation into desired cell types. Another alternative source for MSCs is amniotic fluid, placenta, and amnion membrane which is easy to harvest and has the potential to proliferate and differentiate all three lineages and can be used for the treatment of different kinds of diseases. Amniotic fluid can be harvested through amniocentesis during a routine checkup of fetus in the first trimester for genetic analysis. Placenta and amnion membrane can be harvested after delivery. Amniotic fluid stem cells are more primitive and has proliferative and differentiation ability compared to stem cells derived from placenta and or amnion membrane [9] [10] [11]. The stem cells from amniotic fluid which is pluripotent in nature and expresses OCT-4, and SSEA-4 markers apart from multipotent markers and have been tested for their therapeutic potential against various diseases in preclinical settings [9] [10] [11] [12] [13] [14]. The stem cells from the amniotic fluid is anti- inflammatory, non-tumorigenic, and non-immunogenic in nature which makes them an ideal source for transplant . Secretomes from amniotic fluid stem cells also offer protection against myocardial infarction, ischemic stroke, and neuronal damage [15] [16] [17]. Global myocardial ischemia can be induced chemically in animals by the use of Isoproterenol which causes irreversible cellular damage and infarction in the heart [13] [14]. Structural and functional changes were captured by echocardiography in routine in humans. The non-invasive ECHO- Ultrasound is of great use in the evaluation of the cardiac health of small animals used in translational research [8] [9] [10] [11] [12] [13] [14] [15].

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Rodents are used as models for cardiovascular disease and other disease conditions and the use of non-invasive imaging tools like ultrasound echocardiography led to the translational and therapeutic development to prevent fatal diseases in humans [14]. In our previous work, we have shown the therapeutic efficacy of stem cells from amniotic fluid against the metabolic disorder of Diabetes Mellitus in rats [12].

The current study was undertaken to evaluate the therapeutic role of stem cells from amniotic fluid against global myocardial ischemia about anatomical, morphological, physiological, and biochemical changes that occurred in cardiac tissue during injury.

MATERIALS & METHODS

Amniotic fluid stem cells (AFSC) isolation, ex vivo expansion, and characterization

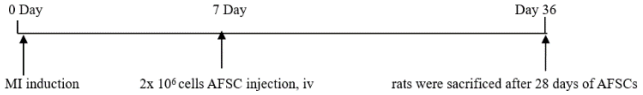
Amniotic fluid samples from 16-20 weeks of pregnancy were collected for prenatal diagnosis through amniocentesis. 0.5 ml of sample is diluted with Phosphate buffered saline and pelleted by low speed centrifugation. The cell pellet was suspended in α-MEM media supplemented with 16.5% FBS, 1% antibiotic solution, and 1% Glutamax and plated in a culture flask at the density of 2000 cells/cm^2 and kept in an incubator. After a few days, adherent cells were cultivated and replated for expansion. Antigenic profiling of hAFSCs was determined by a flow cytometer. For detection and characterization of stem cell surface antigens (SSEA-4, Oct-4, CD34, CD29, CD166, CD 44, CD 45, and CD90), fluorescence-tagged antibodies were used against above surface antigen. Detection and quantitation of cell surface antigen were performed by Flow Cytometer (Becton Dickinson) and Cell Quest software respectively [12].

Animals

Rats (male, adult) were housed in the Animal House facility, Sanjay Gandhi PGIMS, Lucknow, and maintained in standard animal pellets and water ad libitum with exposure to equal hours of light and dark cycle, and approved by the Institute Ethics Committee for Animal Care.

Induction of myocardial injury on rats: Male Wistar rats (n=15) weights 250-300 g were used for the study. An equal number of rats was divided into three groups (n=5 each group). The global Heart Failure Model (MI) was induced by injecting a single dose of Isoproterenol (Sigma, USA;100mg/kg in normal saline) subcutaneously into rats (n=10). Control rats (NC) were injected subcutaneously with normal saline. Tail intravenous injection of AFSCs (2x 10 ^{6} cells, single dose) was given to the MI rats (MI+AFSC group,after 7 days of induction of global myocardial infarction). Rats from all groups were sacrificed after 28 days of AFSCs injection (Day 36).

Experimental outline:



Echocardiographic Studies (Functional studies)

Mildly anesthetized rats were positioned on an imaging platform for 2D Echo ultrasound to assess cardiac functions before (0 days), after (7 days of MI), and after 28 days of AFSCs injection in MI rats (36 days) by using Vevo 2100 ultrasound system (Visual Sonics, Canada). Cross section of heart was imaged in B and M mode for functional and anatomical assessments. Physiological parameters like heart rate, respiration, and echocardiogram of rats were recorded using an imaging platform. Myocardial Infarction was confirmed using ECHO-ultrasound for cardiac functional assay in each group of rats individually.

Histological and Biochemical Analysis

Blood was collected from all the groups of rats for biochemical estimations. Enzymic cardiac biomarker (CK-MB, LDH) were measured in blood by standard methods. To assess the cardiac injury, histochemical analysis was done. Isoflurane anesthesia was given to all experimental rats after completion of experimental design ie 28 days of stem cell treatment period (endpoint 36 days). Histopathology after 36 days of start of experiment , and echocardigraphy, heart tissues were quickly removed following euthanasia under isoflurane, and were weighed to calculate body-weight ratios. Heart tissues were fixed and embedded in paraffin, sectioned for subsequent histological analysis.

Masson's trichrome staining was performed on the heart tissue of all three groups of rats to evaluate ventricular morphology and myocardial fibrosis. Haematoxylin and eosin staining were performed in the heart tissues of all the groups of rats to assess cardiac injury. The heart tissue was examined under an Olympus microscope (magnification: 40x) to assess myocardial tissue injury.

STATISTICAL SIGNIFICANCE

Results are summarised as the mean ± standard deviation (SD). Student's t-test was used when two groups were compared and One-way ANOVA with a post hoc test was used to assess significant differences when more than two groups were compared. $p \geq 0.05$ is taken as statistically significant.

RESULTS

To evaluate the role of AFSC on heart function after global myocardial ischemia, a single injection of AFSC of 2x10^6 cells was given to rats after 7 days of induction of global ischemia. Serial echocardiography by 2D Echo ultrasound reveals changes in heart anatomy and functions in different groups of rats.

Phenotypic and antigenic properties of Amniotic fluid stem cells showed heterogeneous cell populations and

markers of mesenchymal & embryonic origin. AFSCs stained positive (>96%) for several markers such as CD 166, CD 73, CD 90, CD 29 and negative for CD 45, CD 34, and HLA-DR. Pluripotent markers OCT-4 (~43%), and SSEA-4 ((~80%) are found on AFSCs (Table 1).

Effect of stem cell therapy on cardiac parameters

Functional cardiac parameters such as output, Ejection, Fractional shortening, volume, and Left Ventricular mass were recorded and calculation has been accordingly (Table 1). Significant reduction in cardiac output as well as % ejection fraction was found in the MI group (**p>0.01) while cardiac output and ejection fraction is restored towards partial normal after hAFSc injection (#p>0.05) (Table 2). An increase in the LV mass (hypertrophy) was seen in the MI group of rats in comparison to normal control (*p>0.05) rats whereas a decrease in LV mass was observed in the treated group (Table 2).

Effect of stem cell therapy on Body and Heart Weight

A 20% reduction in body weight was observed in MI group in comparison to the control group (*p>0.05). A trend in increase in whole body weight was seen after AFSCs treatment in MI rats. The increase in heart weight was seen in MI rats which was significantly reduced by AFSC treatment (# p< 0.05) (Table 3).

Role of stem cell therapy on enzymic biomarkers CK-MB and LDH

MI animals showed an increase in CKMB level in comparison to normal control (***p>0.001). Amniotic fluid stem cell treatment caused a decrease in the CK-MB level (#p>0.05) as compared to MI animals (Table 2). Similarly, the LDH level was significantly increased (*** p>0.001) when compared to the control. Amniotic fluid stem cells ($2x10^6$ cells, i.v.) treatment significantly decreased the LDH level (## p>0.01) as to MI animals. (Table 4).

Effect of stem cell therapy on cardiac tissue (H&E and Masson Trichrome Staining)

Pathological changes were observed in the heart histology of the MI group with subendocardial necrosis, myocyte vacuolization, macrophage infiltration, intra myofiber edema, and vacuoles whereas in the AFSCs-treated group less vacuolization, edema, and necrosis were observed (Figure 1). Fibrosis in heart tissue reveals the severity of heart failure and cardiac functions. Myofibers are stained in red and collagen is stained in blue after Masson Trichrome staining. Masson Trichrome staining is significantly higher in MI rats suggesting fibrosis and the presence of collagen in heart tissue whereas in AFSC treated rats of MI showed lesser expression of collagen and reduced cardiac fibrosis (Figure 1).

DISCUSSION

In this study, we evaluated the feasibility of AFSCs as therapy in a global Myocardial Infarction in rats using high-frequency ultrasound imaging system. Cardiac parameters are recorded using 2D Echocardiography in all groups of rats with histochemical analysis at the end of experimental time.2 D ECHO-Ultrasound is a standard choice to evaluate the changes in the same animals for continuous assessment of functional and anatomical changes during pathology and treatment strategy.

In cell-based therapy, the most disadvantage of it, is the possibility of cell rejection. The advantages of AFSC are as it is non - tumurogenic, non-immunogenic in nature (low expression of HLA-DR antigen on AFSC, so less chance of cell rejection), no ethical concern, which makes it a good source for stem cells in the treatment of various diseases. The potential of expansion and differentiation of AFSC in different lineages is better as it expresses both mesenchymal stem cells and some embryonic markers. AFSCs showed heterogeneous cell populations and expressed antigens like SSEA-4, transcription factor Oct-4, mesenchymal stem cell markers and do not express hematopoietic s markers and HLA-DR 2 reported previously by us and others also [2] [3] [8] [9] [10] [12].

To assess injury induced by isoproterenol in myocardial tissue, we measured enzymic biomarkers LDH and CK-MB (heart-specific isozyme) for injury in serum and found a significant 5-fold in CK -MB and a 2-fold increase in LDH activity in MI rats in our study which are per other studies [3] [9] [13] [14]. A significant reduction of 28% ejection fraction of the heart was observed in MI rats because of fibrotic damage and collagen deposition in myofiber which affects the contractile ability of the heart [13] [14]. We have evaluated the therapeutic role of stem cells from amniotic fluid in myocardial infarction induced chemically by Isoproterenol on functional, biochemical, and pathological parameters and observed better functional cardiac parameters, reduced LV hypertrophy, low levels of cardiac biomarkers like CK-MB, LDH, and H & E staining as well as Masson Trichrome staining suggests less fibrosis and collagen deposition after single injection of AFSCs treatment in MI rats. Cardiac parameters were significantly improved when stem cells were injected early in acute MI [18] [19] [20] in this study single dose of AFSCs was injected after 7 days of MI induction by Isoproterenol. Increased cardiac biomarkers like CK-MB and LDH suggest the necrosis of cardiac cells and the formation of fibrosis and collagen deposition which shows the loss of the pumping action of cardiac tissue in MI rats. A single dose of AFSCs treatment is protective in the present study which is by a previous study from our laboratory [14]. Previous study showed spindle-shaped amniotic fluid stem cells are protective and proangiogenic against cardiac damage [17].

2D Echo ultrasound is a better technique for the evaluation of cardiac functions in the same animal noninvasively and is useful for studying tissue remodelling and treatment modalities in MI. In the present study, we used non-invasive imaging ultrasound echocardiography for functional cardiac parameters. Amniotic fluid stem cells have capacity to differentiate in different lineage and does

not have immunological and tumorigenic potential making them an ideal source of cell-based therapy.

Though AFSCs expressed markers of multipotent to pluripotent stem cells and have regenerative potential in various diseases but not able to contract spontaneously rather action potential came from host cell membrane current [7] [21] [22] [23]. Several research papers are focused on paracrine properties of AFSCs in several disease models preclinically and also for cardiogenic properties [15] [16] [17] [21].

CONCLUSION

Intravenous AFSC treatment in global myocardial infarction reduces infarction, and fibrosis, and improve ventricular ejection fraction. Our findings suggest that amniotic fluid is a good source of stem cells for the treatment of global myocardial ischemia in a preclinical setting and could be used in a clinical setting for the treatment of various diseases.

Acknowledgment

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Conflict Of Interest

There is no conflict of interest among authors

Table 1. Surface antigens (gene profile) on amniotic fluid stem cells

S No	Markers	Antigen or CD	Presence (+) /Absence (-)	%
1	Embryonic stem cells	Oct-4	+	43
2	Embryonic stem cells	SSEA-4	++	77
3	Mesenchymal stem cells	CD166	+++	96
4	Mesenchymal stem cells	CD73	+++	94
5	Mesenchymal stem cells	CD90	+++	98
6	Mesenchymal stem cells	CD29	+++	98
7	Mesenchymal stem cells	CD45	-	<2
8	Hematopoietic stem cells	CD 34	-	<2
9	Major Histocompatibility complex (MHC)	HLA DR	-	<2

Detection and quantitation of cell surface antigen were performed by Flow Cytometer (Becton Dickinson) and Cell Quest software respectively on amniotic fluid stem cells (n=5).

Table 2. Cardiac parameters in different groups of rats (Functional outcome 2D ECHO)

SNo	Parameters	N Control (n=5)	MI (ISO)(n=5)	MI+ AFSCs(n=5)
1	Heart Rate (BPM)	370±20	340±12	350±10
2	Cardiac output (ml/min)	130.8±15	40.9±18**	70.5±10#
3	Stroke volume (µl)	160.74±12	130.2±14*	140.8±20
4	Ejection fraction (%)	77.6±11	55.8±7*	62.3±18
5	Fraction shortening (%)	43.4±7	20.4±3	36.6±4
6	Left Ventricular mass (g)	0.676± 0.12	0.875±0.15*	0.658±0.14#

Values are mean ±SE; n= number of rats. Echocardiography measures obtained before the time of sacrifice. * $p \geq 0.05$, **$p \geq 0.01$ is considered significant when compared between the control and MI group. # $p \geq 0.05$ is considered significant when a comparison is done between MI and MI + AFSCs group.

Table 3. Role of AFSCs on body and heart weight in MI rats

SNo.	Group	Rat body weight (g) at the end of the experiment	Heart weight (g)
1	Normal control	320.34±10	0.821±0.078
2	MI(ISO 100mg/kg, sc)	256.86 ±12*	1.164 ± 0.057*
3	MI+ AFSC	268.26 ±12.25	0.908 ± 0.053#

Loss in body weight due to ISO treatment whereas significantly increased weight of heart to show hypertrophy due ISO treatment. Hypertrophy is reduced significantly by AFSCs treatment. Values are mean ±SE; *$p \geq 0.05$ when compared between the control and MI group. # $p \geq 0.05$ when a comparison done between MI and MI + AFSCs group

Table 4. Alterations in biochemical parameters (enzyme CK-MB and LDH) in serum:

Sno	Groups	Serum CK-MB (IU/L)	Serum LDH (IU/L)
1.	Normal	21.081±1.23	438.65±36.26
2.	MI(ISO)	120±8.95 ***	948.55±50.41***
3.	MI+ hAFSC	98.18±6.07 #	767.34±27.18##

blood was collected through cardiac puncture. Serum CK-MB and LDH were estimated by auto analyzer using a

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specific reagent kit in different groups of rats. Values are mean ±SE; ***p ≥ 0.001 is considered significant when compared between the control and MI group. # p ≥ 0.05, ##p≥ 0.01 is considered significant when a comparison is done between MI and MI + AFSCs group.

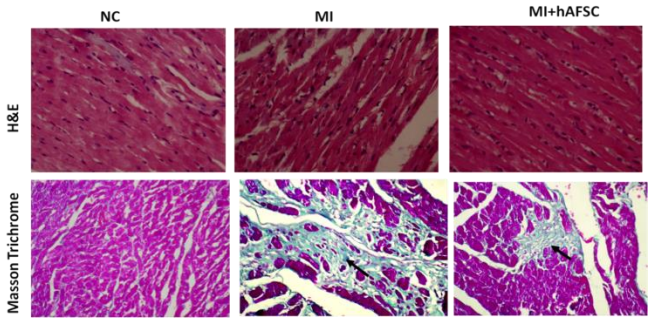

Figure 1. Effect of AFSC treatment on histopathological changes. Heart tissue sections stained with H & E , and with Masson Trichrome of normal, MI and AFSCs-treated MI rats were visualized under a light microscope of 40 x magnification and representative photos of different groups of rat hearts were shown here. Blue-stained collagen was seen in Masson trichrome staining in MI rats suggesting fibrosis and the presence of collagen in heart tissue (black arrow) whereas in hAFSC treated rats of MI showed lesser expression of collagen compared with the MI group

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