Nano-particle engineered atorvastatin delivery to support mesenchymal stem cell survival in infarcted myocardium

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1. Introduction

Myocardial infarction (MI) commonly known as a heart attack, occurs when blood flow stops to a part of the heart causing damage to the heart muscle like myocardium necrosis and cell loss. An MI may be lethal by acute heart failure, an irregular heartbeat, cardiacogenic shock, or cardiac arrest (Zhang et al., 2014). Aspirin is an appropriate immediate treatment for a suspected MI. Nitroglycerin or opioids are used to help with chest pain; however, they do not improve overall outcomes. Nowadays, tissue engineering approaches have gained much attention due to its capability to regenerate new cells of myocardium. In this line, bone marrow derived mesenchymal stem cells (MSC) are of prime importance because they stimulate and restore myocardial regeneration so as to restore functioning of myocardium in post-infarct hearts. Benefit of MSCs is due to their regenerative, paracrine or immunomodulatory functions. However, effective regeneration of cardiac muscles is limited due to the poor cell engraftment and inefficient cardiomyocyte differentiation of MSCs (Takehara et al., 2000; Li et al., 2015). Some reports have shown that, immediate MSC transplantation in MI model of Sprague–Dawley rat does not show significant improvement in cardiac function or infarction size reduction. Damaged tissue of myocardium causes inflammatory reactions, oxidative stress and hypoxia conditions which make the retention/survival of transplanted MSC’s difficult (Zhang et al., 2014). Since, MSCs survival rate is very poor in MI like environment implanted MSC could be too ineffective to improve MI condition. Therefore, there was need to investigate a method that might improve the microenvironment and enhance MSCs survival. Atorvastatin (ATV) is a member of statins and used primarily as a lipid-lowering agent and for prevention of events associated with cardiovascular disease. Furthermore, it may also be used in the secondary prevention of MI like conditions as it is a known anti-inflammatory and antioxidant agent too. Capability of diminishing inflammation, oxidative stress and hypoxia like conditions may help enhance the chance of transplanted MSC survival (Li et al.,...
in infarcted myocardium. It is safe to use and cause no harm to the differentiation and proliferation of MSCs. As ATV is very slightly soluble in water (Kulthe and Chaudhari, 2013), its distribution in MI like microenvironment is limited (Rohilla et al., 2015). Subsequently it undergoes high intestinal clearance frequent first-pass metabolism (Lennernas, 2003). O account of which ATV offers low systemic availability (approximately 30%) (Salmani et al., 2015). The above limitations hamper ATV delivery to the specific site. Changing its physicochemical parameters (Rai et al., 2015) could be the best option to overcome these shortcomings. Variety of nanoparticles has been developed to increase the solubility and avoid the first pass metabolism (Rossi et al., 2013) of drugs like ATV. Nanoparticles are prepared to extend and control the release of the drug in various biological conditions. PLGA has been approved by the FDA for its potential in drug delivery applications in humans. Its biodegradability, biocompatibility, and non-toxicity make it a suitable polymer for drug delivery approaches. PLGA is prepared by copolymerization of poly(lactic acid) and poly(glycolic acid). As the ratio of poly(lactic acid) and poly(glycolic acid) increase, polymer degradation rate decreases and hence release rate decreases. High molecular weight PLGA was recommended to form extended release NPs (Sylvester et al., 2013). PLGA nanoparticles are used to avoid the direct availability of the drug at the site of action in higher amount so as to avoid dose dependent toxicity and unwanted effects. To ensure consistent ATV delivery in systemic circulation in predictable and sustained manner, we hypothesized to investigate the ability of ATV containing PLGA nanoparticles against alone ATV in the survival of MSCs. It was aimed to formulate the ATVNPs to ensure higher availability of ATV in simulated blood fluid for longer duration thereby improving the MSC survival.

2. Materials and methods

2.1. Materials

Materials used in this study were purchased in China only. Atorvastatin. Poly([ε]-lactide-co-glycolide) (PLGA; 85:15 lactide: glycolide), Dimethyl sulfoxide (DMSO) and didodecyltrimethylammonium bromide (DMAB) were from Sigma Aldrich Chemie; Dulbecco’s modified Eagle’s medium (DMEM) was from Invitrogen Pvt. Ltd. Chloroform was from Merck chemicals, Dialysis membrane MWCO 12–14 KDa and buffer tablets were from Himedia, Pvt. Ltd. Alcohol was from Thomas Baker Pvt. Ltd. Rat cardiac H9c2 cells were from Boster Biological Technology, Ltd. Milli-Q water was used throughout the study. All the other chemicals used were of reagent grade. Female Sprague–Dawley rats (200–220 g, about 2 months old) were procured from the Slac Laboratory Animal Co., Ltd. Shanghai. Female rats were chosen as recipients however, male rats (60–80 g, 1 month old) were selected as MSC donors. All the experiments were performed in accordance with the guidelines of care and use of rats Institutional Animal Experimentation Ethics Committee (AEEC) China for Animal Care and Use. About 180 rats were randomly allocated into 6 groups (n = 30). First is normol control group (NC), second is MI control group (MI), third is MI group treated with MSC (MSC), fourth is MI group treated ATV followed by MSC transplantation (ATV + MSC), fifth is MI group treated with ATVPN followed by MSC transplantation (ATVNP + MSC), and sixth is MI group treated with fasudil followed by MSC transplantation (fasudil + MSC).

2.2. UV method

A known concentration i.e. 20 µg/ml of ATV was scanned between 200 and 800 nm using UV–visible spectrophotometer (Shimadzu, Kyoto, Japan) in chloroform. The absorption maximum of ATV was found to be at 246 nm. Consequently, the stock of 1 mg/ml solution of ATV was prepared. Various dilutions of ATV was prepared in the concentration range of 1–50 µg/mL. Absorbance of the dilutions was taken at the respective absorption maxima and the calibration curve was plotted between concentrations and absorbances (Marin et al., 2002; Shah et al., 2012).

2.3. Formulation of ATV loaded nanoparticles

PLGA nanoparticles were synthesized using double emulsion solvent evaporation method. ATV was loaded in nanoparticles as per the procedure reported by Sylvester et al. in 2013(10). Briefly, about 50 mg PLGA was dissolved in chloroform. ATV 5 wt% ratios of ATV: PLGA was mixed in the above organic solvent by sonication (Sonic Vibra cell with 20 kHz ± 50 Hz resonance) at 30% amplitude for 3 min. About 12 ml aqueous phase containing didodecyl dimethyl ammonium bromide (DMAB) as the surfactant (0.25 w/v) was added to the organic phase, sonicated and kept for stirring overnight to evaporate the organic phase. Samples were dried under vacuum prior to the ultracentrifugation at 35,000 rpm for 30 min. NPs pellets were separated and were further washed twice with distilled water, dispersed, centrifuged at 25,000 rpm for 10 min and lyophilized to get powdered NPs of ATV (ATVNP).

2.4. Characterization of ATVNPs

The supernatants of separation and washing steps of nanoparticle synthesis were quantified for any dissolve ATV (indirect method to assess loading efficiency) by reading of the absorbance at 246 nm using UV–Vis spectrophotometer. Entrapment efficiency of nanoparticles was also assessed as per the reported procedures (Mishra et al., 2015). Particle size, poly dispersity index and zeta potential of ATVNPs were determined by dynamic light scattering method using Zeta sizer (Malvern, U.K.). Internal morphology along with the dimensions of the developed ATVNPs was confirmed using transmission electron microscopy (TEM; JEOL, Tokyo, Japan) by clicking digital images at 6000×.

2.5. ATV release from ATVNPs

The release study was carried out using Franz diffusion cell. Donor and receiver compartments were separated by dialysis membrane of 12–14 KDa. Receiver compartment was filled with 50 mL phosphate buffer (containing 2% DMSO) however donor compartment was filled with 3 mL DMSO and PBS (2:98 ratio) solvents containing ATV (300 µg) and ATVPN (equivalent to 300 µg ATV). About 0.5 ml samples were withdrawn from the receiver at 0, 2, 4, 8, 24, 48, 96, 168, 336 & 672 h and replaced with same volume of fresh PBS. Diffusion cell was jacketed with warm water which maintained the temperature at 37 ± 2 °C along with continuous stirring at 100 rpm to simulate physiological conditions. Each sample was quantified for ATV content using UV–Vis spectrophotometer by taking absorbance at 246 nm (Rai et al., 2014; Dubey et al., 2014).

2.6. Isolation of MSC and cell culture

Bone marrow of tibia and femur of male Sprague-Dawley male rats were harvested and seeded into cell culture flask as per the procedure reported for the isolation and culture of MSC by Zhang et al. (2014).
2.7. In vitro cytotoxicity

Rat cardiac H9c2 cells of rat heart were cultured in DMEM enriched with bovine serum penicillin–streptomycin at 37°C in a humidified atmosphere and used to assess the in vitro cytotoxicity of different concentration of ATV given in solution or in the form of ATVPNP dispersion containing ATV (equivalent to 0.01, 0.1, 1.0 and 10.0 mg/100 μl of ATV). The H9c2 cells were exposed to samples under conditions of hypoxia for 24 h after washing with PBS (Nazir and Chat, 2015).

2.8. MI model establishment

Female rats were anesthetized using 10% chloral hydrate (4 ml/kg) injection i.p. and MI was established by permanent ligation of the proximal left anterior descending (LAD) coronary artery as the procedure reported elsewhere by Zhang et al. in 2014. Briefly, success of ligation process was confirmed by myocardial blanching and abnormal movement of the anterior wall. MSC group was administered with 60 μl phosphate-buffered saline injection containing 5 × 10^6 MSCs after LAD ligation. ATV group was administered intravenously with ATV in DMSO solution form at the dose of (20 mg/kg BW) followed by 60 μl phosphate-buffered saline injection containing 5 × 10^6 MSCs after LAD ligation and continued for 4 weak at the daily dose of 10 mg/kg BW. ATVPNP group was administered intravenously with ATVPNP (translating 20 mg/kg BW of ATV in rat) followed by 60 μl phosphate-buffered saline injection containing 5 × 10^6 MSCs after LAD ligation and continued for 4 weak at the daily dose of 10 mg/kg BW. Fasudil group was administered with 20 mg/kg BW fasudil intraperitoneally after LAD ligation followed by MSCs, and continued for 4 weak at the daily dose of 10 mg/kg BW.

2.9. Assessment of cardiac function

2.9.1. Echocardiography

Transthoracic echocardiography was performed to detect left ventricular end-systolic diameter (ESd), end-diastolic diameter (EDd), fractional shortening (FS) and ejection fraction (EF). FS and EF were calculated using the following equations:

\[
LVFS (\%) = \left(\frac{LVEDd - LVESd}{LVEDd}\right) \times 100\%
\]

\[
LVEF (\%) = \left(\frac{(LVEDd)3 - (LVESd)3}{(LVEDd)3}\right) \times 100\%
\]

All the measurements were performed for three consecutive heart cycles and averaged.

2.9.2. Left heart catheterization

After 4 weeks of MI induction, left heart catheterization was performed. Following the procedure, rats were anesthetized (intraperitoneal injection of 10% chloral hydrate; 4 ml/kg) and heparin loaded catheter was inserted into left ventricle through right carotid artery. With the help of left ventricular pressure curve, data of the end-diastolic pressure (EDP), as well as the pressure maximal rate of rise and fall (±dp/dt max) were recorded.

2.9.3. Cytokines estimation

Cytokine i.e. expressed in peri-infarct myocardial tissues were separated by enzyme linked immune-sorbet assay (ELISA). Peri-infarct myocardial tissues were extracted and homogenized in phosphate buffer saline (7.4). Homogenized tissues were centrifuged at 12,000 rpm at 4°C and the supernatant protein solution was taken for estimation of IL-6 and TNF-α using ELISA kit. The procedure used and absorbance taken as per the user’s manual.

2.10. Statistical analyses

The data were analyzed with GraphPad InStat Demo software. All values are presented as mean ± SEM. Differences among groups were tested with one-way analyses of variance followed by post hoc test.

3. Results and discussion

ATV is soluble in methanol and was observed to obey Beer-Lambert’s law in the range of 5–25 μg/ml concentration of ATV. With the help of back calculation, presence of ATV was quantified by reading the absorbance at 246 nm using UV–Vis spectrophotometer and the loading efficiency of the NPs was found to be 17 ± 1.4%.

3.1. Characteristics of ATVPNPs

ATVPNPs were formulated with 0.25% w/v DMAB exhibited hydrodynamic particle size 198.28 ± 16.2 nm, PDI 0.021 and ζ-potentials + 25.6 ± 2.2 mV (Fig. 1). ATV loading did not affect the particle size, PDI and surface charge. TEM study reveals the spherical shape and smooth surface of particles of nano-size (Fig. 2). Particles were observed to be of uniform size. ATV entrapment efficiency of the NPs was observed to be 78.23 ± 3.5%. Due to the biodegradable, biocompatible, and nontoxic nature PLGA has been approved by the FDA in drug delivery applications. For the application of extended release of ATV from NP, 85:15 lactide to glycolide ratio was selected as their increasing ratio leads to decrease in polymer degradation rate and can help in extending duration of drug release. Physicochemical and morphological characteristics of NPs i.e. size and surface charge also play an important role in improving their in vivo efficacy as 100 and 200 nm large PLGA NPs get internalized into the cells, size larger than 500 nm gets localize in the extracellular fluid and induce phagocytosis. Particle size < 100 nm could incite an immune response. As particle size was found to be around 200 nm, it could be helpful in MI like condition by accumulating in extracellular space of infarcted site.

![Fig. 1. Size and surface characteristics (particle size and zeta potential) of ATVPNP using DLS technology.](image-url)
may avoid the instant availability of ATV into the cultured cells and could be proved as the valuable nanocarrier for the delivery of such kind of drugs. This could be due to the extended release behaviour (Fig. 3) of ATVNPs. Initially no release of ATV was observed under all tested conditions. This is perhaps due to the strong packing of ATV within the polymer matrix (Kim et al., 2009).

3.3. H9c2 cell viability after ATV exposure from ATV solution and ATVNPs

Different concentration of ATV either in solution form or in ATVNPs dispersion might affect the survival of H9c2 cells which was observed by cell viability assay. No H9c2 cell death (100% cell viability) was observed at the concentration of 0.1 mg/100 μl of ATV in solution and ATVNPs however at 1.0 mg/100 μl of ATV, solution showed lesser viability of cells i.e. 81% however, ATVNPs did not show any sign of cell death. At 10.0 mg/100 μl, ATV solution showed 14% cell viability however, ATVNPs showed 54% cell viability (Fig. 4). ATV solution was found to be more toxic than that of ATVNPs at the same concentration. No significant difference in viability of the cells was observed in the case of vehicle control. At the therapeutic dose of ATV, no toxicity was observed.

3.4. ATVNPs administration before MSC transplantation improves cardiac function

Based on echocardiography of animals after MSC transplantation at 1 week and 4 week, cardiac function was assessed. One week was considered as baseline however, 4 weeks was endpoint. After baseline correction after 1 week between all the groups (Table 1) cardiac function was assessed in terms of EDD, ESV, EF and FS at the endpoint (after 4 weeks). MI + MSCs group improved the EDD, ESV, EF and FS when compared with the MI control group but the differences were non-significant. EDD and ESV levels were alleviated dramatically in the case of ATV + MSCs which were further improved significantly by ATVNPs + MSC. A significant increase in EF and FS was exhibited in the case of ATV + MSC group which was further improved when ATV was administered in the form of ATVNPs. Left heart catheterization was carried out to assess cardiac function at the endpoint. Only MSC transplantation revealed no sign of significant improvement either in terms of EDP or ±dp/dt max. However, along with ATV it significantly alleviated EDP, increased +dp/dt max and unremarkable increase in +dp/dt max. ATVNPs showed increased potential in alleviating EDP and increasing +dp/dt max. Administration of ATV fabricated NP along with MSC transplantation showed additional improvement as compared to the MI control group.
3.5. ATVNP alleviates the level of expressed inflammatory cytokines

Level of IL-6 and TNF-α were examined by ELISA. Both the cytokines were expressed significantly in the MI control group. ATV + MSC, ATVNP + MSC and fasudil + MSCs groups have significantly reduced the level of IL-6 and TNF-α. MSC transplantation alone had also reduced the level of expression of IL-6 and TNF-α, but the difference was not significant (Fig. 5).

MSCs have been transplanted in many clinical “incurable” diseases such as ischemic heart diseases. Benefit of MSC is due to its regenerative paracrine or immunomodulatory functions. After MI immediate MSC transplantation in rat models of MI is reported to show non-significant improvement in cardiac function or infarction size in infarcted myocardium. This is because MSCs survival rate is very poor in MI like environment on account of which it has not been as effective as to improve MI. ATV is reported to promote the MSC survival by helping in the effective transplantation of MSC in post infarcted myocardium. Pre-administration of ATV alleviates the level of expression of inflammatory cytokines and fibrotic factors on account of which MSCs survival gets improve which ultimately may improve the therapeutic efficacy (Zhang et al., 2014). As ATV is very slightly soluble in water it distribution in MI like microenvironment could be limited. Therefore its NP was developed which was supposed to improve the distribution of ATV into the systemic circulation. Transplantation of MSCs after ATVNP administration leads to further improvement in cardiac function. Further improvement in cardiac function and alleviation in over expressed level of cytokines were attributed to the consistent supply of ATV over the MI site for longer duration.

4. Conclusion

PLGA based ATVNP was fabricated successfully by double emulsion solvent evaporation method to deliver ATV in systemic circulation directly. ATVNP ensured enhanced availability of ATV for longer duration to support MSC survival in a more efficient manner. ATVNP was observed to be non-toxic to the cells as compared to that of ATV alone. Purpose of extending release of ATV for longer duration (at least for 28 h) was fulfilled by PLGA NPs. ATVNP improved the cardiac function by supporting MSC survival in an efficient manner. In conclusion, ATVNP may ensure safe, cost effective, and efficacious treatment of post-infarct myocardium when

| Groups          | NC       | MI       | MSC      | ATV + MSC | ATVNP + MSC | Fasudil + MSC |
|-----------------|----------|----------|----------|-----------|-------------|---------------|
| **EDd (mm)**    |          |          |          |           |             |               |
| Baseline        | 6.7 ± 0.7| 7.8 ± 0.5| 7.63 ± 0.3| 7.1 ± 0.4 | 7.22 ± 0.4  | 7.3 ± 0.3     |
| Endpoint        | 6.7 ± 0.2| 8.9 ± 0.4| 8.26 ± 0.5| 7.29 ± 0.8| 6.9 ± 0.6   | 7.21 ± 0.8    |
| Δ               | 0.0 ± 0.45| 1.1 ± 0.45| 0.63 ± 0.4| 0.19 ± 0.6 | -0.32 ± 0.5 | -0.09 ± 0.55  |
| **ESd (mm)**    |          |          |          |           |             |               |
| Baseline        | 4.4 ± 0.02| 6.3 ± 0.7| 5.6 ± 0.5| 5.69 ± 0.27| 5.21 ± 0.89 | 5.31 ± 0.89   |
| Endpoint        | 4.2 ± 0.06| 7.5 ± 0.5| 6.1 ± 0.3| 5.23 ± 0.93| 4.91 ± 0.33 | 5.20 ± 0.11   |
| Δ               | 0.2 ± 0.04| 1.2 ± 0.6| 0.5 ± 0.4| -0.46 ± 0.6 | -0.3 ± 0.66  | -0.09 ± 0.5   |
| **FF (%)**      |          |          |          |           |             |               |
| Baseline        | 77.7 ± 9.4| 49.7 ± 6.9| 57.5 ± 7.6| 58.96 ± 3.3| 52.36 ± 2.9 | 50.6 ± 2.9    |
| Endpoint        | 76.3 ± 6.8| 45.3 ± 4.5| 53.3 ± 6.5| 67.90 ± 4.2| 74.7 ± 4.76 | 71.7 ± 1.5    |
| Δ               | -1.4 ± 8.1| -4.4 ± 5.7| -4.2 ± 7.05| 8.94 ± 3.75 | 22.34 ± 3.8 | 21.1 ± 2.2    |
| **FS (%)**      |          |          |          |           |             |               |
| Baseline        | 34.8 ± 4.7| 20.8 ± 2.6| 26.0 ± 2.5| 27.7 ± 5.45| 29.20 ± 3.1 | 27.40 ± 4.32  |
| Endpoint        | 36.0 ± 4.3| 19.9 ± 3.2| 23.6 ± 4.2| 29.3 ± 3.27| 34.67 ± 2.2 | 31.34 ± 3.45  |
| Δ               | 1.2 ± 4.5 | -0.9 ± 2.9| -2.4 ± 3.35| 1.6 ± 4.36 | 5.47 ± 2.65 | 3.94 ± 3.85   |
| **EDP (mmHg)**  |          |          |          |           |             |               |
| Baseline        | 97.0 ± 58.0| 2988 ± 302| 3121 ± 390| 3970 ± 619 | 4583 ± 730 | 4343 ± 520    |
| Endpoint        | 4970 ± 530| 2988 ± 302| 3121 ± 390| 3970 ± 619 | 4583 ± 730 | 4343 ± 520    |
| Δ               | 1.2 ± 4.5 | -0.9 ± 2.9| -2.4 ± 3.35| 1.6 ± 4.36 | 5.47 ± 2.65 | 3.94 ± 3.85   |
| **dp/dt max**   |          |          |          |           |             |               |
| Baseline        | 2405 ± 1240| 2430 ± 147| 2682 ± 401| 3387 ± 391 | 3810 ± 212 | 3640 ± 404    |
| Endpoint        | 4970 ± 530| 2988 ± 302| 3121 ± 390| 3970 ± 619 | 4583 ± 730 | 4343 ± 520    |
| Δ               | 1.2 ± 4.5 | -0.9 ± 2.9| -2.4 ± 3.35| 1.6 ± 4.36 | 5.47 ± 2.65 | 3.94 ± 3.85   |

Each value is an average of 10 readings ± SEM. MI, myocardial infarction; MSC, mesenchymal stem cell; ATV, atorvastatin; EDd, end-diastolic diameter; ESd, end-systolic diameter; FF, ejection fraction; FS, fractional shortening, dp/dt_max, left ventricular pressure, EDP, end-diastolic pressure.

* P < 0.05 compared with the MI group.
** P < 0.05 compared with the ATVNP + MSC group.
## P < 0.05 compared with the MSC group.
compared with that of MSC alone and MSC supplemented with ATV solution.

Conflict of interest

The authors declare that they have no conflicts of interest.

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