Sustained Release of Milrinone Using Microparticles: New Strategy for Future Therapy in Heart Failure

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Preface

This Thesis starts with an introduction (Chapter I), which includes review of the literatures on the use of nanotechnology in cardiovascular medicine. This work was published as book chapter:

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Nanotechnology for Cardiovascular Therapy: Current Perspective and Future Outlook

> Book title: Cell based therapy: *New Research* Related Nova publication: Neural Stem Cells and Cellular Therapy Philippe Taupin (Dublin City University, Glasnevin, Dublin, Ireland).

Chapter II was submitted as single manuscript:

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Prolonged action and sustained release of inotropes (milrinone) delivered via microparticles in a rodent model of myocardial infarction

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This work is dedicated to:

My Mother and Father: Fatma and Nasr

My beloved family: Safya and Nasr

My Uncle and best friend: Mansour

Sir Magdi Yacoub; touched my heart, and remains a continuous source of my inspiration in heart surgery and science

Abstract

Purpose:

The aim of this study is to construct a new drug delivery system for milrinone (Mil) using microparticles. This novel technology enhances drug bioavailability, decreases cytotoxicity and dosage frequency with future implication in the treatment of patients with end-stage heart failure.

Methods:

Polylactic-co-glycolic acid microparticles (PLGA-MPs) loaded with Mil were prepared by double emulsion-solvent evaporation technique. In-vitro release kinetics was estimated at T: 37C° and PH: 7.4. Twenty-four female Lewis rats were randomized into one of the three groups (n=8 per group), one week after left anterior descending artery ligation: Group I received intravenous (IV) injection of PLGA-MPs (250 µL); Group II received bolus IV Mil (50µg/kg/min prepared in 250 µL); and Group III received bolus IV Mil-PLGA-MPs with the same dose as Group II. All injections were given intravenously via tail vein over 10 mines. Transthoracic echocardiography and non-invasive heart rate and blood pressure measurements were performed at different pre-determined time intervals for 24hrs after the injection. After that, all rats were sacrificed and plasma was taken for cytokine assays and Mil level using HPLC.

Results:

In-vitro Mil-release kinetic studies showed a slow release of Mil over a 24-hour [Figure1]. Rats in Group III had significantly higher left-ventricular ejection fraction at 90mins, 3hrs, 6hrs and 12hrs post-treatment compared to other groups [Table1]. Plasma level of Mil at 24 hrs. post-injection was significantly higher in Group III compared to other groups (Group I= 0, Group II= 1.7 ±2.4, Group III=9.1±2.2 ng/ml, $P< 0.05$). The levels of ICAM and CINC-1 were lower in Group III compared to other groups ($P<0.05$).

Conclusions:

Inotropic drug encapsulation using microparticles can prolong the clinical effects of milrinone. The current study may provide a new strategy for future drug delivery in patients with end-stage heart failure.

Résumé

Objectif:

Le but de cette étude est de construire un nouveau système d'administration de médicaments pour milrinone (Mil) utilisant des microparticules . Cette nouvelle technologie améliore la biodisponibilité du médicament, diminue la cytotoxicité et la fréquence de dosage avec implication future dans le traitement des patients atteints d'insuffisance cardiaque au stade terminal.

Méthodes:

Microparticules d'acide polylactique -co- glycolique (PLGA- PM) chargés de Mil ont été préparés par la technique de double émulsion évaporation de solvant. In vitro cinétique de libération a été estimé à T : 37C ° et PH : 7.4 . Vingt-quatre rats Lewis femelles ont été randomisés dans l'un des trois groupes (n = 8 par groupe) , une semaine après ligature de l'artère descendante antérieure gauche: Le groupe I a reçu par voie intraveineuse (IV) l'injection de PLGA- PM (250 ul), le Groupe II reçu un bolus IV Mil (50µg/kg/min préparé dans 250 pi) et le Groupe III a reçu un bolus IV Mil- PLGA - députés avec la même dose dans le groupe II . Toutes les injections ont été administrées par voie intraveineuse via la veine caudale sur une période de 10 minutes. L'échocardiographie transthoracique, la fréquence cardiaque non-invasive et la pression artérielle ont été mesurés à différentes intervalles de temps prédéterminés pendant les 24 heures suivant l'injection . Après cela, tous les rats ont été sacrifiés et le plasma a été pris pour mesurer les dosages de cytokines et le niveau du Mil par HPLC.

Résultats:

Les étude de In vitro Mil libération études cinétiques ont montré une libération lente de Mil sur une période de 24 heures [Figure 1] . Les rats du groupe III avaient une fraction d'éjection ventriculaire gauche significativement plus élevée à 90mins , 3h , 6h et 12h post- traitement par rapport aux autres groupes [Table1] . Le niveau de plasma de Mil à 24 heures post- injection était significativement plus élevé dans le groupe III par rapport aux autres groupes (Groupe I = 0 , le groupe II = 1,7 ± 2,4 , le Groupe III = 9,1 ± 2,2 ng / ml , $P < 0,05$) . Les niveaux d' ICAM -1 et CINC étaient plus faibles dans le groupe III par rapport aux autres groupes ($p < 0,05$) .

Conclusions :

L'encapsulation de médicaments inotropes utilisant des microparticules peut prolonger les effets cliniques de milrinone . L'étude actuelle peut offrir une nouvelle stratégie pour l'administration future des médicaments chez les patients atteints d'insuffisance cardiaque au stade terminal.

Chapter I

Nanotechnology for Cardiovascular Therapy: Current Perspective and Future Outlook

Introduction to Cardiovascular disease

Cardiovascular diseases (CVD) remain the first cause of death in the developed countries and have significant economic impact around the world. The American heart association predicts that by 2030, 40.5% of the US population is projected to have some form of CVD and the direct medical costs will triple from $273 billion to $818 billion (3). In the past 30 years, the treatment for CVD had an enormous development by new drugs discovery (-statins, beta blockers), stents discovery, mechanical support devices and refinement of cardiac surgical procedures. Most CVD are preventable, and this highlights the importance of developing policies for prevention and powerful methods for early diagnosis and intervention. The advancement in molecular biology, genomics, proteomics and regenerative medicine helped in shading the light on new and specific targets for cardiovascular disease therapy. The knowledge of these discoveries and realizing their complexities is an essential step before designing any model for diagnosis and treatment.

The common CVDs are atherosclerosis and myocardial infarction. Atherosclerosis refers to the development of atheromatous plaques in the inner lining of the arteries. When the endothelial layer is injured by irritative stimuli, such as smoking, dyslipidemia, hypertension and diabetes mellitus, it expresses adhesion molecules (ICAMs) that capture leukocytes, such as monocytes, which differentiate to macrophages in the sub-endothelial layer. The endothelium also promotes entry of low-density lipoprotein (LDL) particles that will be oxidized and engulfed by macrophages resulting in the formation of foam cells. Then, the macrophages secrete numerous pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor-necrosis factor (TNF). Other cells such as mast cells and T-cells are also involved in the progression of plaque formation. Atheroma formation also involves the recruitment of smooth muscle cells (SMCs) that proliferates in response to mediators such as platelet-derived growth factor (PDGF). The SMCs produce extracellular matrix molecules, including interstitial collagen and elastin, and form a fibrous cap that covers the plaque. This cap typically overlies a collection of macrophage-derived foam cells, some of which die by apoptosis and release lipids that accumulate extracellularly. The inefficient clearance of dead cells can promote the accumulation of cellular debris and extracellular lipids, forming a lipid-rich pool called the necrotic core of the plaque. In advanced atherosclerotic lesions, MMP activity is up-regulated,

and interferon gamma (IFN-γ) produced by inflammatory T-cells down-regulate collagen production, resulting in thinning of the fibrous plaque cap. This process increases the vulnerability of the plaque to rupture. The balance between plaque proliferation and necrosis will determine and predict the probability of plaque rupture which is the leading event in acute myocardial infarction (4).

The vast improvement in treating patients with myocardial infarction by drug therapy, such as thrombolytic drugs, beta-blockers and angiotensin II blockers or by early percutaneous coronary intervention (PCI), leads to the increase in survival but with a dramatic growth in population suffering from heart failure. Myocardial protection and regeneration have a promising role in the future treatment of heart failure. In the setting of myocardial protection, the over-expression of cytoprotective and survival genes, such as antioxidant enzymes, anti-apoptotic proteins, and/or the inhibition of pro-inflammatory cytokines and pro-apoptotic genes has emerged as potential therapeutic targets for cardioprotection from studies in various animal and cellular models of myocardial ischemic injury (5,6). There is an emerging role of micro-RNA (miRNAs) in cardiovascular disease. They are small RNAs acting as negative regulators of gene expression. Some of miRNAs are involved in the processes of cardiac proliferation, hypertrophy, and pathogenesis that act as potential disease bio-markers and therapeutic targets (7-9).

Cellular based therapy has been attractive to many clinicians in the field of heart failure. Several cells such as endothelial progenitor cells (EPC), skeletal myoblasts, embryonic and fetal cardiomyocytes , and mesenchymal stem cells have been used for cellular cardiomyoplasty. This concept has been translated to the clinical side by conducting several clinical trials, which unfortunately showed disappointing and inconsistent results (10-12).

Despite the advanced knowledge in molecular pathways in cardiovascular pathology in the last few decades, little has been translated into bedside. The use of recent development of bio-technology and engineering may help in creating novel and targeted modalities of diagnosis and treatment. Currently, nanotechnology is a fast-rising area of research gaining support from scientists in the academic, industrial, and regulatory sectors. Since its establishment, the cumulative National Nanotechnology Initiative (NNI) program investment totals approximately $16.5 billion (13). Advanced applications of nanotechnology are being used to help in early diagnosis,

imaging tools, drug delivery and cellular cardiomyoplasty. Moreover, there is a trend in using nanoparticles as "theranostic" agents that combine both diagnostic and therapeutic functions. This can improve treatment efficacy, lower drug toxicity, and an overall decrease in morbidity and mortality (13-15).

Introduction to Nanoparticles

"Nano" is derived from the Greek word meaning "dwarf". One nanometer is equal to one billionth of a meter. The concept of nanotechnology was first introduced in 1959 by Nobel physicist Richard Feynmen with his remarkable statement: "There is plenty of room at the bottom" in which he described molecular machines building structures with atomic precision. The term ‘nano’ was given by Professor Norio Taniguchi from Tokyo Science University in 1974 in a paper on ion-sputter machining (16). The first nanoparticles made were liposomes in 1960s (17). After that, various types of nanoparticles have evolved, and some of them were approved by Food and Drug Association (FDA) as potential aid for disease diagnosis and therapy. In this section, we will discuss different types of nanoparticles and their various properties (Figure 1).

Liposomes

Liposomes are self-assembled lipid bilayer vesicles, with 20-150nm diameter to a few micrometers in diameter. They are formed from natural nontoxic phospholipids and cholesterol that make them highly biocompatible and suitable for in vivo use. These vesicles have aqueous core surrounded by hydrophobic lipid bilayer, hence they are suitable for encapsulating hydrophobic agents in the lipid shell, hydrophilic agents in the aqueous core and amphiphilic agents distributed through the hydrophobic hydrophilic domains. Furthermore, the liposome's outer corona can be decorated with materials such as polyethylene glycol (PEG) to enhance the circulation time, or with cell receptor specific antibodies in enhanced targeted therapy (18-20).

Micelles

Micelles are self-assembled colloidal nanostructures with a hydrophobic core and a hydrophilic shell. This allows the incorporation of hydrophobic or lipophilic bioactive agents within the micelles. Micelles can remain longer in circulation and spontaneously target body areas using the effect of enhanced permeability and retention effect by diffusing in regions with leaky vasculature, such as cancer, infracts and inflammation. The shell can be modified with targeting ligands to facilitate specific delivery to the diseased organ or tissue (20-22).

Polymer nanospheres

Polymers have become an essential class of materials in the biomedical area because of their mechanically plasticity, biocompatibility and biodegradability (23, 24). Biostable polymers are used in clinical applications, such as vascular grafts, pacemaker lead coatings, orthopedic fixation devices and intra ocular implants. Moreover, biodegradable polymers are used in sutures, wound-healing devices, implantable drug-delivery systems, fully polymeric stents as well as tissue engineering. The shape of Polymer nanoparticles can be solid, dense, porous structures or hollow structures. The shape of these particles has a significant impact of the enhancing target distribution and cell internalization (20, 24).

Ultrasound-sensitive nanoparticles

Ultrasound-based sonographic imaging modalities are well established in detection and diagnosis of physiological and pathological tissues. Based on the rationale that ultrasound can be used to destabilize nano-structures to induce drug loading, several attractive research strategies have been developed for nano-based ultrasound-mediated imaging and drug delivery in vascular diseases. Microbubbles are gas-filled bubbles that vibrate when a sonic energy field is applied and can reflect ultrasound waves. They are used for contrast ultrasound imaging agents in cardiology and radiology. These particles can be further modified by adding ligands to visualize the target diseased organ and promote early detection (20, 25-28).

Metal nanoparticles

Noble metal-based structures have been of great interest in biomedical applications such as, gold and silver. Gold-based nanoparticles have unique features and properties. Gold (Au) is resistant to oxidation under physiological conditions, which permits unrestricted interaction with the biological environment. They are usually less than 50 nm in size and have high surface area; hence they are used to deliver drugs in high doses. Moreover, Au-based nanoparticles exhibit unique optical properties that provide light absorption that eventually is converted to heat. This key feature is used in developing various photothermal therapy (PTT) strategies (20). However, gold nanoparticles can penetrate placenta barrier and testis-blood barrier with potential toxicity on the reproductive system (29).

Magnetic nanoparticles

Magnetic nanoparticles (MNPs) are novel and important tools in targeted therapeutic and diagnostic applications. They are 5-100 nm in size and are made from magnetic sensitive materials such as iron and cobalt. They exhibit a unique feature of lacking magnetization when exposure to the magnetic field is lost, that enable them to maintain their colloidal stability and biocompatibility. They can also undergo surface modification that can be applied in targeted imaging and therapy using MRI (30).

Super-paramagnetic iron oxide nanoparticles (SPION) are widely used MNPs and they are FDA approved. The first-generation of these agents such as Feridex and Resovist have the tendency to form polycrystalline clusters that are rapidly cleared by the retculo-endothelial system. Recently, several clinical trials are using new generation of these agents (ex: Ferumoxtran) that have longer circulation time. This will allow time for deeper tissue penetration in the myocardium and atherosclerotic plaque which eventually will enhance their therapeutic potential (31).

Quantum dots (QDs)

Quantum dots (QDs) are semi-conductor nanocrystals with the size of 1-5nm and contain ~1000 to 10000 atoms. These particles emit a spectrum of energy

and light according to their size. When the size of QDs increases, the light emitted will have less energy and change from blue to red. Further surface modifications of these particles and attachments of bioactive materials have been intensively explored for future application in cell imaging and targeted therapy (32, 33).

Viral particles and nanohybrids

Gene therapy using viral nanodelivery system has become an important approach in the treatment of myocardial infarction (34). This is because, in contrast to non-viral nanoparticles, viruses show much higher gene delivery efficiency with higher and prolonged gene expression for successful therapeutic outcomes. Extensive pre-clinical and clinical trials have been successfully performed with direct angiogenic gene delivery technique to the myocardium using viral delivery systems. It has been reported that intramyocardial delivery of Ang-1 and VEGF can further improve the cardiac function and attenuate ventricular infarct area as reported in other studies using mammalian vectors (35, 36). Recently, cell based gene delivery is under intense research in order to alleviate the risk of host immune rejection and unwanted inflammatory response commonly associated with direct viral gene delivery. Intravenous administration of hMSCs transfected with the angiopoietin-1 and VEGF gene using a fiber-mutant adenovirus vector has shown encouraging results for the treatment of ischemia (37). Although mammalian viral vectors are most commonly used for gene delivery, we have developed insect cell originated biologically safe baculovirus based angiogenic delivery vector (38, 39)The recombinant baculovirus was hybridized with vascular gene carrying nanoparticles by an ionic bond to form unique nanobiohybrid gene delivery system that brings together the advantages of both non-viral (lack of specific immunogenicity, simplicity of use, and ease of large-scale production) and viral gene therapy (efficient transduction, easy scale-up) systems. Direct and stem cell based intramyocardial delivery of this new kind of gene delivery nanocomplex has shown to reduce scar area, promote angiogenesis and significantly reduce cardiac dysfunction (38, 40).

Use of nanotechnology in cardiac regenerative medicine

The main strategy in cardiac tissue regeneration is to deliver stem cells and/or growth factors to the site of injury. This is defined as cellular cardiomyoplasty. Catheter or direct injection of stem cells around the infarct region has been used by different studies. The main challenge that faces this strategy is the low rate of stem cell retention following implantation (41, 42). However, increasing the number of cells did not have a significant impact to overcome this problem. In fact, it may be associated with decrease heart function and diastolic failure (43). Optimization of cell environment is an important consideration which can be done by providing essential growth factors, blood supply and extracellular matrix.

Microencapsulation of the stem cells delivered via intramyocardial injection post myocardial infarction was first reported by our lab (44). The semi-permeable membrane provides a stable microenvironment for stem cells by allowing oxygen and nutrients to diffuse into the cell environment, while permitting waste and by-products to exit. The size of the micropores allows stem cells to secrete cytokines to the exterior of the membrane while simultaneously preventing the destruction by immunological or mechanical factors (44). However, it has been demonstrated that encapsulation of stem cells can affect them by losing their differentiation ability (45). This could be attributed to cell leakage associated with breach in the poly-lysine (APA) membrane lining these particles. Recently, Alginate-chitosan polymeric microcapsules (GCAC) have shown superior features in housing stem cells and their growth. In addition, they were more resistant to rotation, osmotic pressure and myocardial contractions than (APA) particles (46). Mores studies are needed to further improve the delivering capacity of these particles which will have a significant impact on stem cell therapy.

Several growth factors were used in cardiac regenerative therapy, such as angiopoietin (Ang), fibroblast growth factor (FGF) insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). Self-assembling polypeptide nanoparticle**s** **(**SAPN**)** have been used to enhance the delivery of IGF along with transplanted cardiomyocytes or cardiac progenitor cells in infarcted heart model. This helped in reducing the apoptosis and

increasing the growth of the transplanted cells (47, 48). Similar approach has been used in different studies to deliver VEGF (49, 50), basic FGF (51-53) and chemokine stromal cell derived factor-1 (54) that showed promising results. Recently, our lab studied the effect of intramyocardial injection of Placental Growth Factor (PlGF)-loaded chitosan-alginate nanoparticles on ventricular remodeling post acute myocardial infarction. These particles allowed sustain release of PlGF more than 120 hrs. There has been a significant improvement in ejection fraction and decrease in the scar area compared with the control group. In addition, there was a decrease in pro-inflammatory cytokine (TNF α and IL-6) and increase in anti-inflammatory markers (IL10) (55).

One of the important strategies in regenerative therapy is to construct scaffolds that mimic the extracellular matrix (ECM) that will provide a three-dimensional structural and functional framework for cells to proliferate. Ideally, scaffolds should be biocompatible with the host tissue and degrade at a rate parallel to the formation of native ECM. In addition, they should have unique properties to encourage cellular retention, recruitment, proliferation and differentiation. Various biological (such as fibrin, collagen, hyaluronic acid and sodium alginate) and synthetic materials (such as polyesters and elastomers) have been used for scaffold construction. There is a rapid growing interest in using nanoscaffolds for tissue regeneration. Nanoscaffolds are made from nanofibers and have a greater surface area for cell adhesion than the conventional materials. Nanofibers are fabricated to resemble the most abundant fibers in ECM which are the collagen fibers that have diameter of nanometer to sub-microns. There are three methods for the development of nanoscaffolds: electrospinning, molecular self-assembly and thermally induced phase separation (TIPS). **Electrospinning** provides uncomplicated and inexpensive method of producing nanoscaffolds. This process produces a scaffold with nanofiber diameters ranging from 100-500 nm in size which overlap those of native EC. These types of scaffolds have been primarily used with mesenchymal stem cells, and allow cells to differentiate into different cell lineages. **Molecular self-assembly** is considerably a more complex procedure than electrospinning. Using co-polymers, DNA structures, lipids and proteins, these bio-molecules will self-assemble into stable structures without the interference of external sources. The self-assembly process is imperfect and produces fragments of fibers which are susceptible to endocytosis. Another

technique that has been used for several years to fabricate synthetic porous scaffolds is **thermally induced phase separation** (TIPS). TIPS involves the formation of a porous scaffold to mimic the fibrous structure of natural collagen. TIPS technique has been used to fabricate nano-fibrous matrices by using synthetic biodegradable polymers. Many of the polymers that have been developed for tissue engineering have been more suited for hard tissue engineering due to their elevated glass transition temperature and high modulus. For soft tissue, it is preferable to produce elastic scaffolds, which are malleable but maintain mechanical strength (56).

Recent studies demonstrate the capability of self-assembled nanofibers in creating the microenvironment of cardiac tissue. Endothelial cells and cardiomyocytes cultured in-vitro within nanoscaffolds lead to the growth cardiomyocyte outside the endothelial cells in capillary-like fashion along with the formation of electrical synchrony (57). This illustrates that nanofibers can help in cell assembly to form myocardium-like structures. In-vivo studies showed that by injecting these biomaterials in infarcted left ventricle of mice model, they formed hydrogel micro-environment that gradually get populated with endothelial cells and eventually merge with the host vasculature (58). These distinctive features of nanofibres lead to further development and modifications of their structure to be used in sustaining the growth factors of stem cells and retention of essential growth factors (59). Lin *et al* created nanofibres combined with VEGF and inject them in infarcted heart both in rats and pigs model. This combination creates optimal micro-environment for healing and promoting arteriogenesis and recruiting endogenous myofibroblast and cardiomyocytes to the damaged area. Eventually, this led to scar area reduction and rapid functional recovery of the heart (60).

Use of nanotechnology in cardiovascular imaging

The use of nanoparticles in medical imaging has a great potential in early and specific diagnosis of cardiovascular diseases. The promising feature of nanoparticles for imaging is their ability to deliver large numbers of imaging agents per targeted event. These particles have considerable capacity for internal packaging capacity, as well as a sufficient surface area for the presentation of multiple types and numbers of active elements that can target the area of interest (23, 61-63). Several vascular biomarkers have been studied to design nanoparticles that target thrombosis and atherosclerosis. For example, αVβ3 integrin is a well known biomarker for angiogenesis that gets up-regulated in certain diseases such as cancer, cardiomyopathy and atherosclerosis (64, 65). In-vivo studies done on hind limb ischemia model showed that dendritic nanoprobes labeled with positron or γ emitters and targeting αVβ3 integrin have 6 to 10 fold increases in signal in ischemic regions (66-68).

Ultrasound imaging is a widely used non-invasive imaging tool in modern medicine. Operator skills and patient factors such as obesity are important factors that limit its diagnostic yield. Microbubbles are intravascular contrast particles with a size of several micrometers that allow recirculation in the bloodstream. It is composed of a shell made of different materials, such as silver, surfactant, protein, biopolymer, galactose or lipids, and a center filled with different gases (25, 26, 28, 69). These agents can be equipped with different ligands to target various vascular or cardiac receptors. ICAM-1 and VCAM-1 are essential markers of early inflammation and using microbubbles to target these markers can guide to localize early inflammation and plaque formation (70-75). Glycoprotein IIb-IIIa receptors on the surface of activated platelets are useful markers for Clot imaging. Microbubbles targeting these receptors successfully bind to thromboemboli which may aid in fast localization of the areas at risk for thrombosis and ischemia (76-78). In addition, using high ultrasonographic impulses with these targeted microbubbles can help in recanalization and improvement of the microcirculation after myocardial infarction as shown in animal models (79, 80). The use of microbubbles has been expanded to target ischemic memory. Diagnosis of patient with chest pain of uncertain cardiac origin remains a challenge to emergency physicians. Acute myocardial ischemia/reperfusion

is associated with endothelial up-regulation of endothelial selectin that persists even after the resolution of ischemia. This concept has been utilized to construct ultrasound microbubbles to target P-selectin which showed a strong correlation between the area of the contrast enhancement and the area of ischemic risk (81, 82).

Contrast agents used in MRI and CT scan have dose-related toxicity especially in patients with kidney dysfunction. The use of nanoparticles can be further expanded in non-targeted applications by taking advantages of their unique biodistribution characteristics and biocompatibility. For example, Gadolinium contrast agents can be incorporated in liposome nanoparticles which lower the dose by 22-23 folds with same or better image resolution. This modification can improve the safety of these contrast agents especially in patients with renal impairment (83, 84). Super-paramagnetic iron oxide nanoparticles have been studied recently in assessing patients with myocardial infarction. After MI, the uptake of these particles occurs around infarcted myocardium which will help in assessing cellular myocardial inflammation and left ventricular remodeling.

The imaging of apoptosis in the heart was accomplished by using nano-based imaging agents. This was applied in the setting of acute ischemia and chronic heart failure which have different apoptotic pattern. Imaging apoptosis in heart failure is challenging because it is sparse and the capillaries are not leaky making the delivery of the imaging agent very difficult. Imaging apoptosis was performed using AnxCLIOCy5.5 nanoparticles. Each particle contains three to five annexin groups per super-paramagnetic cross-linked iron oxide (CLIO). The MRI signal provided by CLIO and near infrared fluorochrome Cy5.5 permits fluorescence imaging and microscopy of the agent to be performed. Several in-vivo studies in mice proved the ability of AnxCLIOCy5.5 nanoparticles in imaging cardiomyocyte apoptosis which correlate well with caspase-3 activity in the myocardium. While the translation of nanoparticle-based imaging agents is significantly more complex than that of radio-labeled imaging agents, this technology will provide an excellent platform for investigating the evolution of heart failure as well as the efficacy of novel therapies (85).

Use of nanotechnology in drug and gene delivery: focus on vascular stenosis

Several nanoparticles (NP) drug delivery systems been approved by the US Food and Drug Administration (FDA). Most of these drugs are used in the field of oncology, such as PEG-stabilized liposomal doxorubicin (Doxil, Evacet), Protein bound paclitaxel (Abraxane) and Pemetrexid (Alimta) (86). The advantage of NPs is that it can help to increase circulation time due to their small particle size. It has been shown that particles under 200 nm had longer circulation time regardless of any modification on the surface marking (87, 88). Clearance of nanoparticles from the circulation is usually by opsinization and further destruction by reticuloendothelial system (RES). It has been shown also that coating nanoparticles with hydrophilic polymers such as PEG can increase the circulation time and decrease their clearance by RES. Hence, using nanoparticles can perhaps solve issues related to drug compliance due to frequent dosages, dose adjustment in renal or liver failure patients and drug toxicity (89-91).

The administration of drugs as a single dose rather than multiple doses has recently been made possible using controlled-release formulations. This enables a constant level of the drug pharmacokinetics and ultimately increases the clinical efficacy. Using drug nanocomposites as sustained-release vehicles provides a breakthrough in novel drug delivery in cardiovascular disease therapy. Layered double hydroxides (LDHs), also known as anionic nanoclays or hydrotalcite-like compounds, have an attractive role in sustained drug delivery. They have unique properties such as ease of preparation, low cost, good biocompatibility, low cytotoxicity, and full protection of the drugs loaded (92, 93). This led to the development of controlled release formulation, such as antihypertensive drugs (lisinopril, enalopril, ramipril, captopril and perindopril), low molecular weight heparin (LMWH) and statins (pravastatin and fluvastatin) (94-99).

Targeted therapy is a concept that has been introduced by Paul Ehrlich, a German physician and scientist, with his famous "magic bullet" theory. This forms the bases for the recent development of immunotherapy. Nanoparticles can be used for

targeted therapy either in passive or active form. The passive targeting feature of nanoparticles lies heavily on the size of these particles. These agents have greater penetration and retention in regions with "leaky capillaries" such tumors, ischemic myocardium and atherosclerotic arteries. Moreover, nanoparticles can be structurally modified by adding surface ligands to target the diseased tissue as mentioned in the application in medical imaging (100, 101). These two mechanisms of targeted therapy have led to numerous studies on the treatment of atherosclerosis and myocardial infarction.

Percutaneous transluminal coronary balloon angioplasty (PTCA) is a common procedure used to treat coronary artery stenosis. This procedure is usually accompanied by deploying stents that prevent the vessel from recoiling and reduce the incidence of restenosis. However, this procedure is accompanied by injury to the endothelium, neointimal hyperplasia and eventually in-stent restenosis. Restenosis occurs due to multifactorial factors that involve smooth muscle proliferation, extracellular matrix elaboration and vascular remodeling. An intact endothelium leads to fast endotholization of the vessel and prevents further stenosis (102). This led to the design of drug-eluting stents which are stents medicated with anti-proliferative drugs such as sirolimus and paclitaxel,everolimus and zotarolimus. Although the incidence of in-stent restenosis has declined using these stents, high cost and prolonged dual anti-platelet therapy with its associative risk of bleeding are essential drawbacks of these stents (103, 104). One of the most critical challenges in targeting injured vessel after stent placement is the high shear pressure which leads to short residence time for the therapeutic agent at the target arterial wall cells (105). Several nano-based preparations have been revolutionized to treat in-stent restenosis which can be delivered either systemically or incorporated in stent structure. Paclitaxel eluted stent coated with porous carbon–carbon nanoparticles showed promising results with respect to rapid re-epithelization and reducing neointimal hyperplasia when compared to drug-eluting and bare metal stents in animal models (106-108). Using single injection of systemic nanoparticle paclitaxel showed almost complete resolution of intimal hyperplasia in rabbit model up to 28 days (109). These promising results led to the first clinical trial (SNAPIST-I) that evaluates the safety and feasibility of using this drug in human application (110). In addition, other drugs have

also shown a beneficial effect in treating in-stent restenosis using nano-based delivery systems, such as sirolimus (111) and prednisone (112).

Gene therapy is another strategy in treating in-stent restenosis. Several pre-clinical studies have delivered different genes using plasmid or viral vectors (113, 114). As expected, the efficiency of delivering these molecules was decreased by high shear stress and other factors that have been previously mentioned. Significant amount of work has been done in the field of nanoparticle-based gene delivery. Most of them utilized commercially-available cationic liposomes to deliver different genes, such as endothelial nitric oxide synthase (eNOS), chloramphenicol acetyl transferase (CAT), C-type natriuretic peptide (CNP), prostacyclin synthase (PGIS) and vascular endothelial growth factor (VEGF). (105) Catheter based VEGF gene delivery was performed in human clinical trial: Kuopio Angiogenesis Trial (KAT). In this trial, VEGF was delivered via viral or liposome vectors. This study demonstrated the safety of intra-coronary gene delivery. In addition, a significant increase in angiogenesis was detected in myocardial perfusion in patients treated with VEGF carried by viral vectors. However, no differences in clinical restenosis rate were present after 6 months follow-up (115). The possibility of insufficient VEGF cytokine concentration at the stent site along with the short half-life might lead to the suboptimal results of this trial. Recently, there has been development of a new nano-bioactive hydrogel coated vascular stent device using single walled carbon nanotubes (CNT). VEGF and ANG1 genes were combined using nanoparticles (NPs) and incorporated in the stent. The stent was tested in-vivo in a dog model and showed a significant reduction in intimal hyperplasia with fast re-epithlization of the injured artery (Figure 2). (2) This will potentially lead to the discovery of new generations of vascular stents.

Theranosis: the future medicine

The attractive features of nanoparticles and its application in diagnosis and therapeutic delivery lead to the establishment of the field of "theranosis" (Figure 3). The goal is to create particles that can be used both for imaging and therapy at the same time. This will facilitate treatment effect monitoring and reduction of the disease burden simultaneously (116). Visualization of theranostic particles can provide instant information that can help in drug dose, drug choice and duration of treatment. This can also lower off target toxicity, improve the efficacy and decrease in morbidity and mortality. This technology will lead to the creation and evolution of personalized medicine (14).

This strategy can be applied in monitoring the progression of atherosclerosis. Paramagnetic nanoparticles targeted against αvβ3-integrin and loaded with anti-angiogenesis drug fumagillin were studied in rabbit model of atherosclerosis. MRI showed initial enhancement of the plaque region which got significantly reduced in 7 days, demonstrating the decrease in angiogenesis and positive response to treatment (117). This effect has been shown to be prolonged by adding statin therapy and can represent a potential method to monitor atherosclerotic angiogenesis which is a sign for plaque instability (118). In another study, iron oxide nanoparticles were loaded with near-infrared fluorophores and phototoxic agents to attack macrophages in the atherosclerotic plaque. These particles are activated by light and lead to eradication of the inflammatory macrophages (119). Similarly, evaluation of in-stent restenosis (ISR) can be optimized and early detected using this technology. Perfluorocarbon nanoparticles loaded with anti-proliferative drugs (doxorubicin and paclitaxel) have been used to target tissue factors expressed in smooth muscle cell (SMC) that play a major role in the pathogenesis of ISR. Sophisticated MRI studies can allow detecting the uptake of these particles and distinguish them from the surrounding tissues (120). Other potential application of the concept of theranosis is the treatment of thrombosis and guiding the thrombolytic therapy (121-123).

Figures

Figure 1

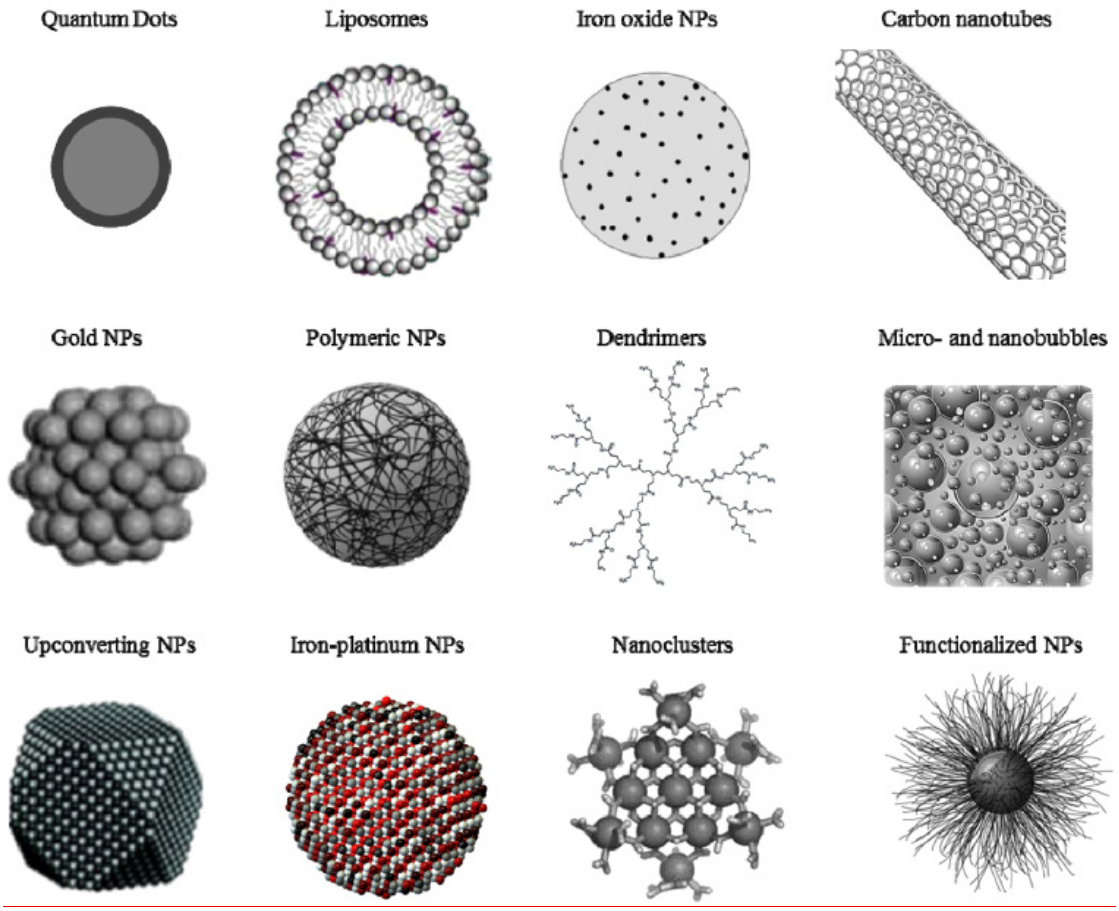

Figure 1:

Different types of nanoparticles used in nanomedicine.

Figure reproduced from (1)

Figure 2:

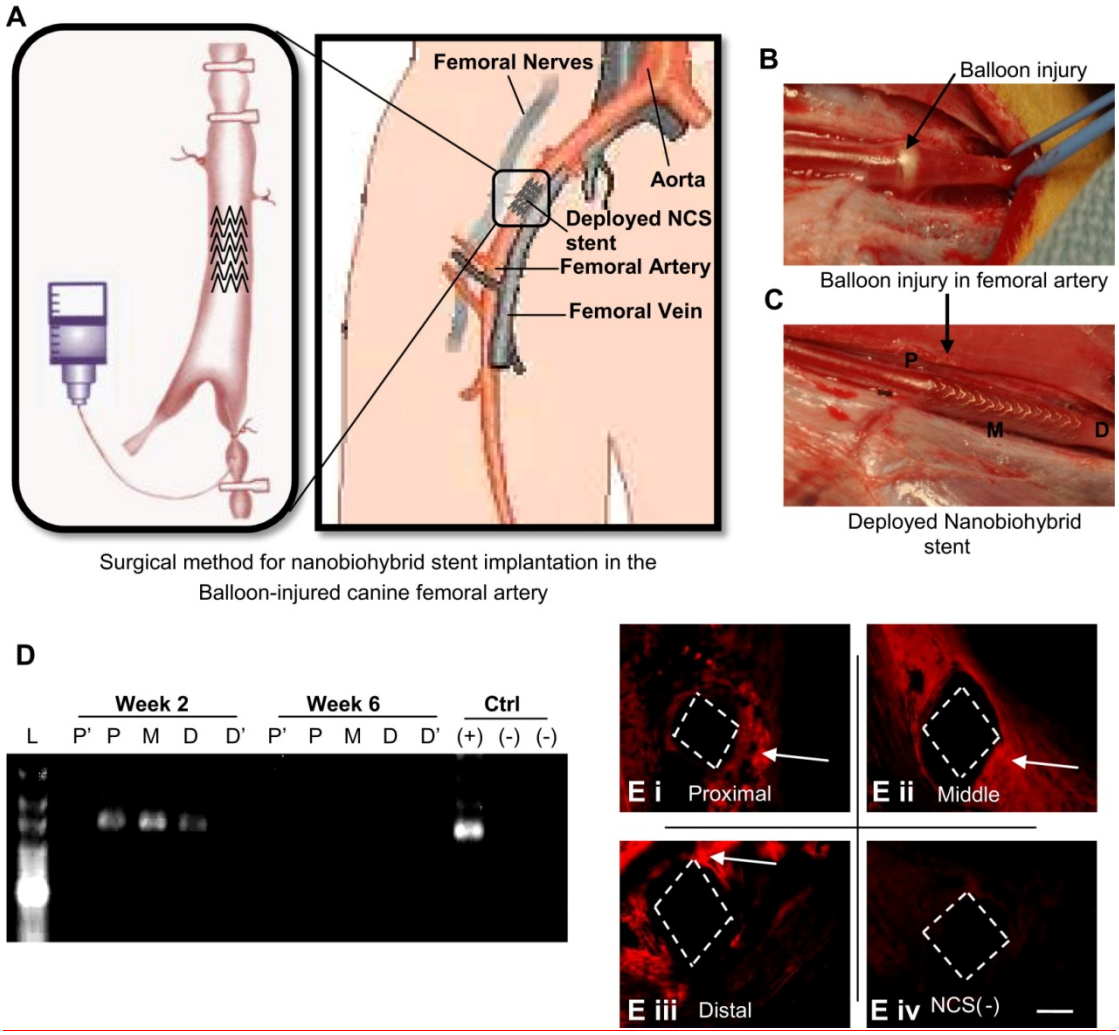

Figure 2:

Stent mediated gene delivery efficacy in dog femoral artery. (A) Schematic of surgical procedure for femoral stent implantation. (B) Denudation of femoral artery by creating balloon injury. (C) Deployed stent inside the denuded artery. (D) RT-PCR analysis confirmed NCS_{gfp} based gfp expression in the stented artery 2 weeks post stent implantation. Expression was not undetected in the artery sections 1 cm proximal (P′) and distal (D′) to the stented portion at both week 2 and 6. Expression of gfp disappeared by week 6 confirming the transient nature of the biotherapeutic stent. NCS (−) group was taken as the negative control E. Immunohistochemical localization of gfp in stented femoral artery at week 2. Transgene expression was localized at the strut area (white dotted) where the stent surface touched the artery inner lining with no expression in NCS (−), as indicated by the white arrows. Scale bar: 50 μm. Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; P, proximal; M, middle; D, distal portions of the stented artery.

Figure reproduced from (2)

Figure 3:

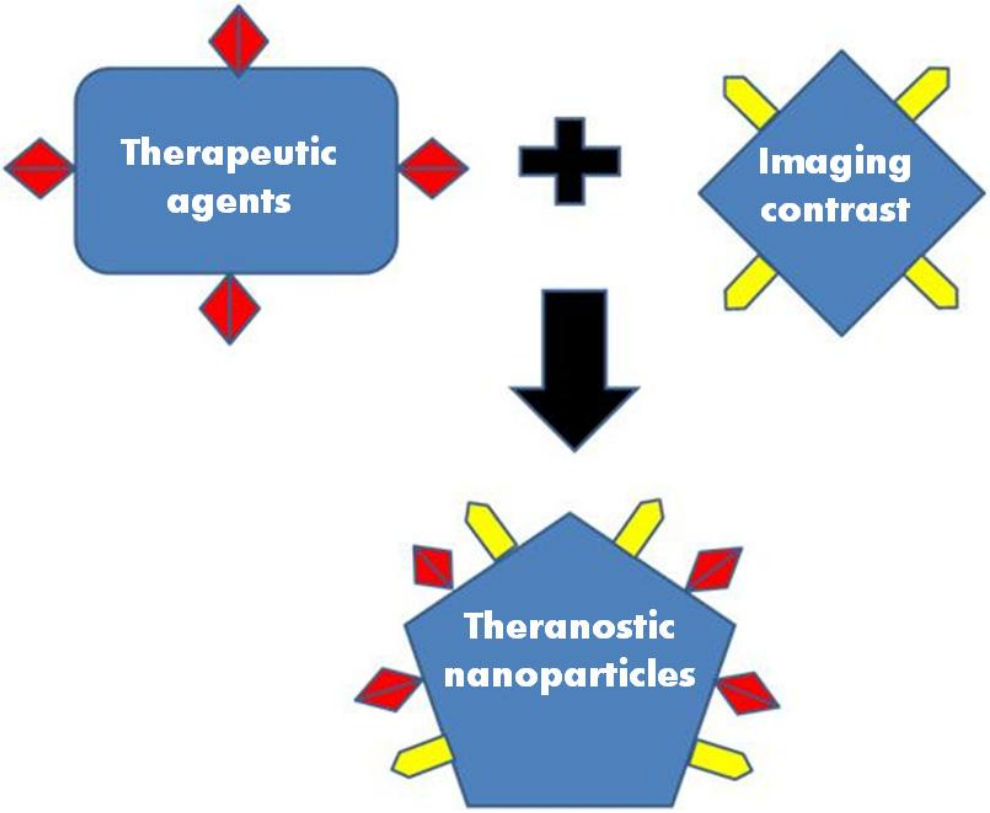

Figure 3:

Theranostic nanoparticles can be used both for imaging and therapy at the same time.

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Chapter II:
Sustained Release of Milrinone Delivered via Microparticles in a Rodent Model of Myocardial Infarction

Introduction

Nanomedicine is a new multidisciplinary field that has demonstrated promising potential in medical therapeutic and diagnostic applications. This evolving field deals with micro-and nanoparticles (MNPs) whose structures exhibit distinct physical, chemical and biological properties due to their small size. These features make them highly valuable in biomedical applications as biological markers, contrast agents for biological imaging and drug delivery systems (1). Drug encapsulation using MNPs can have the advantages of increasing drug circulation time, and passing across biological barriers that allow subsequent internalization and distribution within tissues of interest through passive and active targeting mechanisms (2).

Milrinone is a type III phosphodiesterase inhibitor that has strong inotropic and vasodilator effect. It has little effect on heart rate and oxygen consumption, which offer an advantage over other cardiac inotropes (3). Milrinone is used commonly to treat low cardiac output and high pulmonary arterial pressure (4). It also exhibits an anti-inflammatory effect, such that its role in the management of systemic inflammatory response syndrome (SIRS) following cardiopulmonary bypass (5) and/or sepsis has become more apparent (6, 7). The half-life of Milrinone in human is approximately 2 hrs and hence, it is usually given via central line as continuous infusion under monitored setting (8). Intravenous Milrinone had been reported to be given as an outpatient therapy for end-stage heart failure patients who required frequent re-hospitalizations, inotropic dependent, awaiting either heart transplantation or implantation of ventricular assist devices (9, 10). The logistic of outpatient, intravenous administration of a potent inotrope requires tremendous paramedical support, costly and potentially hazardous. Oral form of Milrinone is less effective with predictable outcome such that it is practically rarely used clinically (11).

The objective of this study was to construct a new drug delivery system for Milrinone using biodegradable microparticles to enhance drug bioavailability with controlled pharmacokinetics, and to decrease cytotoxicity and dosage frequency. This will potentially offer a novel therapeutic strategy, similar to Insulin administration in Diabetics, for the treatment of patients with end-stage heart failure. We hypothesized that intravenous injection of Milrinone prepared in biodegradable microparticles may prolong its inotropic effect by sustained slow-release mechanism that enhances myocardial function in a rat model of ischemic cardiomyopathy. To the best of our knowledge, this is the first study that delivers cardiac inotropes using biodegradable microparticles technology.

Methods

In-vitro studies

Preparation of Microparticles

Biodegradable PLGA [poly (lactic-co-glycolic acid] microparticles (MPs) were loaded with Milrinone using double emulsion-solvent evaporation technique. Briefly, Milrinone solution (2.5 mg) was added to PLGA (50mg) dissolved in 1 ml DCM (Dichloromethane). The primary emulsion was generated by a high-speed homogenizer (PowerGen 125, fisher Scientific, USA) for 2 mins. The double emulsion was achieved by adding the emulsion to 10ml of 0.5% (w/v) PVA (Polyvinyl Alcohol) and homogenizing again for another 1 min. To remove the organic solvent, the resultant W/O/W emulsion was placed under magnetic stirring for 3 hrs at room temperature. After 3hrs, the hardened PLGA MPs were then washed three times with distilled water by centrifugation. The MPs were prepared in three different batches and frozen at -20^{o}C for further use.

Morphology and Structure Characterization of Microspheres:

The morphology of the microspheres was studied by scanning electron microscopy (SEM) (Hitachi S-4700 FE). The size was confirmed using particle sizer instrument. The diameter of the microparticles was measured by the technique of electrophoretic laser Doppler anemometry using a Zeta Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY). The ZetaPlus Particle Sizer software (version 4.11; Brookhaven Instruments Corporation) was used to determine the size distribution of the microparticles. The particle sizes were measured for three batches of microparticles, and each measurement was obtained after taking the average of the three runs.

Milrinone Encapsulation Efficiency and Release Kinetics

The percentage of encapsulated Milrinone drug was estimated by the 2,3 dichloro-5,6-dicyanobenzoquinone (DDQ) test as described elsewhere (12). Briefly, DDQ was used here as a oxidative coupling and charge transfer reagent to quantify the Milrinone concentration using spectrophotometer. The drug loading efficiency was determined by dissolving accurately weighed amounts of drug carrying microparticles (approximately 15 mg) in 5mL acetonitrile and subsequent quantification by adding DDQ solution (0.05% w/v) in methnaol and reading absorbance at 356nm. To detect the release kinetics, the drug-loaded microparticles were placed in 10 ml phosphate buffer into glass vials. The containers were horizontally shaken at 37ºC. At pre-determined time intervals, 1mL samples were withdrawn (replaced with fresh PBS) and treated with DDQ as mentioned above for quantification using spectrophotometry. Each experiment was conducted in triplicate.

In-vivo studies

All experiments were performed on female Lewis rats (200—250g, Charles River,Quebec, Canada) in accordance with the guidelines set forth by the Canadian Council on Animal Care and were approved by the institutional ethics committee.

Ligation of the Left Anterior Descending Coronary Artery and Intravenous Microparticles Injection

Ligation of the left anterior descending artery (LAD) was performed through left thoracotomy as previously described (13). One week after ligation, all rats were randomized into one of three groups: Group I, received intravenous tail vein injection of empty PLGA microparticles (250 µl); Group II; received bolus intravenous tail vein injection of Milrinone (50µg/kg in 250 µl) slowly over 10 mins (14, 15); and Group III, received intravenous tail vein injection of Milrinone prepared in PLGA microparticles in equivalent dose and volume as Group II. The reason for using empty microparticles as a control was to ensure that these particles per se had no impact on cardiac function or other inadvertent toxicity. Various end point measurements were taken as described below. Twenty-four hours after tail vein injection, all rats were euthanized. The hearts were washed with PBS solution to remove excess blood and clots and then fixed in neutral-buffered 4% formalin. Blood was collected and centrifuged at 1200 rpm for 20 mins and the serum plasma was stored at -80ºC for further analysis.

Echocardiography, Heart Rate and Blood Pressure Measurements

Echocardiographic examinations, heart rate and blood pressure measurements were performed under inhaled isoflurane anesthesia (2.5% in oxygen, 500–700 mL/minute). Baseline trans-thoracic echocardiography was performed on day 4 post-

LAD for each rat and followed by serial measurements ligation, after tail vein injection at the following intervals: 30mins, 90mins, 3hrs, 6hrs, 12hrs and 24hrs. Serial echocardiographies were performed in a blinded fashion with a commercially available system (Micromaxx P04224; SonoSite, Bothell, WA), equipped with a linear probe 7–13 MHz 25 mm footprint turbo transducer (P06519.11; SonoSite). Left ventricular end-diastolic diameters (LVEDD) and end-systolic diameters (LVESD) were measured with M-mode tracings between the anterior and posterior walls from the para-sternal short-axis view just below the level of the papillary muscles. The time of end-diastole was defined as time of maximum diameter of the LV in one heart cycle. Accordingly, end-systole was defined as the minimum diameter. Left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) were estimated following the American Society of Echocardiology leading-edge method as previously described (16).

Blood pressure and heart rate measurements were obtained using CODA standard non-invasive blood pressure system from Kent Scientific Corporation. The measurements were done serially after intravenous tail injection at the following intervals: 5 mins, 15 mins, 30 mins and 90 mins.

Measurment of cytokines

In order to determine the pro/anti-inflammatory cytokines ratio, we measured plasma level of TNFα and IL-10 using ELISA kits (Biosource, Invitrogen, USA) according to manufacturer's recommendations. In addition, the relative cytokines level was detected in rat plasma using Proteome Profiler™ Rat Cytokine Array Panel A (R&D Systems, Minneapolis, MN, USA) as instructed by the manufacture. The average signal (pixel density) of the pair of duplicate spots representing each cytokine

was determined and subtracted from the background according to the manufacture recommendation (17).

Measurement of Milrinone Level using HPLC

Materials

Milrinone lactate 1mg/mL solution for injection was prepared from Sandoz Canada Inc. (Boucherville, Quebec, Canada); Amrinone used as internal standard was obtained from Sigma-Aldrich (Oakville, Ontario, Canada); HPLC grade methanol, HPLC grade acetonitrile, phosphoric acid and dipotassium phosphate were from Fisher Scientific Inc. (Ottawa, Ontario, Canada). Water was purified on site by reverse osmosis and Milli-Q.

Chromatography:

The HPLC chromatography system included Beckman System Gold 126 binary pump, Beckman System Gold 166 variable wavelength detector, Beckman System Gold 508 autosampler and Flatron TC-50 column thermostat. The chromatographic data were collected and processed using 32 Karat software. The separation of Milrinone lactate and Amrinone was achieved by Waters Symmetry C18 5um 4.7X150mm column (Milford, MA, USA). The mobile phase composition was made of 37.5% methanol and 62.5% 5mM K2HPO4 buffer in Milli-Q water, and was adjusted to pH 7.4 with H3PO4. Mobile phase was filtered on Teflon 0.45µm filter and degassed prior to the use. It was used as isocratic elution with the flow rate of 1.4mL/min. Internal standard solution was prepared by dissolving 1.0 mg of Amrinone in 500 mL HPLC grade acetonitrile. The Amrinone solution has two functions – to introduce the internal standard into the sample and to precipitate plasma proteins. The standard solution of Milrinone lactate was prepared by dilution of 1 mg/mL original Milrinone lactate solution in Milli-Q water. A 200µL of plasma

aliquot was transferred into 1.5 mL polypropylene microcentrifuge tube then 800µL of Amrinone solution was added to it before the tube was closed, vortexed at a high speed for 1 minute and then centrifuged at 12000g for 3 minutes. Following centrifugation the supernatant was transferred into a clean glass test tube (10X75mm) and evaporated at 60°C under a gentle flow of dry nitrogen. After complete liquid evaporation 100 µL of mobile phase was added to the test tube, then vortexed for 30 seconds and the liquid was transferred into HPLC vial with 100 – 150 µL insert for analysis. Injection volume was 27 µL. Standard samples were prepared exactly the same way with the only difference that instead of plasma, the standard solution of Milrinone lactate with concentrations in the range of 1 – 1000 ng/mL was used.

Method Validation

Recovery of Milrinone was estimated as 84%, which is acceptable for the internal standard method, the detection limit was 2.5ng/mL and quantitation limit was 5ng/mL. The method is linear in the wide concentration range (5–1000 ng/mL), with LSQ weighting 1/response^2 the goodness of fit (r^2) was 0.997.

Statistical Analysis

All statistical analyses are carried out using the SPSS program version 16. All statistical tests are two-tailed. **P**-values of <0.05 are considered statistically significant. All data are expressed as mean ± standard deviation (SD). Repeated echocardiographic variables, heart rate and blood pressure measurements are compared by means of one-way repeated-measures analysis of variance (ANOVA). In addition, ANOVA is used to compare the cytokines and Milrinone level among the groups at 24 hrs post-injection. If a significant F-ratio is obtained, a Bonferroni post-hoc test will be used to assess pair-wise differences.

Results

Morphology and Structure Characterization of Microspheres

Electron microscopic analysis confirmed the presence of microparticles and provided morphological information on typical Mil-loaded PLGA microparticles (Figure 1A). The particles were about 4-5 μm in diameter. They have an oval shape and smooth surfaces. Loading PLGA microparticles with Milrinone did not result in any significant change in shape and size of the microparticles.

Milrinone Encapsulation Efficiency and Release Kinetics

The encapsulation efficiency of Milrinone with different PLGA concentration is shown in (Figure 1B). At Mil: PLGA concentration ratio of 1:10 and 1:20, the encapsulation efficiency is 16.45%±4.3 and 23.62%±3.3, respectively and there was no significant change in size compared to free microparticles. Increasing Mil:PLGA concentration ratio to 1:40 resulted in improvement of the encapsulation efficiency to 27.96%±5.6 with increase in size of the microparticles to 7.38±5.6.

The Percentage of Milrinone release from PLGA particles over 24 hrs is illustrated in (Figure 1C). There was an initial burst effect release phase followed by lag release phase, which is characteristic to PLGA microparticles (18, 19). Changing the (Mil:PLGA) ratio from 1:10 to 1:40 decreased the amount of Milrinone during the burst and the lag phase. A Mil:PLGA ratio of 1:20 was selected for the in-vivo experiments because the encapsulation of Milrinone at this concentration result in minor changes in microparticle size and shape with gradual and slow release of Milrinone over 24 hrs.

Echocardiography, Heart Rate and Blood Pressure Measurements

Figure 2 shows the means and standard deviations of LVEF and LVS% measured for all the groups at predetermined time intervals. The baseline and post-LAD ligation LVEF and LVFS were not significantly different among the groups (P >0.05). At 30mins; the mean LVEF and LVFS measured in Group III **(LVEF: 73±9%**, **LVFS: 52±6%)** and Group II **(LVEF: 69±3%**, **LVFS: 44±3%)** were significantly higher than Group I (**LVEF: 47±2%**, **LVFS: 26±0.6%; P<0.05)**. At 90mins, the mean LVEF and LVFS in Group III **(LVEF: 72±8%**, **LVFS: 51±6%)** were significantly higher than both Group I **(LVEF: 46±3%**, **LVFS: 25±1%)** and Group II **(LVEF: 60±7%**, **LVFS: 36±5%; P<0.05)**. At 3hrs, 6hrs, and 12hrs: the mean LVEF and LVFS in Group III were gradually decreased over time but were nevertheless significantly higher compared to the other groups **(P<0.05)**. At 24 hrs, Group III had a tendency of higher LVEF and LVFS recovery post-injection compared to Group II and I but did not reach statistical significance (P>0.05). One-way ANOVA showed no significant difference in heart rate and blood pressure among the groups over pre-determined time intervals (P>0.05).

Serum Cytokines Measurements

The mean pro-inflammatory/anti-inflammatory cytokines ratio (TNF-α/IL-10) measured in the plasma of all rats is illustrated in Figure 3A. Serum TNF-α/IL10 ratio was significantly higher in Group I (**6.2±1.1**) compared to Group II and III (**4.2±0.4**, **3.8±0.8, P<0.05**). Group III has lower TNF-α/IL-10 compared to Group II but did not reach statistical significance (P=0.5).

The relative multi-cytokines panel is shown in Figure 3B. Vascular endothelial growth factor (VGEF) and macrophage inflammatory protein-3α (MIP-3α) signals were absent in both Groups II and III. The relative pixel density of cytokine-induced

neutrophil chemoattractant-1 (CINC-1) and intercellular adhesion molecule-1 (ICAM-1) in Group III (CINC-1: **0.86±0.02**, ICAM-1: **0.18±0.01**) were significantly lower compared to Group I (CINC-1: **1.03±0.02**, ICAM-1: **1.01±0.04**) and II (CINC-1: **0.8±0.02**, ICAM-1: **0.31±0.01**, $P<0.05$).

Milrinone Assay using HPLC

Both Milrinone lactate and Amrinone were detected at wavelength 325 nm. The retention time of Milrinone was 6.15 min and IS (Amrinone) was 3.93 min. The total run time was 8 minutes. HPLC analysis (Figure 4) showed that the Milrinone plasma level at 24 hrs post-treatment in Group III is significantly higher than Group I and II (Group I: 0 unit, Group II: 1.7±2.4, unit Group III 9.1±2.2 ng/ml, $P<0.05$).

Discussion

The technologies of micro-and nanoparticles (MNP) have advanced tremendously over the last two decades for their potential applications in various health care fields. MNPs promote the innovative creation of drug delivery systems that increase drug bioavailability, decrease toxicity and enhance drug targeting by passive or active mechanisms (20). The US Food and Drug Administration (FDA) have approved several drug delivery systems, using these particles. Most of these drugs were used in the field of oncology, such as PEG-stabilized liposomal doxorubicin (Doxil, Evacet), Protein bound paclitaxel (Abraxane) and Pemetrexid (Alimta) to deliver a highly toxic drug in a target-specific site with minimal side-effects (21). In cardiovascular drug delivery, MNPs has been used in different settings that focused mainly on the treatment of in-stent re-stenosis (ISR) (22, 23). In this current study, we have demonstrated the feasibility of using microparticles to prolong the inotropic effect of single injection of Milrinone by a slow drug release mechanism attributed to the semi-permeable properties of the polymer. To our knowledge this is

the first report that utilized microparticles to deliver cardioactive drugs for the treatment of heart failure.

The microparticles used in this study for Milrinone encapsulation was prepared from Poly (lactic-co-glycolic acid) (PLGA) polymer which is one of the most widely used FDA approved biodegradable polymers. This polymer has the following attractive features: (a) biocompatibility, (b) well described formulations and methods of preparation adapted to various types of hydrophilic (e.g.: Milrinone) or hydrophobic drug applications, (c) possibility of sustained release preparation, and (d) protecting drugs from rapid degradation (24). The size of the PLGA microparticles (PLGA MPs) used in this study is about 4 μm, which is ideal for intravenous route and provides sustained systemic release of the drug without the risk of embolization. Particles > 10 μm will persist at the site of injection and would be appropriate for local delivery (25), however, particles less < 1 μm will be ideal for targeted therapy because of their ability to cross mucous barrier, endothelial cell, and even blood-brain barrier (26). The release kinetics curve of Milrinone in-vitro showed an initial burst effect, which is characteristic of the PLGA microparitcles. The initial burst release is the quantity of the drug that escapes from microparticles prior to the onset of polymer erosion-mediated drug release (27). Here, we found that the burst effect in-vitro was at 6-7 hrs, however, the rise in EF after injecting Mil-PLGA MPs in the rat was early in the first 30 mins. This discrepancy could be explained by the fact that Milrinone is a hydrophilic drug and that will facilitate its diffusion to the surface of the particles especially after entering the blood stream (28). The fast burst effect could be modified by wide particle size distributions or coating MPs with materials that can provide a protective layer that will help in delaying the burst effect and increasing the circulation time (29). As such, many MNP designed options are technically available

to obtain the optimal pharmacokenetics desirable for any drugs for different clinical purposes.

Milrinone has been widely used clinically in the treatment of low cardiac output, pulmonary hypertension and right ventricular failure. It is indicated (class IIb) for continuous long-term support in patients with end stage heart failure who are inotropic dependent and awaiting either transplantation or ventricular assist device implantation (30). In this current study, we have shown a significant recovery in left ventricular contractility in the group receiving Mil:PLGA microparticles compared to the other groups for at least 12 hours or potentially 24 hours. The effect was prolonged at least 4 times longer than Milrinone as free drug given in the same dose and route. Apart from its strong inotropic effect, Milrinone has also an essential anti-inflammatory effects reported in many experimental and clinical trials in the settings of myocardial infarction, congestive heart failure, cardiopulmonary bypass and sepsis. These effects are probably indirectly related to the improvement of hemodynamics after administering the drug. However, direct effects via elevating cAMP and inhibiting the NF-kb pathway, hence will decrease the production of pro-inflammatory cytokines have been suggested (31, 32). Similar to previous reports, we have shown that giving single dose Milrinone, either as free drug or prepared in microparticles, could result in a significant reduction in the pro-inflammatory markers (TNFα, chemokines: CINC-1and MIP-3α ICAM and VEGF). In addition, encapsulation of Milrinone using microparticles prolonged its effect on the production of these cytokines as suggested by a significant lower level of CINC-1 and ICAM in Group III despite having received the same dose of Milrinone in both Group II and III.

There are several limitations associated with this study. First, this is the first application of NMPs in delivery of cardioactive drugs. The dose-responsive characteristics, optimal hemodynamic effects, and determination of unacceptable hemodynamic side effects need to be defined. Nevertheless, we have shown the feasibility to prolong the inotropic effect of Milrinone without increasing toxicity or side effects. Second, there was no invasive hemodynamic monitoring to obtain objective data regarding cardiac output, blood pressure, heart rate, systemic resistance and pulmonary pressure etc. Trans-thoracic echo may potentially create a bias because it is operator dependent. To minimize this bias, the echocardiographic operator and analysis in this study were blinded to the type of injection and treatment. Third, there was no detailed in-vivo pharmacokinetics study to document Milrinone release over time. Finally, the variation of PDE III expression across different species should also be considered. This may account for the differences in inotropic effects on human versus rats. It has been reported that more inotropic effect is seen on the same dose of Milrinone in humans than rats (33). This reiterates the need for further studies to investigate the dose-response characteristics and species differences of Milrinone.

In conclusion, the encapsulation of Milrinone in microparitcles may provide new strategies in delivering these hemodynamically potent drugs in more gentle and prolonged fashion while minimizing hemodynamic side effects. Future studies are needed to optimize the encapsulation and Milrinone release in predictable manner. In addition, using PLGA microparitcles technology can potentially develop different sustained release Milrinone formulation, such as intramuscular, subcutaneous or oral form that can be delivered via various routes. Inhaled Mil-PLGA microparticle is one such example that sustained Milrinone delivery can be achieved by nasal administration with minimal systemic side effects and toxicity. The application of

MNPs in drug delivery has proven to be effective and feasible. Further exploitation for cardiovascular application may hold great potential for the treatment of patients with congestive heart failure.

Figure legends

1. **Figure 1:** Characterization of microparticles: (**A**) Transmission electron microscopy was used to obtain the size characterization. The PLGA microparticles measured ≈4 μm in diameter. Most microparticles were spherical in shape. (**B**) The encapsulation efficiency using various Mil-PLGA concentrations. (**C**) In-vitro release kinetics of Milrinone (Mil)-loaded PLGA microparticles over time.

   Note: Mil-PLGA concentration of 1:20 was used in this study.

2. **Figure 2:** Echocardiographic analysis. Data are presented as mean ± SD. (**A**) Graphs of left ventricular ejection fraction determined by 2D images. (**B**) Graphs of percentage fractional shortening determined by 2D images.

   Note: *P<0.05 Group III vs Group I and II ; $^{\Psi}$ P< 0.05 Group III vs Group I; **P<0.05 Group II vs Groups I.

3. **Figure 3:** Plasma cytokine levels for Group I (empty microparticles), Group II (Milrinone), and Group III (microparticles+Milrinone). (A) Ratio of serum tumor necrosis factor (TNF)-alpha to IL-10 levels.Note: *P< 0.05 Group I vs Groups II and III. (B) Rat Cytokine Panel A: Representative array images are shown on the *upper panel*. Profiles created by quantifying dot densities, background-subtracted, and normalized to positive controls, are presented in the *lower panel*. Note the signal intensities of sICAM, and CINC-1 in Group III are significantly less than other groups. MIP-3α and VEGF are absent in Group II and III.

Note: *P<0.05 Group III vs Group I and II; **P<0.05 Group II vs Groups I; $^{\Psi}$ P< 0.05 Group I vs Group II and III.

Abbreviations: CINC-1: Cytokine-induced Neutrophil Chemoattractant-1; sICAM: Intercellular Adhesion Molecule; LIX: Lipopolysaccharide-induced CXC chemokine; MIP-3α: Macrophage Inflammatory Protein-3α; RANTES: Chemokine ligand-5; TIMP-1: Tissue Inhibitors of Metalloproteinases-1; VEGF: Vascular Endothelial Growth Factor.

4. **Figure 4:** HPLC of Milrinone in rat plasma: **(A)** Group I (empty microparticles); (**B**) Group II (Milrinone); and (**C)** Group III (microparticles Milrinone); **(D)** The mean of plasma Milrinone level in all three groups at 24 hrs post-tail vein injection.

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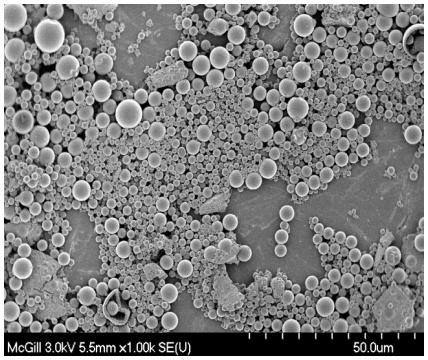
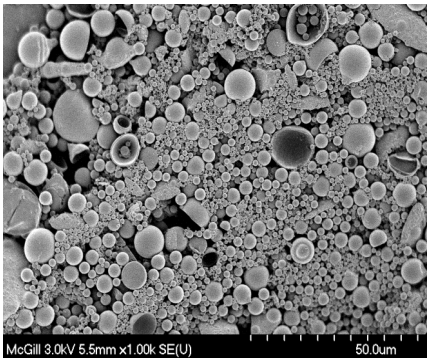
Figures

Figure 1: Characterization of microparticles

1A
Structure Characterization of Microparticles



Empty MP



MP+ Milrinone

Milrinone encapsulation efficiency

Milrinone: PLGA	Homogenization	Diameter
wt/wt	% Encapsulation	µm
1:10	16.45±4.3	4.20±1.5
1:20	23.62±3.3	4.81±2.3
1:40	27.96±5.6	7.38±5.6

1C
Milrinone release kinetics

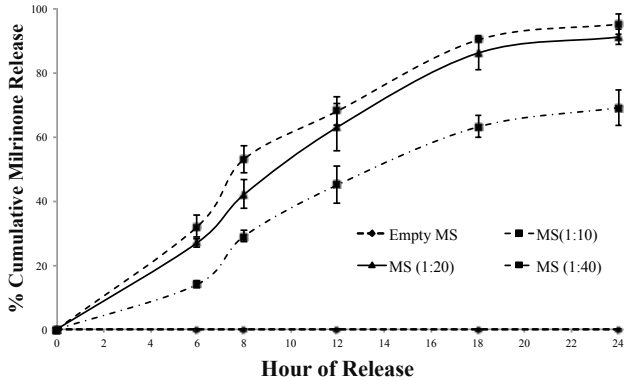
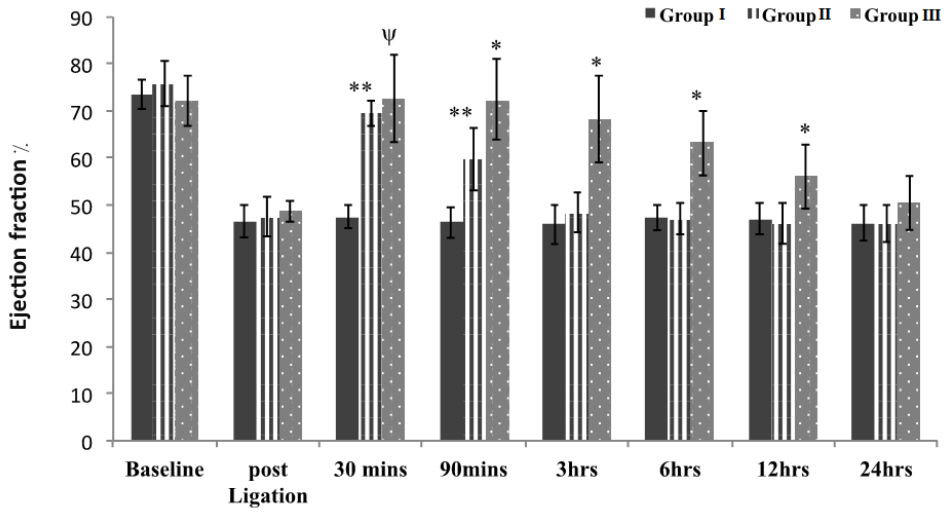


Figure 2: Echocardiographic analyses

2A
Left Ventricular Ejection Fraction



2B
Left Ventricular Fractional shortening

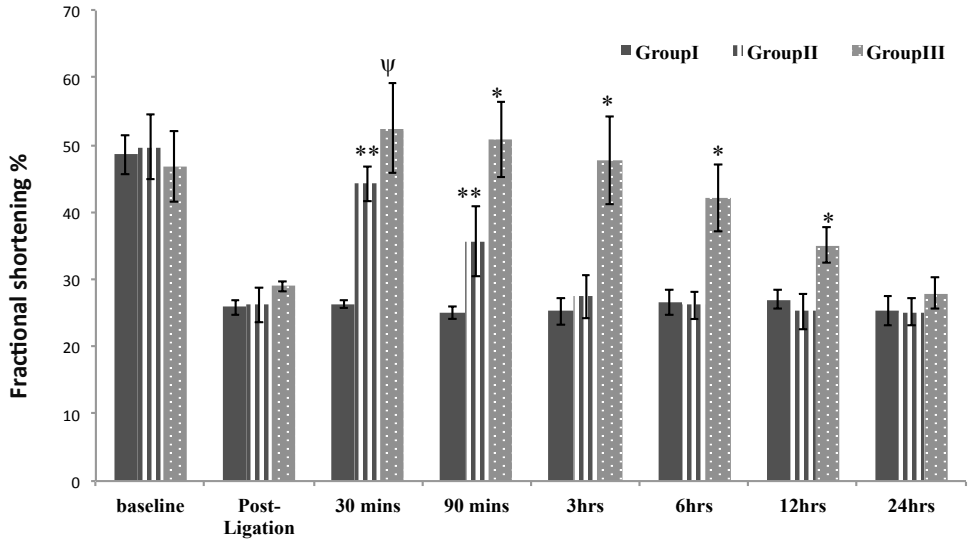
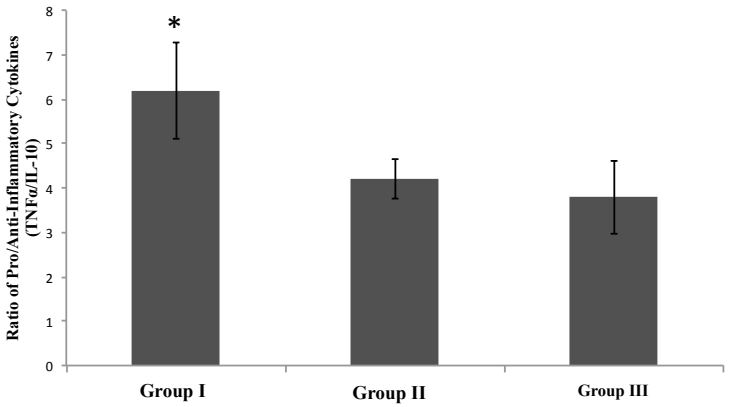


Figure 3: Plasma cytokine analyses

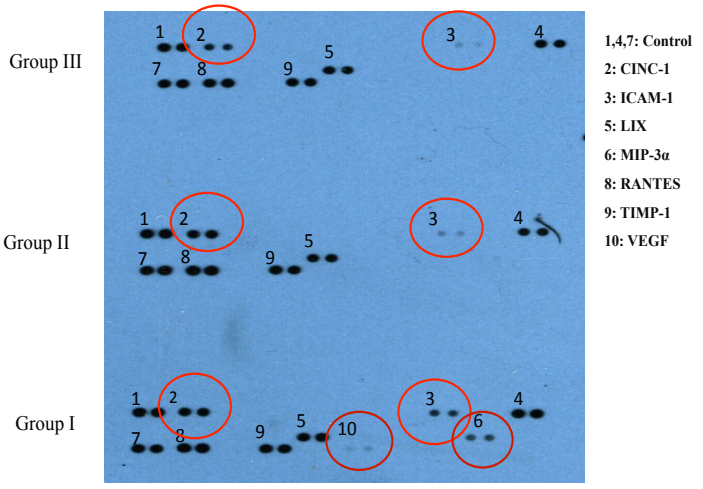
3A

Pro/anti-inflammatory cytokines ratio



3B

Rat Cytokine Array Panel A



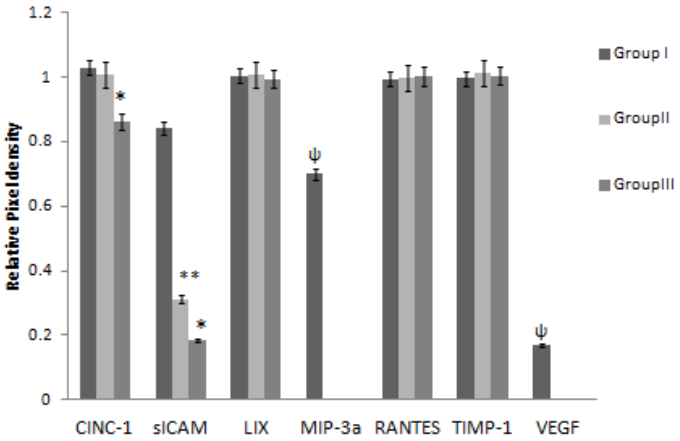
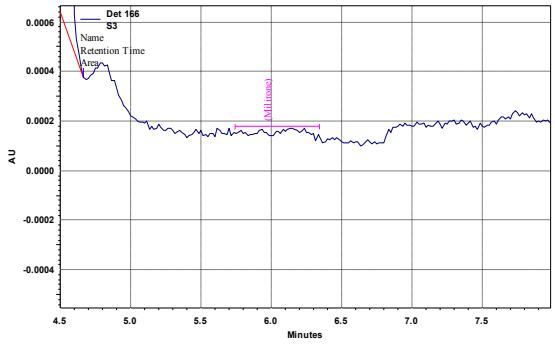


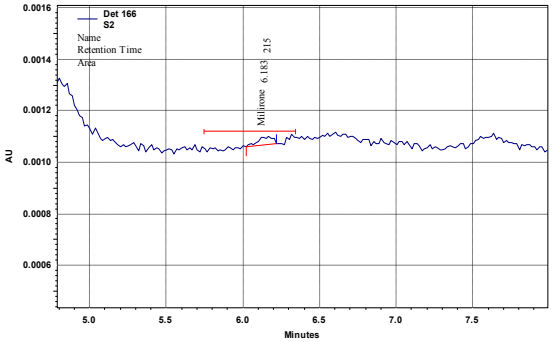
Figure 4: HPLC of milrinone in rat plasma

4A



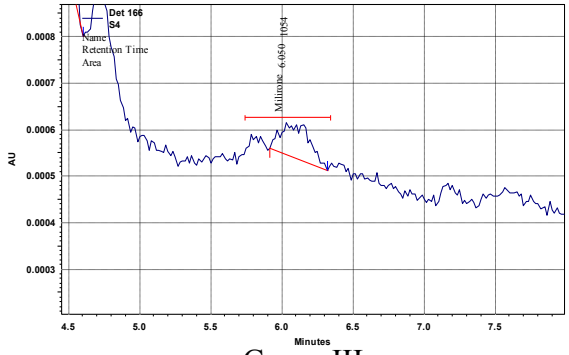
Group I

4B



Group II

4C



Group III

4D

Mean± SD	Group I (n=8)	Group II (n=8)	Group III (n=8)	P value
Mil level (ng/ml)	0	1.7±2.4	9.1±2.2	P<0.05 (Group III vs Group I &II)

Closing remarks

The applications of nanotechnology in the management of cardiovascular disease are expected to increase rapidly in the near future. Nanotechnology provides several advantages in drug delivery by increasing effective concentration at the disease site and reducing systemic toxic effects. Sustained delivery of growth factors and stem cells along with the fabrication of extracellular matrix will play an important role in the field of regenerative medicine. This will eventually help in optimizing care for the individual patient and contribute to the field of personalized medicine. Increased awareness of this technology by clinical and health expertise can help in identifying areas of greatest need for this technology and facilitate a cost-effective transformation of these innovations to human health care across the world.

To our knowledge, this is the first study that describes the use of microparticles to deliver cardiac inotropic drugs. The Encapsulation of milrinone in microparitcles may provide new strategies in delivering these powerful drugs in more gentle and prolonged fashion. Future studies are needed to optimize the encapsulation and the release of milrinone in slower and predicted manner. In addition, using PLGA microparitcles can help in developing different sustain release milrinone formula that can be delivered via various routes, such as IM, SC or oral route. Using Inhaled PLGA microparticles is another promising route for sustained milrinone delivery with minimal systemic side effects and toxicity. This eventually will aid in the discovery of new drug delivery systems for patients with end-stage heart failure.