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# Targeting and modulating infarct macrophages with hemin formulated in designed lipid-based particles improves cardiac remodeling and function

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ABSTRACT

Uncontrolled activation of pro-inflammatory macrophages after myocardial infarction (MI) accelerates adverse left ventricular (LV) remodeling and dysfunction. Hemin, an iron-containing porphyrin, activates heme oxygenase-1 (HO-1), an enzyme with anti-inflammatory and cytoprotective properties. We sought to determine the effects of hemin formulated in a macrophage-targeted lipid-based carrier (denoted HA-LP) on LV remodeling and function after MI.

Hemin encapsulation efficiency was ~100% at therapeutic dose levels. In vitro, hemin/HA-LP abolished TNF-α secretion from macrophages, whereas the same doses of free hemin and drug free HA-LP had no effect. Hemin/HA-LP polarized peritoneal and splenic macrophages toward M2 anti-inflammatory phenotype. We next induced MI in mice and allocated them to IV treatment with hemin/HA-LP (10 mg/kg), drug free HA-LP, free hemin (10 mg/kg) or saline, one day after MI. Active in vivo targeting to infarct macrophages was confirmed with HA-LP doped with PE-rhodamine. LV remodeling and function were assessed by echocardiography before, 7, and 30 days after treatment. Significantly, hemin/HA-LP effectively and specifically targets infarct macrophages, switches infarct macrophages toward M2 anti-inflammatory phenotype, improves angiogenesis, reduces scar expansion and improves infarct-related regional function.

In conclusion, macrophage-targeted lipid-based drug carriers with hemin switch macrophages into an anti-inflammatory phenotype, and improve infarct healing and repair. Our approach presents a novel strategy to modulate inflammation and improve infarct repair.



## 1. Introduction

Despite significant progress in the treatment of acute myocardial infarction (MI), many patients develop adverse left ventricular (LV) remodeling, a maladaptive process associated with progressive heart failure and death [1–3]. The adult myocardium is unable to regenerate new cardiomyocytes to replace significant cell loss after MI. Currently, there are no therapies that can form new functional cardiomyocytes in the infarcted heart. Thus, there is a need to develop novel treatments for post-MI repair.

The immune system, particularly macrophages, governs both the healing and repair process after MI [4–7]. In acute MI, failure of transition from a pro-inflammatory (M1) macrophage to a reparative (M2) macrophage may induce prolonged M1 macrophage activity and affect the outcome of infarcted myocardium [4,8–10]. Impaired resolution of inflammation poses a major threat for a variety of cardiovascular diseases [11]. Thus, modulation of pro-inflammatory macrophages to anti-inflammatory macrophages is proposed as a new therapeutic approach [9,12,13]. In the past, most approaches have focused on minimizing the inflammatory response after MI. However, recent studies have demonstrated that tuning the balance between pro- and anti-inflammatory responses can benefit infarct repair strategies by activating endogenous healing and repair [5,14,15]. Thus, understanding the role of macrophages and its importance in the context of myocardial regeneration and repair is now considered a great translational challenge, about which little is known [6,13,16].

Controlled drug delivery, applying particular drug carriers that can enable the targeting of macrophages in the infarcted heart, could provide great potential to boost efficacy of MI repair. Lipid-based drug

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carriers offer great potential for improving therapeutic efficacy in acute MI [13,17–19]. While the entry of drugs to the infarcted myocardium is highly restricted, drug-carrying particles can significantly improve the pharmacokinetics and bio-distribution relative to free drugs.

We sought to test the hypothesis that activation of heme oxygenase-1 (HO-1) in macrophages would improve infarct healing and repair. We chose to load the lipid drug carriers with hemin. The rationale for our approach was that hemin is a potent HO-1 inducer as well as an HO-1 substrate. HO-1 exerts its protective effects through the degradation of heme and the subsequent production of anti-inflammatory, anti-apoptotic and anti-oxidative molecules [20]. Heme-mediated induction of HO-1 reduces inflammation selectively by suppressing M1 macrophages and activating M2 reparative macrophages [21]. Development of a therapeutic approach that improves infarct healing and repair would reduce post-MI morbidity and mortality that result from severe cardiac injury, and would be of enormous importance to global health.

## 2. Materials and methods

The study was performed in accordance with the guidelines of the Animal Care and Use Committee of the Sheba Medical Center, Tel-Aviv University.

### 2.1. Preparation of lipid-based drug carrier

Regular lipid-based particles (RL-LP) were composed of soy phosphatidylcholine (SPC):cholesterol:1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE) at the mole ratio of 55:40:5 and the total lipid concentration of 100 mg lipid/ml. The lipids were dissolved in ethanol and subjected to evaporation in a rotary evaporator under reduced pressure. The dry lipid film was incubated (in a shaker bath) with the swelling solution (0.1 M borate buffer at pH 9) for 2 h at 65 °C. To modify RL-LP into HA-LP, hyaluronan (1.5 MDa) was dissolved in acetate buffer (0.1 M, pH 4.5) at a concentration of 2 mg/ml and pre-activated by incubation with the cross-linker EDC for 2 h at 37 °C. The activated HA was mixed with the RL-LP suspension at the ratio of 1:1 (v/v), bringing the pH back to 9. This reaction mixture was incubated for 24 h in a shaker bath at 37 °C [22–24]. The HA-LP was freed from excess materials and by-products by centrifugation for 30 min at 4 °C and a g force of 160,850, followed by several successive washes and re-centrifugations in 0.1 M ammonium bi-carbonate, after which the final pellets were suspended in this salt solution. The HA-LP suspension was divided into aliquots of 200 μl and frozen for 2 h at −80 °C, followed by lyophilization. [The resultant liposome powders were stored at −18 °C for further use.] This produces powders of salt-free buffer-free HA-LP since, in the course of lyophilization, the $NH_4HCO_2$ salt turns into ammonium and $CO_2$ gases which are then pumped out of the system. Lyophilize powders of RL-HP were similarly prepared.

To prepare the hemin-encapsulating HA-LP, the lyophilized powder of the drug free HA-LP was rehydrated in an aqueous solution of hemin (Sigma, Rehovot, Israel) in PBS pH 7.6, and the system was incubated in a shaker bath for 24 h, at 37 °C.

The liposomes containing the encapsulated hemin were separated from the excess non-encapsulated hemin by ultracentrifugation and washings. Hemin concentration in both the supernatant and re-suspended pellet (back to original volume) was assayed by its absorbency at 420 nm, which was found to be linear in both buffer and 5% decoxycholate over the hemin concentration range of 0–100 μg/ml. The encapsulation efficiency, calculated from these data, together with the initial known hemin concentration, was 91(±6)%.

### 2.2. Targeting primary cultured macrophages by HA-liposomal rhodamine (rhodamine/HA-LP)

Testing lipid-based drug-carrier efficacy in vitro was done with "green" macrophages, from Csf1R-icre-Rosa TdTomato-EGFP Rosa mouse (Jackson Laboratory, Bar-Harbor, ME, USA, stock no. 007576). This mouse allows direct live fluorescent visualization of EGFP in monocytes and macrophagese. ROSAmT/mG is a cell membrane-targeted, two-colour fluorescent Cre reporter allele, which expresses cell membrane-localized red fluorescence in widespread cells/tissues prior to Cre recombinase exposure, and cell membrane-localized green fluorescence in Cre recombinase-expressing macrophages (and future cell lineages derived from these cells). Breeding and genotyping of *cfms-icre* [25] *and* $Rosa^{mT/mG}$ [26] were performed as previously described.

Macrophages were aspirated from the peritoneum by flushing with saline. Peritoneal cells were incubated for 2 h with 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel) RPMI (Biological Industries, Kibbutz Beit Haemek, Israel) with 1% penicillin-streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel), adhering to the dish surface. Then, for macrophage enrichment, medium was aspirated and 0.25% Trypsin-0.05%EDTA (Biological Industries, Kibbutz Beit Haemek, Israel) was added twice, each time for 4 min at 37 °C. Intact cells in the culture were referred to as macrophages and cultured for 24 h with 100 μl of HA-LP doped with PE-rhodamine.

### 2.3. Cytokines and gene expression

The anti-inflammatory activity of hemin/HA-LP was evaluated in vitro. LPS (Sigma, Rehovot, Israel) RAW264.7 macrophages in 96-well plates were divided into 5 groups (6 wells per group). All groups received 100 μg/ml LPS (Sigma, Rehovot, Israel) for stimulation and were given the following treatments: free hemin, drug free RL-LP, hemin/RL-LP, drug free HA-LP or hemin/HA-LP. Hemin and liposome doses were 50 μg/ml and 4 mM lipid, respectively. Wells receiving cell growth media alone served as controls. Incubation was for 24 h at 37 °C. TNF-α was measured by a commercial ELISA kit (R&D Systems, Minnesota, MN, USA) according to the manufacturer's instructions. Next, primary macrophages were aspirated from mouse peritoneum by saline, centrifuged for 10 min, 1800 rpm, 4 °C and seeded in 48 well plates for ELISA (Biolegend, San Diego, CA, USA) or in six well plates for RNA purification (RNeasy plus mini kit, QIGEN GmbH, Hilden, Germany). Cells were incubated for 2 h with 10% FBS RPMI medium, adhering to the dish surface. Then, for macrophage enrichment, RPMI medium was aspirated and Trypsin-EDTA was added twice, each time for 4 min at 37 °C. Intact cells in the culture were referred to as macrophages. Macrophages were then incubated with hemin/HA-LP, drug free HA-LP, free hemin, or RPMI medium only. Hemin dose for 48 well plates was 3.75 μg per well and for six well plates - 15 μg. After three days in culture, cell media were collected for IL-12 level detection by ELISA, where the cells went through lysis for RNA purification (RNeasy plus mini kit, QIGEN GmbH, Hilden, Germany), followed by reverse transcription reaction to cDNA (high capacity cDNA reverse transcription; Applied Biosystems, City CA, USA). Real-time PCR was carried out in the Step one system and software (Life technologies, City NY, USA) according to the manufacturer's instructions. The PCR protocol consisted of 40 cycles of denaturation at 95 °C for 30 s and annealing at 58 °C for 30 s. We used the comparative Threshold Cycle (Ct) method for quantification analysis. The Ct values from HO-1 were normalized to the Ct value of GAPDH. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method [27] and expressed as fold change.

To determine hemin/HA-LP ability to modulate macrophages toward M2 cells, 1.5 ml of thioglycolate (Hy labs, Rehovot, Israel) were IP injected into Balb/C mice. Three days later, mice were treated by IP injection of hemin/HA-LP (2 mg/kg), HA-LP (10 mg lipid/kg), free hemin (2 mg/kg) or saline (100 μl). After three days, primary macrophages were aspirated from mouse peritoneum by saline, centrifuged for 10 min, 1800 rpm, 4 °C and seeded in six well plates for RNA purification. Cells were incubated for 2 h in 10%FBS RPMI medium, adhering to dish surface. Then, for macrophage enrichment, medium was aspirated and Trypsin-EDTA was added twice, each time for 4 min at 37 °C. Intact cells in the culture were referred to as macrophages and went