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Therapeutic Ultrasound: Increased HDL-Cholesterol Following Infusions of Acoustic Microspheres and Apolipoprotein A-I Plasmids

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Abstract

Background—Low levels of HDL-C are an independent cardiovascular risk factor associated with increased premature cardiovascular death. However, HDL-C therapies historically have been limited by issues relating to immunogenicity, hepatotoxicity and scalability, and have been ineffective in clinical trials.

Objective—We examined the feasibility of using injectable acoustic microspheres to locally deliver human ApoA-I DNA plasmids in a pre-clinical model and quantify increased production of HDL-C *in vivo*.

Methods—Our novel site-specific gene delivery system was examined in naïve rat model and comprised the following steps: (1) intravenous co-administration of a solution containing acoustically active microspheres (Optison™, GE Healthcare, Princeton, New Jersey) and human

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Competing financial interest

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ApoA-I plasmids; (2) ultrasound verification of the presence of the microspheres within the liver vasculature; (3) External application of locally-directed acoustic energy, (4) induction of microsphere disruption and *in situ* sonoporation; (4) ApoA-I plasmid hepatic uptake; (5) transcription and expression of human ApoA-I protein; and (6) elevation of serum HDL-C.

Results—Co-administration of ApoA-I plasmids and acoustic microspheres, activated by external ultrasound energy, resulted in transcription and production of human ApoA-I protein and elevated serum HDL-C in rats (up to 61%; p-value < 0.05).

Conclusions—HDL-C was increased in rats following ultrasound directed delivery of human ApoA-I plasmids by microsphere sonoporation. The present method provides a novel approach to promote ApoA-I synthesis and nascent HDL-C elevation, potentially permitting the use of a minimally-invasive ultrasound-based, gene delivery system for treating individuals with low HDL-C.

Keywords

ApoA-I; Optison™; acoustic microspheres; ultrasound; drug delivery; HDL-C; hypoalphalipoproteinemia

Introduction

Low levels of high-density lipoprotein cholesterol (HDL-C) represent an independent risk factor associated with premature cardiovascular disease in patients who receive LDL-C therapy (1, 2). In addition, low HDL-C is associated with familial hypoalphalipoproteinemia and premature cardiovascular disease with mortality rates comparable to those found in individuals with marked elevations of low-density lipoprotein cholesterol (LDL-C) (3–5). Although numerous approaches have been used to increase HDL-C, currently there are no corresponding therapeutic options for increasing clinically effective HDL-C to reduce atherosclerotic events.

Previous clinical studies have supported the hypothesis that increased levels of serum HDL-C are related to a reduction of systemic atherosclerosis and a decrease in cardiovascular events (6–8). However, recent unsuccessful clinical trials of various investigational HDL-C therapies cast doubt on the cardiovascular protective effects of HDL-C, or at least suggest greater complexity than was previously appreciated. The recently described identification and differentiation of the functional status of HDL-C sub-particles may provide new insight into the protective effects of HDL-C for cardiovascular disease (9, 10). In support of this, human and rabbit studies have shown that recombinant human ApoA-I (r- ApoA-I) proteins used to increase HDL-C can reduce intra-plaque lipid content and induce plaque regression (11–13).

In the current study we examined a novel mechanism for increasing HDL-C via delivery of human ApoA-I plasmid DNA (pDNA), effectively decoupling the toxicity of the drug from the efficacy of the therapy. We hypothesized that venous co-infusions of human ApoA-I plasmids and acoustically active microspheres, when exposed to ultrasonic energy (i.e. ApoA-I Therapy), would increase the natural hepatic production of HDL-C. Preclinical studies were performed in rats in which microsphere perfusion of the liver vasculature was

confirmed by the presence of a visual contrast effect within the liver parenchyma. Subsequently, pulsed sequences of ultrasound energy were applied to disrupt the microspheres *in situ* to induce sonoporation resulting in local delivery of human ApoA-I plasmids to the liver. Following treatment, we assessed translation of human messenger RNA that coded for ApoA-I protein and resulted in elevated serum HDL-C.

Methods

Animals

Male Sprague-Dawley rats (180–250g) were purchased from Charles River Laboratories (Wilmington, MA). The experimental protocol conformed to the *Guide for the Care and Use of Laboratory Animals* and was approved by the IACUC at General Electric Global Research (Niskayuna, NY). Rats were housed in standard cages, provided with *ad libitum* access to standard commercial feed and water, and were maintained on an alternating 12-hr light:dark cycle in rooms with controlled temperature and humidity.

Human ApoA-I plasmid (pDNA)

The ApoA-I expression plasmid (approximately 5 kb) was constructed by subcloning an 804 bp human ApoA-I PCR cDNA product into expression vector pMIR0125 (Mirus Bio Corp, Madison, Wisconsin) containing human apoE hepatic control region (HCR), human ubiquitin C promoter and first intron, which is preferentially expressed in liver. The final construct, pMIR0332-HCRUbC-h ApoA-I was sequenced and the resulting clone matched the reported human ApoA-I sequence.

In addition, a control plasmid that encoded for enhanced green fluorescence protein (GFP), a full-length FP sequence followed by a SV40 polA signal was constructed solely to measure the effectiveness of DNA delivery in pilot studies. The GFP expression plasmid used a cytomegalovirus (CMV) promoter. The GFP and corresponding ApoA-I DNA plasmids were of similar size (approximately 5kb).

In vivo Study designs

Animals were anesthetized with a gas mixture of oxygen and 1.5–3.0% isoflurane for procedure preparation, blood sampling and treatment period (15 minutes total) while a warming bed maintained body temperature at 37°C.

Infusions were made via a lateral tail vein. In all injections, 1ml of acoustic microspheres (Optison™, GE Healthcare, Princeton, New Jersey) and 1ml of pDNA (8mg in 1mL) were mixed gently within the injection syringe for 30 seconds. The 2ml total infusion volume was administered over approximately 100 seconds and contained $5.0 - 8.0 \times 10^8$ microspheres. During the peripheral infusion of the pDNA/microsphere mixture, ultrasound imaging of the liver was performed and was used to confirm contrast perfusion of the vasculature. Following treatment, blood specimens were collected at set time points. For each blood collection, rats were anesthetized.

Delivery of microsphere and pDNA mixture

Similar to the method described in a recent publication, the effectiveness of DNA hepatocyte transfection was assessed using pDNA encoding GFP (15). Rats were injected with a solution containing acoustic microspheres and plasmids encoding GFP delivered via tail vein infusions. Rats were euthanized and their livers were excised three days post-delivery of GFP pDNA. The liver lobes were bisected and scanned for fluorescence on a Typhoon 9410 (GE Healthcare Life Sciences, Piscataway, NJ) scanner at 488 nm wavelength emissions to confirm the delivery and expression of pDNA. (Figure 1).

Human ApoA-I mRNA and protein expression post treatment—A separate group of rats received a single treatment of ApoA-I Therapy for analysis of mRNA and production of human ApoA-I protein (ApoA-I Therapy n=6, Control n=8). At 12 hours post treatment, blood was collected and rats euthanized for liver collection and analysis. Livers were excised, formalin fixed, paraffin embedded and sectioned. The presence of human mRNA in these tissue sections was assessed by the View RNA ISH Tissue assay developed by Affymetrix (Panomics Solutions, Santa Clara CA). An ELISA assay (ApoA-I (HUMAN) KA0460, Abnova, Walnut, CA) was used for the determination of circulating human ApoA-I protein production according to manufacturer protocol.

ApoA-I plasmids - single treatment—The single treatment rat protocol for ApoA-I plasmid delivery was studied with a total of 37 animals, blocked randomly into one full therapy and seven control cohorts: (a) microspheres + ApoA-I pDNA + ultrasound (ApoA-I Therapy, n=15); (b) microspheres (Optison, n=3); (c) ApoA-I pDNA (pDNA, n=3); (d) ultrasound (US, n=3); (e) isoflurane anesthesia (Anesthesia, n=4); (f) ultrasound with microspheres (US+Optison, n=3); (g) ultrasound with ApoA-I pDNA (US+pDNA, n=3); and (h) ApoA-I pDNA with microspheres (pDNA+Optison, n=3).

ApoA-I plasmids - multiple treatments—Two cohorts of animals were used for the multi-treatment rat study: (a) ApoA-I Therapy (n=15) and (b) isoflurane inhalant anesthesia control (n=15). Following baseline blood collection from all animals, treated rats were administered the microsphere/ApoA-I pDNA mixture via tail vein injections with subsequent application of ultrasound energy. Anesthesia control animals received no injections or acoustic energy. Duration of isoflurane exposure was identical in both cohorts. The dosing schedule consisted of identical treatments on day 0, 10 and 20. Post-procedure blood samples were collected at 24 hours and at five days following each treatment to assess HDL-C production.

Ultrasound equipment and acoustic parameters

For all *in vivo* studies, a Vivid *i* ultrasound imaging system (GE Healthcare Systems, Milwaukee, Wisconsin) was used in conjunction with a 3S cardiac phased array probe. The system was unmodified other than a software change to enable the system to programmatically switch between two user-defined transmit levels. The user-selected imaging settings corresponded to a 1.6 microsecond (2.5 cycles long) transmit waveform centered at 1.54 MHz. All acoustic transmit settings remained within the range of the standard product capabilities and FDA guidelines for human diagnostic use (14). Prior to

performing *in vivo* studies, tests were run to determine the system settings and acoustic parameters sufficient to generate microsphere cavitation and collapse (inertial cavitation), as monitored by passive cavitation detection.

The ultrasound imaging and cavitation was performed as follows; After an infusion of pDNA mixed with microspheres was initiated, during the initial 20 seconds of imaging, low transmit power setting (mechanical index setting, $MI = 0.4$) was used to confirm perfusion of the acoustic microspheres within the liver parenchyma. Once confirmed, the transmit power setting was programmatically cycled between a MI of 1.3 for two seconds of microsphere disruption followed by a MI of 0.4 for eight seconds to allow reperfusion. This sequence was repeated ten times, with a total treatment time of approximately 100 seconds. A single operator performed all ultrasound imaging and microsphere disruption throughout the study and was not blinded to treatment.

Analytic methods

Three blood samples were collected over 6 days to establish a pre-treatment baseline HDL-C level for each rat. In both treated and control animals, a blood sample was collected at 24 hour intervals at the same time of day to minimize confounding effects of daily metabolic fluctuations. Samples were centrifuged and the separated serum samples were analyzed for HDL-C by Charles River Labs using the Beckman Coulter HDL-C Reagent Immunoassay, (Model OSR6195) with an AU analyzer. This assay yields a selective measurement of serum HDL-Cholesterol in the presence of other lipoprotein particles. To assess potential adverse effects of treatment, blood samples were evaluated using a comprehensive veterinary diagnostic blood analyzer (VetScan VS2, Abaxis Inc, Union City, California) that quantified 14 parameters of blood (albumin, alkaline phosphatase, alanine aminotransferase, amylase, blood urea nitrogen, calcium, creatinine, globulin, glucose, potassium, sodium, phosphorus, total bilirubin, and total protein). Additionally, gross histological analysis of the liver tissue was conducted to identify negative effects of microsphere cavitation.

Statistical analysis

Graphical data are reported as arithmetic mean + standard error of the mean. Statistical significance between Average Control and ApoA-I Therapy cohorts was $P\text{-value} < 0.05$ as determined by two-sample t-test. Evidence for the statistical significance of HDL-C elevation post treatment was determined using repetitive measures ANOVA. Tukey pairwise comparison was performed between all cohorts and timepoints. As specified, change in circulating HDL-C is reported as a comparison of baseline and post-treatment levels or expressed as absolute values. All statistical analyses were carried out using Minitab17 (Minitab Inc., State College, PA USA).

Results

Demonstration of pDNA delivery to the liver using a reporter gene (GFP)

Representative images of the effective uptake of GFP pDNA and protein expression detected by the green fluorescent signal are shown in Figure 1. Visual analysis of the GFP distribution pattern revealed non-uniformity that may have been influenced by a number of

factors including placement of the external ultrasound transducer, local organ perfusion variability, or other micro-environmental effects (15). In these proof-of-concept studies it was observed that using multiple planes of insonation produced a more diffuse GFP expression post microsphere cavitation (15).

Human ApoA-I mRNA and protein expression

Results summarized in Figure 2 demonstrate a high degree of human mRNA expression present throughout the histological section from the treated liver. Translation of human ApoA-I derived mRNA resulted in quantifiable circulating human ApoA-I protein in four of six ApoA-I Therapy animals measured by ELISA, averaging 0.24ug/mL plasma. Ultimately this leads to increased serum HDL-C values measured at 24hours post treatment and beyond. Conversely, tissues of control animals collected at the same time point as ApoA-I Therapy animals expressed no human ApoA-I mRNA and exhibited no appreciable increase in human ApoA-I protein or HDL-C. These null results were indicative of and consistent across each of the four control cohorts examined: pDNA+microspheres (no ultrasound), ApoA-I pDNA+ultrasound (no microspheres), microspheres+ultrasound (no ApoA-I pDNA) and saline only.

Serum HDL-C decrease in control cohorts

Although individual baseline values ranged from 39 to 95mg/dL, a progressive reduction in serum HDL-C from baseline values was consistently observed in rats in all control cohorts at each sample collection (Table 1 and 2). To convey this, serum HDL-C levels post-treatment are expressed as a percentage of individual animal baseline (percent change) as well as a ratio of the HDL-C value of ApoA-I Therapy to matched controls. The temporally associated serum HDL-C levels in controls served as the comparator for each test cohort at each time point for all animals. Although the mechanism for the acute reduction in HDL-C is uncertain, it may be attributed to several factors (*vide infra*).

Single treatment

The response of serum HDL-C levels after treatment is shown for ApoA-I therapy and all control cohorts in Figure 3. Compared to baseline values HDL-C was elevated an average of 11.5mg/dl in the ApoA-I therapy cohort. In contrast, a consistent decrease in serum HDL-C levels was observed in all other animals at 24 hours after treatment, and averaged -5.8mg/dL.

Following treatment, 15 of 30 (50%) rats that received ApoA-I Therapy exhibited a significant elevation of serum HDL. Sixty-three percent of these rats exhibited a peak HDL-C elevation one day post treatment; 87% of the rats had reached an HDL-C peak within 48 hours. No rats revealed an HDL-C peak beyond four days following a single ApoA-I treatment. Results were confirmed using repetitive measures ANOVA of absolute HDL-C values ($p < 0.05$). These data demonstrated a rapid incorporation of the plasmid into the hepatocytes with an efficient elevation of serum ApoA-I protein and HDL-C.

Multiple treatments

A treatment protocol was designed to elicit a prolonged HDL-C elevation in response to serial treatments performed over three weeks. To minimize negative effects of anesthesia/bleeding, HDL-C was sampled at day one and five following each treatment cycle. As in single treatment studies, a consistent reduction of HDL-C from baseline was recorded in the control group (anesthesia only) while the ApoA-I therapy cohort experienced an average increase of 61% above mean control HDL-C after the first treatment (min = 24.7% max = 116.0%) (Figure 4). The average increase following treatments two and three at 24 hours post treatment was 29 and 28% respectively. The average increase measured in the ApoA-I therapy cohort HDL-C was 16.2mg/dL over baseline on day one, while the average control decreased by -9.9mg/dL. 93% of the animals treated (14 of 15 rats) responded with a greater than 10% increase in HDL-C. This improvement in treatment efficacy, compared to the corresponding single treatment experimental cohort is most likely attributed to technique refinement, including factors such as probe placement and injection rate.

Safety

To monitor for adverse effects of the treatment protocols, serum was analyzed for a variety of biomarkers of hepatotoxicity. The sole notable response to treatment was a transient (~20%) decrease in amylase in the rat that paralleled increases in HDL-C levels and returned to baseline within three days after treatment. Published reports indicate that elevated serum lipid levels interfere with enzyme testing, resulting in false-low reports, and may have contributed to the observation of changes in serum amylase (16). Gross histological analysis showed no significant abnormal morphological findings in any liver sections; no evidence for cavitation induced damage, inflammation or other liver pathology. All liver sections demonstrated normal liver morphological features in treated and untreated animals in both single and multi-treatment study designs.

Discussion

The present study demonstrates that a novel ultrasound-directed, site-specific delivery system, consisting of co-infused human ApoA-I pDNA and acoustic microspheres with externally applied ultrasound energy, increased the production of nascent HDL-C in rats. This was in contrast to a significant and progressive decrease in endogenous HDL-C in control animals that did not receive the combination plasmid/microsphere infusion. Expression of mRNA for human ApoA-I protein was observed throughout the liver of rats receiving ApoA-I therapy, resulting in increased circulating ApoA-I protein as a precursor to elevated levels of serum HDL-C. The level of HDL-C peaked within 72 hours after a single infusion in rats, and following serial infusions at day 0, 10 and 20, the HDL-C remained elevated at day 25. No abnormalities were observed in extensive laboratory tests and histology following infusion, attesting to the safety of the procedure. The present technology provides a potential method to increase HDL-C as a therapeutic option for the treatment of systemic atherosclerosis, and potentially for the treatment of monogenetic diseases including familial hypo-alphalipoproteinemia and Tangier Disease (17).

An important observation in this study was that endogenous HDL-C was decreased in response to the protocol in all animal cohorts other than in the ApoA-I Therapy group. Although the mechanism for the acute reduction in HDL-C is uncertain, it may be attributed to several factors associated with experimental stress, inflammation, or anesthesia effects (18–24). Recently, Chen *et al.* linked the reduction in serum HDL-C in mice to acute phase reactants resulting in blockage or diminution of the reverse cholesterol transport (RCT) mechanism (25). As noted above, all animals (ApoA-I Therapy and control) were handled identically; it is therefore reasonable to assume the mechanisms leading to reductions in serum HDL-C levels was experienced similarly for all subjects. In view of the decrease observed in the controls, it follows that the increase in HDL-C observed ApoA-I Therapy group was actually greater by the absolute increase relative to the temporally matched control values (as depicted by the gray bars in Figures 3 & 4). These factors support the concept that such treatment may provide clinically significant elevations of HDL-C in humans, particularly during periods of stress; i.e., acute coronary syndromes (22).

Our site specific, delivery system was designed to facilitate uptake of the ApoA-I plasmids into the liver parenchyma. The use of alternating periods of low and high acoustic energy allowed sufficient time for reperfusion of the liver microvasculature, resulting in adequate intravascular targets for subsequent acoustic pulses. At higher acoustic amplitudes, individual microspheres underwent stable and inertial cavitation creating local disturbances, including acoustic streaming, and/or microsphere rupture with accompanying microjets (26). When these disturbances occur in close proximity to the endothelium, the shear forces are hypothesized to induce transient porosity within the cell membranes and interstitium, permitting entry channels into the hepatocytes (27). Once in the hepatocyte, passive diffusion and/or active transport are believed to provide mechanisms for plasmid access to the cell nucleus. Notably, as evidence for parenchyma ApoA-I plasmid uptake and transcription, human ApoA-I protein was increased in the serum within hours of treatment and was accompanied by a corresponding increase in *nascent* serum HDL-C. With regard to technical execution, we observed optimal results when the infusion rate and acoustic microsphere concentration permitted full visualization of the liver parenchyma without significant signal attenuation. Additionally, repositioning the ultrasound transducer during the low amplitude portion each acoustic pulsing sequence provided increased exposure to a greater portion of the total liver parenchyma volume, thus yielding more effective delivery of the plasmids.

Several recent clinical trials related to the reduction of cardiovascular events by elevating serum HDL-C were unsuccessful and have called into question the value of HDL therapies (28–31). Based upon recent publications, it is clear that the complex nature of the HDL-C sub-particles confer both functional and dysfunctional metabolic activities (32, 33). Several of the HDL-C sub-particles exhibit beneficial anti-atherosclerotic, anti-inflammatory, anti-oxidant and anti-apoptotic properties while other HDL particles appear to be ineffective and “dysfunctional” and may actually be pro-inflammatory (34, 35). It is possible that the enhanced expression of natural HDL-C produced in this study offers the potential to achieve the anti-atherosclerotic benefits observed with high native levels of HDL-C but not obtained with prior pharmacologic interventions. Moreover, while other studies have infused

recombinant apoproteins to transiently increase HDL-C, often measured in hours, our system produced more sustained elevations of HDL-C and did not rely on the use of viral vectors, or foreign proteins or peptides.

To assess possible hepatotoxicity we performed postmortem histology of the liver and quantified 14 individual biomarkers in all animals; no abnormalities were observed. We acknowledge that additional studies are required to evaluate the safety of this delivery system if used in patients. In addition, potential safety issues generally associated with traditional plasmid/gene therapies may exist. Early clinical trials of gene therapy used viral mediated vectors based on their high transfection efficiency and persistence (36–39). However, the inherent properties of a virus presented limitations and viral proteins potentially producing immune responses, thus limiting reinjection potential. The demonstrated plasmid delivery system is not viral mediated. Plasmid based therapies appear to have a benign toxicity profile and are not considered insertional (40–43). This position is reflected in the FDA guidance for gene therapy (44).

It should be noted that the key components of this site-specific plasmid delivery system have been separately approved by the FDA for other clinical uses, and the safety of those components has been established in those contexts. Optison™ acoustic microspheres are FDA-approved for left ventricular chamber enhancement during echocardiography, and the acoustic parameters used in our study were within FDA guidelines for diagnostic applications. In addition, therapeutic ultrasound and acoustic microspheres are now being used as adjunctive therapy for the treatment of pancreatic cancer (45). Acoustic microspheres have also been proposed as a delivery vehicle for siRNA and DNA therapy (46). Given the minimally invasive nature of this technology and the large installed base of ultrasound users, microsphere delivery, if clinically validated, may be relatively economical and effective.

Several limitations exist in regard to the studies performed and further improvements could be made in future investigations. All data were acquired in experimental animals, and the response in humans may be different. Clinical application of this approach may require periodic treatments to maintain an elevated level of HDL-C. The transient effect seen in the rat model could possibly be prolonged using techniques such as transposon systems or nuclear localizing agents to increase delivery. Additionally, the transition to future clinical studies will need to incorporate ultrasound hardware and software system improvements such as volumetric 3D imaging capabilities as well as automation to trigger microsphere disruption. This would enable larger volumes of the liver to be treated in a more efficient manner. This translation and design validation must include larger, more clinically relevant *in vivo* models in which HDL metabolism and target organ size closely matches that of humans.

Future studies will include optimization of plasmid dosing and acoustic designs with subsequent testing of genetically modified (knock out) mice to assess efficacy of nascent HDL therapy in atherosclerotic animals. As a first step toward validating the efficacy of newly generated HDL-C, a preliminary examination by *in vitro* cholesterol efflux testing (in conjunction with Alan T. Remaley, MD, PhD, Senior Investigator, Lipoprotein Metabolism

Section, NIH) suggested a positive correlation between ApoA-I Therapy and functionality of the nascent HDL-C. A modest increase in efflux via the ABCA1 transporter was noted (3.6% in two of three animals tested vs. -0.45% in Control animals). While this result gives hope for efficacy, expansion of such functionality studies and characterization of the *de novo* HDL-C and a more thorough quantitation of ApoA-I protein is imperative. If future studies support the generation of a functional HDL-C, the aggregate data may be sufficient to warrant testing the hypothesis that our ApoA-I Therapy produces clinically beneficial elevations of HDL-C in humans.

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Abbreviations and Acronyms

ApoA-I	apolipoprotein A-I
HDL-C	High density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
MI	Mechanical index
GFP	Green florescent protein
pDNA	plasmid DNA

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Highlights

Therapeutic Ultrasound: Increased HDL-Cholesterol Following Infusions of Acoustic Microspheres and Apolipoprotein A-I Plasmids

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- Sonoporation, via acoustic microspheres with ApoA-I pDNA , resulted in increased HDL-C
- HDL-C therapy using localized, acoustic delivery is devoid of viral-mediated vectors
- A novel therapeutic, gene delivery system was developed for hypo-alpha-lipoproteinemia

Figure 1. Acoustic microsphere delivery of Green Fluorescent Protein (GFP) Plasmid DNA
Fluorescence detection of green fluorescent protein (GFP) was examined in bisected livers of naïve rat (A) and a GFP treated rats (B) single-plane insonation and (C) multi-plane insonation). The treated rats received a tail vein injection of 1ml Optison with 1ml GFP DNA (8mg) followed by a series of acoustic pulses. Green fluorescence detected in the bisected liver of the GFP treated rat confirmed delivery and expression of the plasmid.

Figure 2. Representative expression of Human ApoA-I mRNA and resultant translation of human ApoA-I protein in the rat post treatment

The upper left panel demonstrated an absence of human messenger RNA identified in rat liver parenchymal cells and a subsequent absence of human ApoA-I protein in serum, representative of all control animals. The lower panel exhibited a significant presence of human ApoA-I mRNA (FastRed labeled) contained within intact liver parenchymal cell at 12 hours post therapy in a rat serum sampled immediately before liver collection at 12 hours post therapy. Images are 20x Brightfield.

Figure 3. Circulating serum HDL-C in rats following microsphere delivery of ApoA-I plasmid DNA

The control cohorts represent subsets of the three requisite components of microsphere plasmid delivery: Ultrasound (US), pDNA, and acoustic microspheres (Optison). The ApoA-I Therapy (Blue) and Control Average (Red) are expressed as a percentage of individual rodent baseline values (pre-treatment serum HDL-C). The Therapy/Control Ratio (Gray bars) represents the increased nascent HDL-C in distinction to the reduction in endogenous HDL-C observed in control animals. The consistent and progressive decrease in endogenous HDL-C was observed at each experimental condition in all control groups, whereas there was a significant, persistent increase in nascent HDL-C observed in the ApoA-I therapy group (US+pDNA+Optison). Note: $p < 0.05$ for all points as compared to Control Average (*)

Figure 4. HDL-C results during a series of three treatments, occurring on day zero, 10 and 20 in rats

Following each of three identical ApoA-I treatments performed on day 0, 10, and 20 (Blue), the average HDL-C level of treated animals increased above matched controls with maximum increase of 61% 24 hours after treatment 1. A corresponding significant elevation of HDL-C was observed at 24 hours after treatments (day 10 and 20) averaging 29 and 28% above baseline, respectively. Therapy/Control Ratios (Gray bars) relate the measured increase in nascent HDL-C levels observed in the therapy group. Note there was a consistent and progressive decrease in endogenous HDL-C was observed at each experimental condition in the control groups (n=15) (Red).

Table 1**Change in circulating HDL-C following a single treatment**

The ApoA-I Therapy was the only cohort to result in an increase above baseline serum HDL-C post treatment. All other conditions resulted in decreased HDL-C.

	Control Average (n=22)	ApoA-I Therapy (n=15)	US Only (n=3)	Optison Only (n=3)	US+pDNA (n=3)	US+Optison (n=3)	pDNA+Optison (n=3)	pDNA Only (n=3)	Anesthesia Only (n=4)
Baseline	63.0	61.6	64.7	67.0	58.7	68.8	72.0	58.9	53.9
Day1	57.2	73.1	58.3	57.0	53.7	54.3	67.7	57.3	53.5
Day 2	57.7	70.7	57.7	53.7	57.0	56.7	72.7	57.3	51.0
Day 3	51.0	63.6	51.0	49.7	54.3	61.3	57.3	42.7	43.0
Day 4	54.5	59.5	52.7	56.7	52.0	67.7	60.0	47.7	47.0
Day 5	48.9	62.1	47.7	50.3	45.7	51.7	56.7	47.0	44.8
Day 6	48.5	57.1	51.7	47.0	44.0	50.3	54.3	45.7	46.8
Day 7	53.4	56.4	51.3	54.0	55.7	65.0	61.0	46.7	43.5

Table 2
Change in circulating HDL-C following multiple treatments

The ApoA-I Therapy was the only cohort to result in an increase above baseline serum HDL-C post treatment. As in the single treatment study, the control cohort (Anesthesia only) resulted in decreased HDL-C.

	Anesthesia Control (n=15)	ApoA-I Therapy (n=15)
Baseline	56.6	53.1
Day1	46.7	69.3
Day 5	44.2	51.3
Day 11	48.3	57.9
Day 15	52.1	43.6
Day 21	47.4	55.5
Day 25	47.7	55.6