Diabetic Complications Consortium Final Technical Report – 3/1/2018

Project Title: BH4-Loaded Nanoparticles for Diabetes-Associated Cardiovascular Disease

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1. Project Accomplishments:

This project is still a work-in-progress due to a number of unforeseen issues that significantly delayed its start as well as its completion. Initially there was a delay in gaining approval from our Institutional Animal Care and Use Committee, which resulted in approval granted months after the initial start date for the grant period. We were instructed that the grant period could not be modified due to the reporting requirements of the parent grant and *we were instructed to start the grant period on the required date and then request a no-cost extension at the end of the initial reporting period.* As instructed, we requested the no-cost extension. Unfortunately, the process required that the original grant award be terminated, and a new subaward agreement drafted and approved. This caused several months of delay. A further delay occurred when the granting entity subsequently changed its name to Augusta University. This required the grant award to be terminated yet again and a third subaward agreement approved, which took six months to complete. At the current time, we are completing the studies outlined in Specific Aim 2 and should have the results, analysis and a second manuscript for submission in the next few months. Results of the initial part of the study have been presented at scientific meetings and a manuscript describing the proof of concept studies submitted for publication.

2. Specific Aims:

Specific Aim 1: Determine the optimal treatment schedule for orally administered tetrahydrobiopterin-loaded (BH4-loaded) solid lipid nanoparticles to provide long-term reversal of endothelial dysfunction in a model of diabetes-associated atherosclerosis.

Initial studies investigating the ability of our nanoparticles to reverse endothelial dysfunction were carried out in the streptozotocin-induced type I diabetes mellitus rat model [while we waited for approval for our diabetic mouse model]. We found that orally delivered, BH4-loaded solid lipid nanoparticles (SLNPs) were able to normalize the BH4 levels in coronary endothelial cells of diabetic rats. We assessed the levels of both tetrahydrobiopterin (BH4) and dihydrobiopterin (BH2), as the human clinical trials of orally delivered, non-encapsulated BH4 failed because much of the BH4 that was absorbed into the systemic circulation from the gastrointestinal system was oxidized to BH2, which blocks endothelial nitric oxide production and exacerbates endothelial dysfunction. We demonstrated that encapsulating the BH4 in SLNPs allows BH4 levels to be restored in the endothelial cells of the coronary circulation (Figure 1, below), while no significant increase in plasma level of BH4 is detected (not shown). This data supports the conclusion that maintaining BH4 in its reduced state within the nanoparticles allows uptake of BH4 within nanoparticles by endothelial cells, followed by release of BH4 inside the endothelial cells. Importantly, this overcomes the oxidation issue (i.e., conversion of BH4 to BH2 in the plasma when BH4 is given in a non-encapsulated form), which was the cause of the human clinical trial failure.

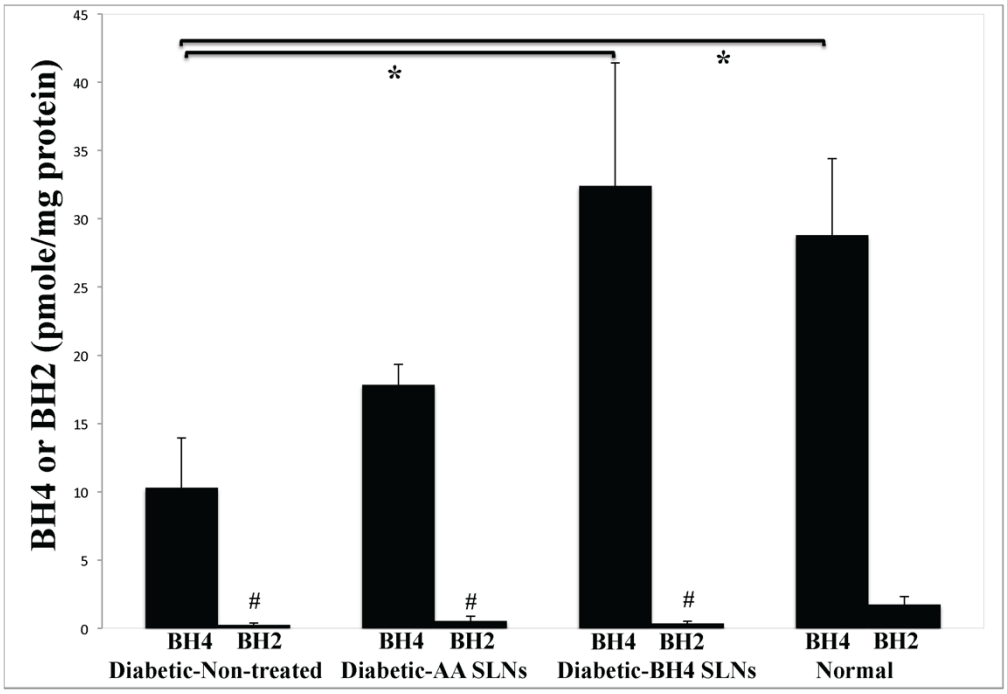


Figure 1: Orally delivered, BH4-loaded solid lipid nanoparticles (SLNs) normalized BH4 levels in coronary endothelial cells. BH4-loaded SLNs or ascorbic acid-loaded (AA, vehicle-loaded) SLNs were administered to diabetic rats via oral gavage at 0 and again at 24 hours. At 48 hours, animals were euthanized, hearts removed and coronary endothelial cells were isolated for immediate measurement of BH4 levels. Levels of BH4 and BH2 in coronary endothelial cells were compared between non-treated, vehicle-treated, and BH4-treated diabetic rats vs. the levels in normal (non-diabetic, non-treated) rats. Data are presented as mean ± SEM. * $p<0.05$ vs. BH4 in non-treated diabetic rats; # $p<0.05$ vs. BH2 in normal (nondiabetic, nontreated) rats; analyzed using one-way ANOVA with Fisher's least significant difference post hoc analysis.

To begin to understand the pharmacokinetics of BH4 delivery via SLNPs and due to the fact that BH4 encapsulated in our SLNPs is NOT detected in BH4 assays of lymph or plasma (data not shown), we utilized the same SLNPs loaded with a fluorescent 6-coumarin dye to follow the movement of the nanoparticles from the gastrointestinal system into the mesenteric lymphatic system and finally into the plasma of the systemic blood circulation. Control nanoparticles contained saline only. While fluorescent SLNPs were clearly visible in the intestinal lumen, fluorescent NPs were not visible in blood vessels draining the wall of the small intestine, indicating preferential uptake via mesenteric lymphatic vessels (Figure 2A). To verify that fluorescent SLNPs entered the lymphatic system via intestinal lacteals, a single mesenteric lymphatic vessel was cannulated to collect pre-nodal lymph (Figure 2B). The lymph node draining this vessel [and two other non-cannulated vessels] was identified and a single post-nodal lymphatic vessel was then cannulated to collect post-nodal lymph. Plasma samples were prepared from blood drawn following collection of postnodal lymph samples. The fluorescence of lymph and plasma samples was measured (**Figure 2C**). We found that BH4 encapsulated in SLNPs is not detected in plasma using the standard extraction method for BH4 analysis (data not shown). To perform pharmacokinetic analysis of BH4-loaded SLNP delivery to the systemic blood circulation following oral delivery of SLNPs, we utilized oral gavage to deliver 6-coumarin-loaded SLNPs to the gastrointestinal tract. Analysis of plasma fluorescence indicated peak appearance of SLNPs in the plasma at 6 hours post-gavage, with fluorescence levels returning to baseline by 48 hours after gavage (Figure 2D).

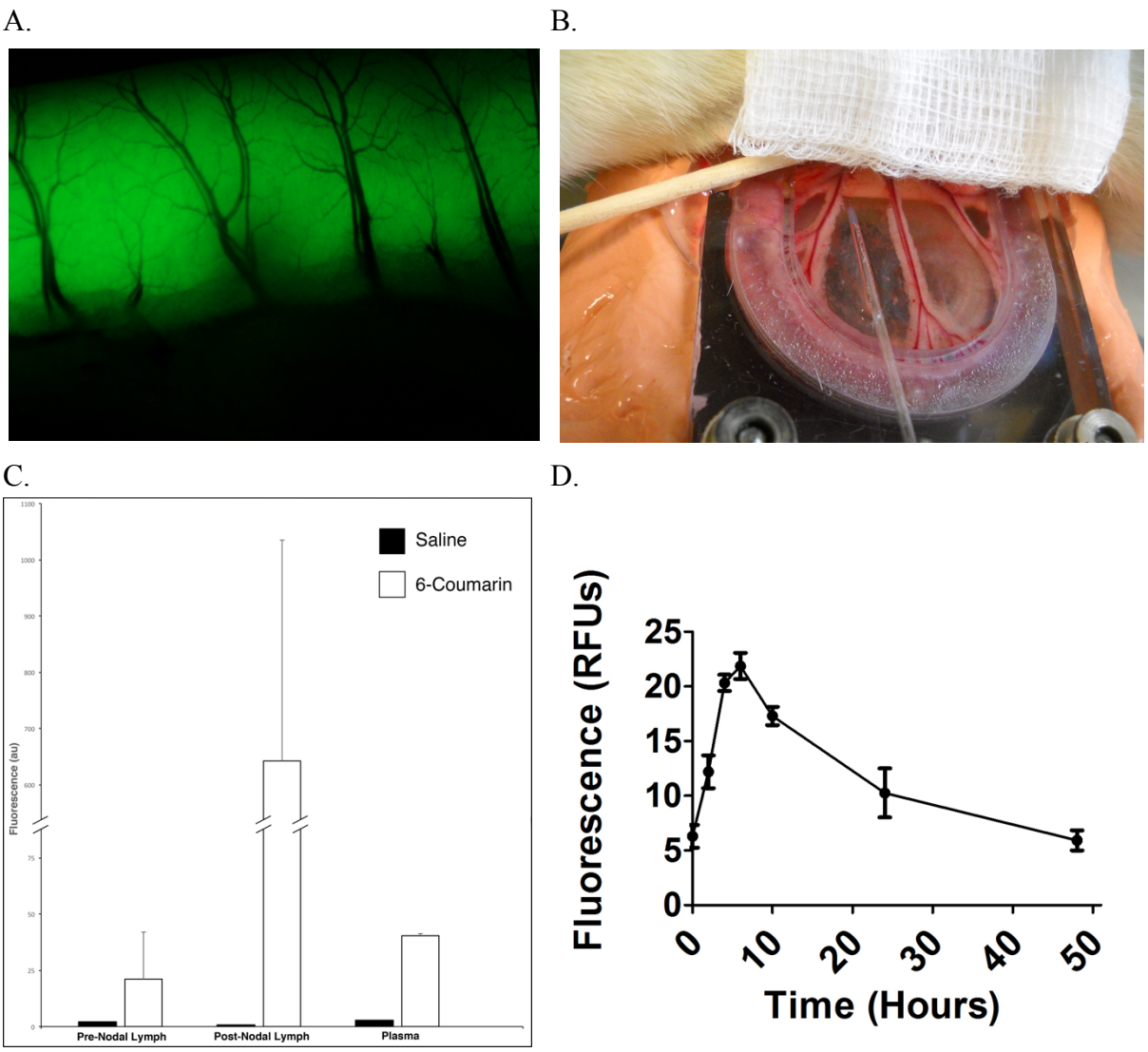
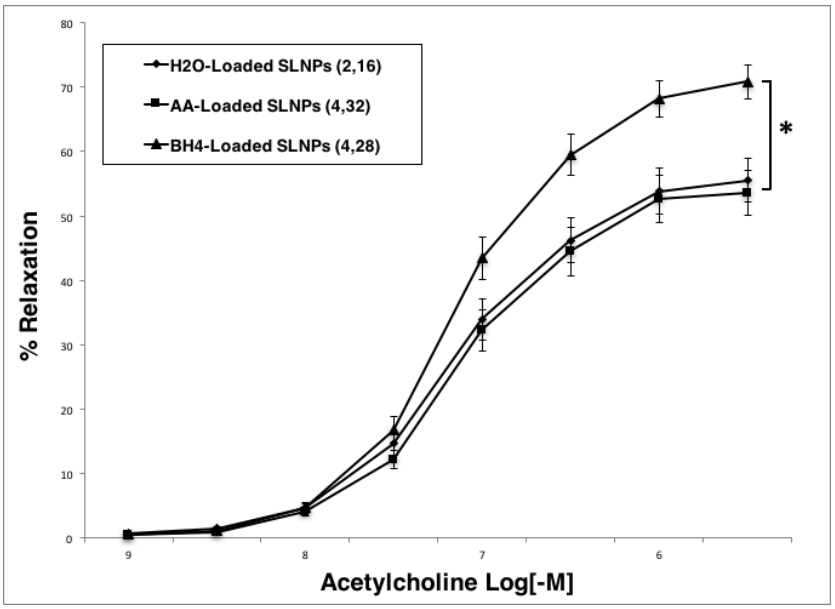


Figure 2: SLNPs are taken up by mesenteric lymphatic vessels but not intestinal blood vessels. (A) Fluorescent SLNPs in the small intestinal lumen and associated blood vessels were imaged using an Olympus CZX16 stereomicroscope equipped with corresponding filters and an Olympus DP72 fluorescence camera. **(B)** Cannulation of a pre-nodal mesenteric lymphatic vessel for collection of lymph. **(C)** Fluorescence (in arbitrary units [au]) of plasma and lymph samples was measured after cannulating a pre-nodal mesenteric lymphatic vessel, followed by cannulating a post-nodal mesenteric lymphatic vessel, collecting lymph for 1 hour at each site. Plasma samples were then obtained following completion of the post-nodal vessel cannulation. Data are presented with background fluorescence subtracted (duplicate samples were analyzed in two separate experiments, n=2 animals per experiment, data shown as mean ± SEM). **(D)** Pharmacokinetics of a single oral gavage (50 µl) of 6-coumarin-loaded NPs, showing fluorescence in plasma from blood samples taken at the indicated times (n=3 animals).

To demonstrate that uptake of BH4-loaded SLNPs results in nitric oxide production and thus endothelium-mediated vasodilation [a measure of endothelial function], BH4-loaded SLNPs were administered to diabetic rats via oral gavage. SLNPs delivering 65 µg (approximately 0.2 mg/kg) of BH4 were administered once each day for 2 days and rats were euthanized 24 hours after the second gavage. Nitric oxide-mediated vessel relaxation was studied in rats receiving BH4-loaded SLNPs, SLNPs made with the ascorbic acid (AA) vehicle, or SLNPs made with H_2O only (Figure 3A). Acetylcholine concentration-response curves from aortic rings of rats receiving BH4-loaded SLNPs were also compared to the curves generated with aortic rings from non-treated diabetic rats as well as non-treated normal rats (non-diabetic control animals) (Figure

3B). *Endothelium-dependent (i.e., NO-mediated) dilation returned to the level of a normal (non-diabetic) rat after just two days of treatment with BH4-loaded SLNPs.*

A.



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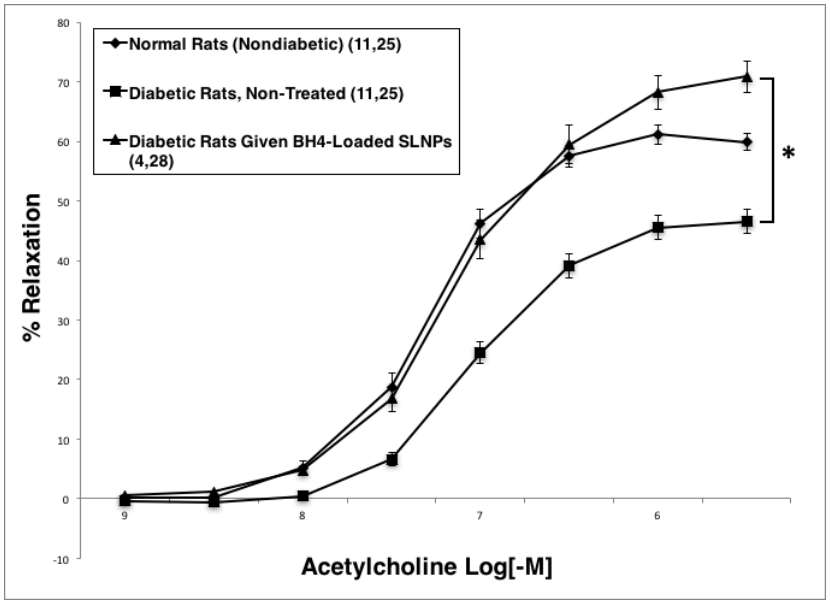

Figure 3: Endothelium-dependent relaxation of arterial rings from diabetic rats ingesting SLNPs. (A) Aortic rings were dissected from animals receiving SLNPs containing BH4, ascorbic acid (AA) vehicle, or H_2O only and endothelium-dependent relaxation (i.e., NO synthesis) was assessed. **(B)** The NO-mediated response of vessel rings from rats receiving BH4-loaded SLNPs was also compared to the response of vessel rings from non-treated control (non-diabetic) rats as well as vessel rings from non-treated diabetic rats. The % relaxation is expressed relative to the maximal relaxation obtained in the presence of nitroprusside (100 μM). Data are presented as mean ± SEM with number of animals followed by number of vessel rings analyzed shown in parentheses. * $p < 0.05$, analyzed using two-way ANOVA for repeated measures and Tukey's honest significant difference post hoc test for multiple comparisons.

Specific Aim 2: Demonstrate orally administered BH4-loaded solid lipid nanoparticles reduce the risk of atherosclerosis associated with type 1 diabetes. T

These studies are currently underway and all animals have not completed the 10-week treatment period. The purpose of this study is to evaluate the ability of BH4-loaded SLNPs to delay and/or attenuate the development of atherosclerosis in nanoparticle-treated ApoE knockout (ApoE-/-) mice maintained on a high-fat diet. Some mice were induced to become diabetic at 6 weeks of age by i.p. injection of streptozotocin. Both diabetic and vehicle-control (non-diabetic) ApoE-/- mice were randomized to 3 groups (n=10 mice/group; 6 groups total): (1) Non-treated (water gavage), (2) Vehicle-treated (ascorbic acid-loaded SLNs), and (3) BH4-treated (BH4-loaded SLNs). Based on the data from Specific Aim 1, our long-term efficacy study for Specific Aim 2 was initiated with SLNPs being administered every 72 hours for the 10-week treatment period.

Data from animals that have completed the treatment period (n=5) support the ability of BH4 SLNPs to improve endothelium-dependent (nitric oxide-mediated) vessel relaxation in ApoE-/- mice, when compared to the same mice receiving SLNPs containing only the ascorbic acid vehicle (Figure 4). A smaller number of diabetic ApoE-/- mice administered the BH4 nanoparticles showed a similar improvement in vessel function (data not shown).

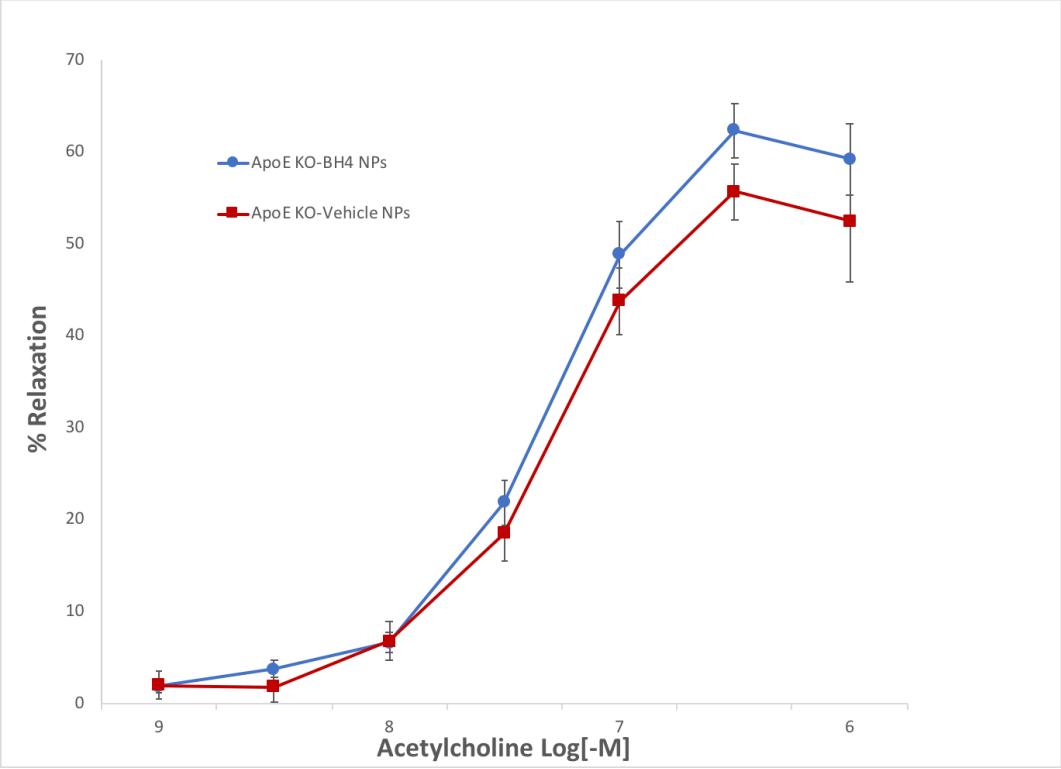

Figure 4: Endothelium-dependent relaxation of arterial rings from ApoE-/- mice ingesting SLNPs. Aortic rings were dissected from animals receiving SLNPs containing BH4 or ascorbic acid (AA) vehicle and endothelium-dependent relaxation (i.e., NO synthesis) was assessed. Preliminary data (n=5 animal, 10 vascular rings assessed).

In addition to measuring coronary BH4 levels and assessing abdominal aortic vessel reactivity, we will also compare: (a) aortic root atherosclerotic lesion size, (b) inflammatory cell infiltration, and (c) adhesion molecule expression.

3. Publications:

Abstracts presented:

Kelly, K.A., Stees, M.P., Wang, W., Gashev, A.A., Wu, G., Labhasetwar, V., and Meininger, C.J. Nanoparticle-mediated delivery of tetrahydrobiopterin restores endothelial function in diabetic rats. Presented at the International Vascular Biology Meeting, Boston, MA, November 2016.

Kelly, K.A., Stees, M.P., Rao, K.S., Vasir, J.K., Dimitrejevic, S., Li, X., Heaps, C.L., Wang, W., Gashev, A.A., Wu, G., Labhasetwar, V. and Meininger, C.J. Nanoparticle-mediated delivery of tetrahydrobiopterin restores endothelial function in diabetic rats. Presented at the Gulf Coast Vascular Research Consortium Meeting, Shreveport, LA, May 2016.

Manuscripts submitted:

Kelly, K.A., Stees, M.P., Rao, K.S., Vasir, J.K., Dimitrejevic, S., Li, X., Heaps, C.L., Wang, W., Gashev, A.A., Wu, G., Labhasetwar, V. and Meininger, C.J. Nanoparticle-mediated delivery of tetrahydrobiopterin restores endothelial function in diabetic rats. Submitted to Journal of Physiology.