RESEARCH ARTICLE

AGE EXACERBATES NEOINTIMA GROWTH IN CAROTID ARTERIES OF ZUCKER DIABETIC FATTY RATS.

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Abstract:

Background:

Diabetes is a pervasive disease that can affect all organs and tissues of the body. Hyperinsulinemia, hyperglycemia, dyslipidemia and advanced glycation end products are but a few of the anomalies of diabetes that impinge upon the vasculature and are causal agents for vascular inflammation and atherosclerosis. The purpose of this study was to determine the effects of age, obesity and hyperinsulinemia on carotid arteries after mechanical injury and compare the parameters of intima media thickness, lumen diameter and blood flow velocity between 8-week and 16-week, control (CON)(lean) and experiment (EXP)(fat) litter-mate male Zucker Diabetic Fatty rats.

Methods:

The injured carotid arteries of 8-week and 16-week old male Zucker Diabetic Fatty rats and their CON littermates were catheterized for endarterectomy at Charles River Laboratories. Rats were transported to our vivarium one week after endarterectomies were completed. Using ultrasound imaging, internal lumen diameter, dorsal and ventral walls, external artery diameter and blood flow velocity of the injured carotid arteries (injured) were compared to the same parameters in the non-injured carotid arteries (internal non-injured) within the same age group, between CON and EXP rats of the same age group and between 8-week and 16-week age groups. One week after receiving the rats, each rat was anesthetized for 30 minutes for ultrasound measurements using an isoflurane-oxygen solution. Ultrasound images were collected on injured catheterized and non-injured CON carotid arteries on days 14, 21, 42 and 62 post surgery.

Results:

As age progressed, there were significant differences in the injured carotid lumen and injured total diameter measurements between the 8- and 16-week cohorts over time in CON and EXP rats. We noted that the injured carotid arteries of the 8-week cohorts never improved, while the same parameters of the 16-week cohorts did exhibit recovery. Interestingly, the 8-week rats demonstrated no change of the injured lumen over time after damage, while the 16-week rats demonstrated increased lumenal space, indicating recovery. In contrast, there were no differences in the non-injured carotid lumens and total diameters of 8-week and 16-week CON and experiment (EXP) rats. There were no significant differences between the blood flow velocities of the injured carotid arteries of the 8-week and 16-week cohorts over time. However, compared to the 8-week cohort, the non-injured carotid velocity of the 16-week rats was significantly lower in both CON and EXP rats. There were no significant differences in injured carotid measurements when comparing CON to EXP rats within the same age group.

Keywords: Carotid arteries, Inflammation, Ultrasound imaging, Atherosclerosis

Introduction

The health of the circulatory system is dependent upon the integrity of the vascular wall, the state of its endothelial barrier and physical injury to the vessels themselves. Many factors impinge upon the vessel walls. These include, but are not limited to monocytes, cytokines and hormones. Biochemical and physical insults to the vessel wall can activate a sequela of events that compromise the endothelium whereby the lumen, the vessel diameter and blood flow are altered from normal conditions [1].

Diabetes is a risk factor for cardiovascular disease. In its wake are the insults to the vasculature due in part to the presence of hyperphysiological concentrations of insulin (i., e., hyperinsulinemia, HI) in the serum, advanced glycation end-products (AGE), hyperlipidemia and hypertension [2,3]. All of these contribute to atherosclerosis. Obesity and cardiovascular disease are not limited to people in the US, but can be seen in other cultures such as Persian [4], Asian [5] and multi-ethnic groups [6]. The events that comprise the foundation of damage to the endothelium are many. Since the factors that influence damage to the endothelium have not been fully examined, it behooves us to find the mechanisms that drive the pathophysiology of arterial inflammation. This report addresses the effects of age, obesity and hyperinsulinemia on the vasculature.

We demonstrated in this study that age, but not hyperinsulinemia, contributed to the pathophysiology of the endothelium, including lumen diameter, intima media thickness, and blood flow velocity in carotid arteries of Zucker Diabetic Fatty (ZDF) rats as measured by ultrasound imaging and confocal microscopy. Since individuals with diabetes do not benefit by angioplasty to the extent as individuals without diabetes, we utilized the ZDF rat to model this issue. We measured these

parameters in EXP and CON littermate ZDF rats by comparing injured and non-injured carotid arteries over time (14 – 62 days post-surgery) in CON and EXP rats that had their injured carotid artery internally injured by balloon catheterization at 8 and 16 weeks of age. This procedure was carried out to simulate a postangioplasty scenario. We report here the impacts of these insults on the vascular health and recovery of these carotid arteries.

METHODS:

Materials:

Zucker Diabetic Fatty rats (8 and 16 weeks old, at the time of surgery) were obtained from Charles River Laboratories (Wilmington, MA, USA) ten days after carotid artery injury (ATH-EMB) (a.k.a. endarterectomies) were performed on their injured carotid arteries. Briefly, a balloon tip catheter was passed from the external carotid artery into the injured common carotid artery. Once positioned, the catheter bulb was inflated and the catheter was advanced and retracted multiple times to induce endothelial injury. Upon completion, the catheter was removed and the animals recovered. Male CON (genotype 380, CON Fa/+, lean) and littermate male EXP (genotype 370 obese) were utilized for these experiments. The rats were housed in the veterinary medical unit at the Eastern Colorado Health Care System (ECHCS), Denver VA Medical Center, Denver, Colorado, USA. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, United States Department of Agriculture (USDA) and Public Health Service (PHS) and with the approval of the ECHCS Institutional Animal Care and Use Committee (IACUC). AlexaFluor647(AF647)-conjugated anti-bodies to Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1) were both obtained from Novus Biologicals (Denver, CO, USA).

Diet and Housing:

Rats were allowed to acclimate to our facility for one week after receipt and before studies began. Rats ate Teklad 2018 chow (Harlan/Envigo, Indianapolis, IN, USA) and drink water *ad libitum.* Male rats were housed two per cage on a constant 12 h light/dark cycle under controlled temperature and humidity conditions. Male rats were used in these experiments because male rats, but not female rats, become obese with the Teklad 2018 chow.

Equipment and Imaging Programs:

Ultrasound imaging was performed using the VEVO 2100 (FujiFilm VisualSonics, Inc., Toronto, Ontario, Canada). The MS250 probe was used for all carotid measurements. VEVO 2100 software (v.1.6.0) was used for imaging and analyses. The following carotid parameters were assessed: carotid intima media thickness (dorsal and ventral) and carotid lumen diameters using M-mode, and blood flow velocity using Doppler Pulse Wave.

Experimental Design:

Rat cohorts were delineated into 8-week (n = 5 CON, 5 EXP) and 16-week old (n = 6 CON, 6 EXP) groups. Ultrasound evaluations of the injured and non-injured

carotid arteries were performed on days 14, 21, 42 and 62 post surgery, and were made under isoflurane-oxygen mixture. Unconsciousness was determined by a change in breathing and toe pinch. The neck area near the location of the carotid arteries was cleared of hair using a depilatory agent and then followed by a careful washing and drying of the area. Each rat was secured to the examination table and monitored for respiration velocity and internal temperature by foot-pad and rectal probe. Ultrasound gel was applied to the neck area for ultrasound measurements. The probe was first located perpendicular to axis of the spine in the carotid artery region in order to locate the carotid arteries. Subsequently, the probe was turned 90 degrees to measure the lumen diameter, intima media thickness and the blood flow velocity of the designated carotid.

Confocal Microscopy:

Injured and non-injured carotid arteries were explanted from rats immediately following the Day 62 ultrasound measurements. Explanted carotid arteries were frozen in Optimal Cutting Temperature (OCT) compound (Electron Microscopy Sciences, Hatfield, PA. USA) and then sectioned into 10 micron samples for immunofluorescent preparation. Tissue sections were fixed for 10 minutes at room temperature using 1% (v/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS. The sections were washed twice with PBS and blocked for 30 min at room temperature using 1% (w/v) Bovine Serum Albumin (BSA, Sigma-Aldrich Inc., St. Louis, MO, USA). After the blocking procedure, the blocking solution was aspirated and the sections incubated with anti-VCAM-1 and anti-ICAM-1 antibodies overnight at 4˚C. Both antibodies were diluted 1:100 in 1% BSA. Following this incubation, the sections were washed five times with 1% BSA and then 5 times with PBS. Fifty percent glycerol (Fisher Scientific, Fair Lawn, NJ, USA) mounting medium was applied to the sections and a coverslip was affixed. A Leica SP8 (Buffalo Grove, IL, USA) confocal microscope was used to visualize the presence of cell surface VCAM-1 and ICAM-1.

Statistical Analysis:

All carotid measurements were performed using VEVO 2100 software analyses and graphing were performed using Graphpad Prizm (ED.5v.f.09, La Jolla, CA, USA). A Student *t*-test was performed on data at the same time point, comparing non-injured to injured and 8-week-old versus 16-week-old parameters.

RESULTS:

TABLE AND GRAPHS –

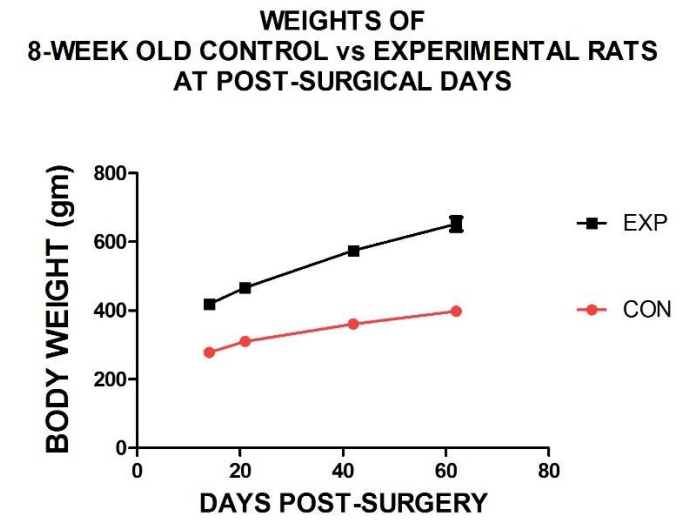
Table 1 and Graphs A (week 8 cohort) and B (week 16 cohort) indicate 8-week and 16-week body weights of CON and EXP rats over the 62-day period of research.

Table 1 and Graphs A. and B. – Table and graphs indicating weights of 8-week and 16-week CON (lean) and EXP (fat) rats at 14, 21, 42 and 62 days. Table and graphs indicating average weight of CON and EXP rats at indicated days post-surgery.

TABLE 1 AND GRAPHS A. AND B.

Day	Week 8 CON	Week 8 EXP		Week 16 CON	Week 16 EXP
14	278.5±7.0	419±2.7		390.2±15.6	565.5±13.1
21	310±5.8	467.8±3.6		398.3±14.8	592.3±12.7
42	361.6±4.3	575.6±12.3		469.2±15.3	691.2±15.1
62	398.4±4.2	652±19.7		496.2±14.2	744.3±26.0

A.



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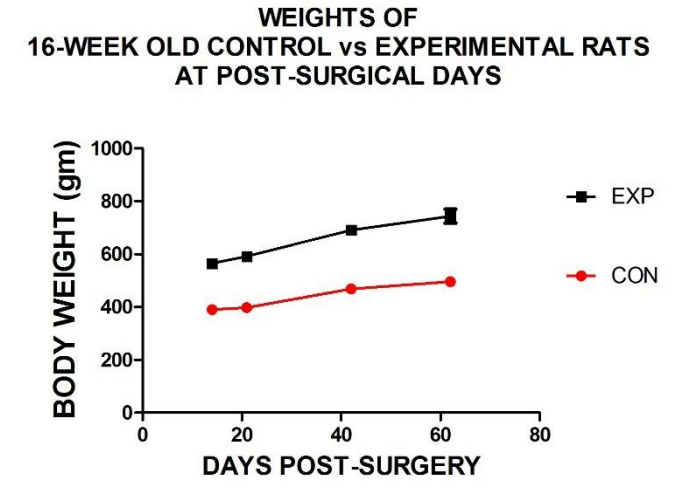

ULTRASOUND IMAGING –

Injured balloon-catheterized and non-injured untouched carotid arteries of live, age-matched male obese (EXP) Zucker Diabetic Fatty rats and their CON littermates were analyzed *in vivo* using ultrasound technology to measure (1) lumen

and vessel diameters, (2) dorsal and ventral intima media thickness and (3) blood flow velocity of injured and non-injured carotid arteries. We observed that there were significantly different dimensions between injured and non-injured carotid arteries in age-matched rats.

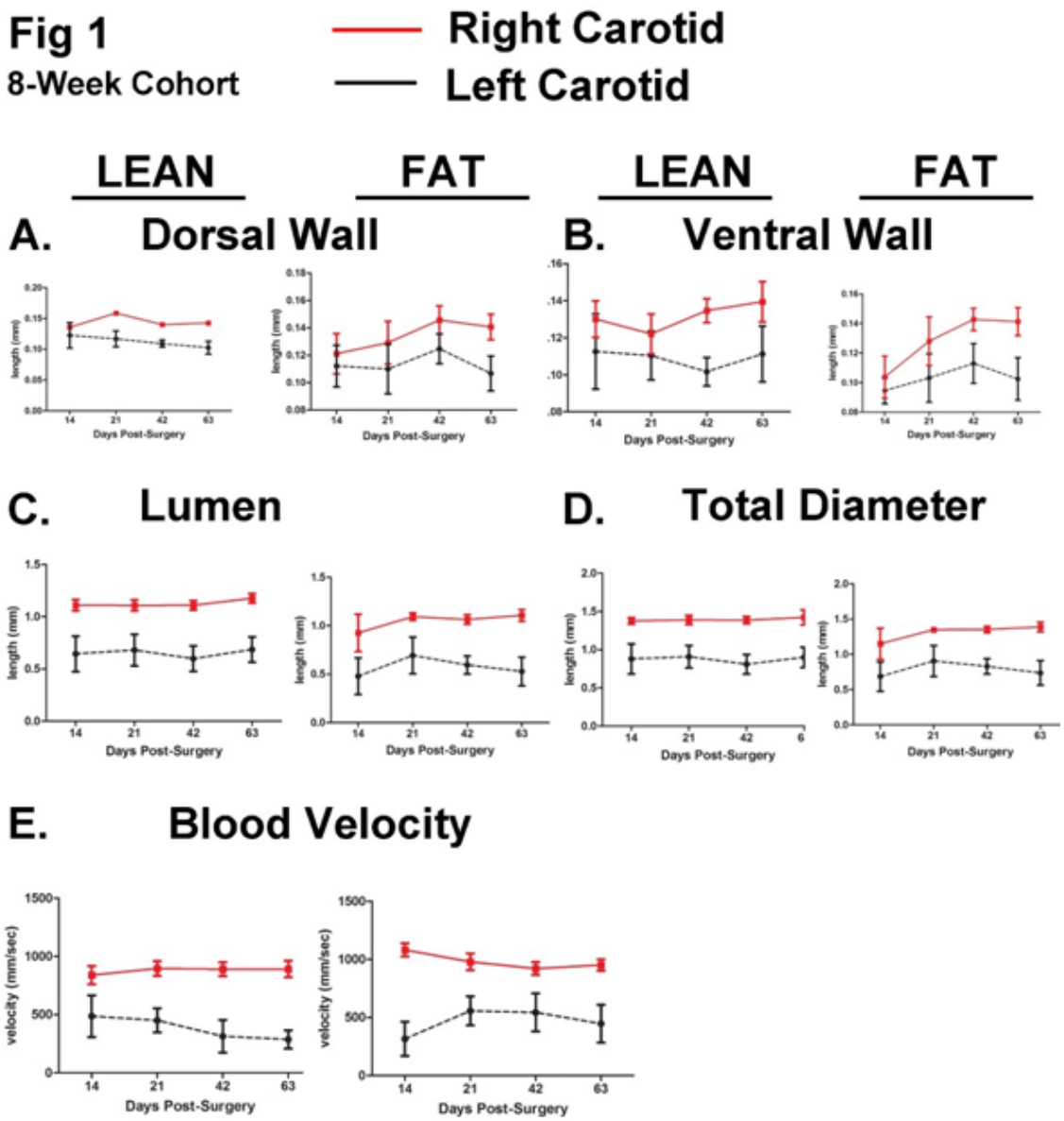

Figure 1 – Injured and non-injured carotid arteries of CON and EXP 8-week cohort rats were measured over time using ultrasound measurements. Injured carotid arteries of eight-week old CON and EXP rats were injured by balloon catheterization and visualized by ultrasound over 62 days. Dorsal and ventral intima media thickness, carotid lumen and total diameters and blood flow velocities were measured in injured carotid arteries (black lines) and compared to the same parameters of their respective internal CON non-injured carotid arteries (red lines).

8-week cohort – (Figure 1)

In Figure 1, the measurements of the non-injured carotid arteries (red lines) of the CON rats did not change significantly over time. Nor were they significantly different at any time point from the EXP rats. The dorsal (Fig 1A) and ventral (Fig 1B) intima

media thickness of the non-injured carotid arteries (red lines) of the EXP rats increased over time, while those seen for the injured carotid arteries (black lines) remained the same. Measurements of the non-injured carotid arteries (red lines) were significantly different versus the injured carotid arteries (black lines) throughout the entire timeline in both the CON and EXP rats. While all dimensions were less in the injured carotid arteries as compared to their respective internal CON non-injured carotid arteries in both CON and EXP rats, there were no significant differences when comparing the identical carotid arteries of the CON and EXP animals (i.e., CON injured vs. EXP injured and CON non-injured vs. EXP non-injured).

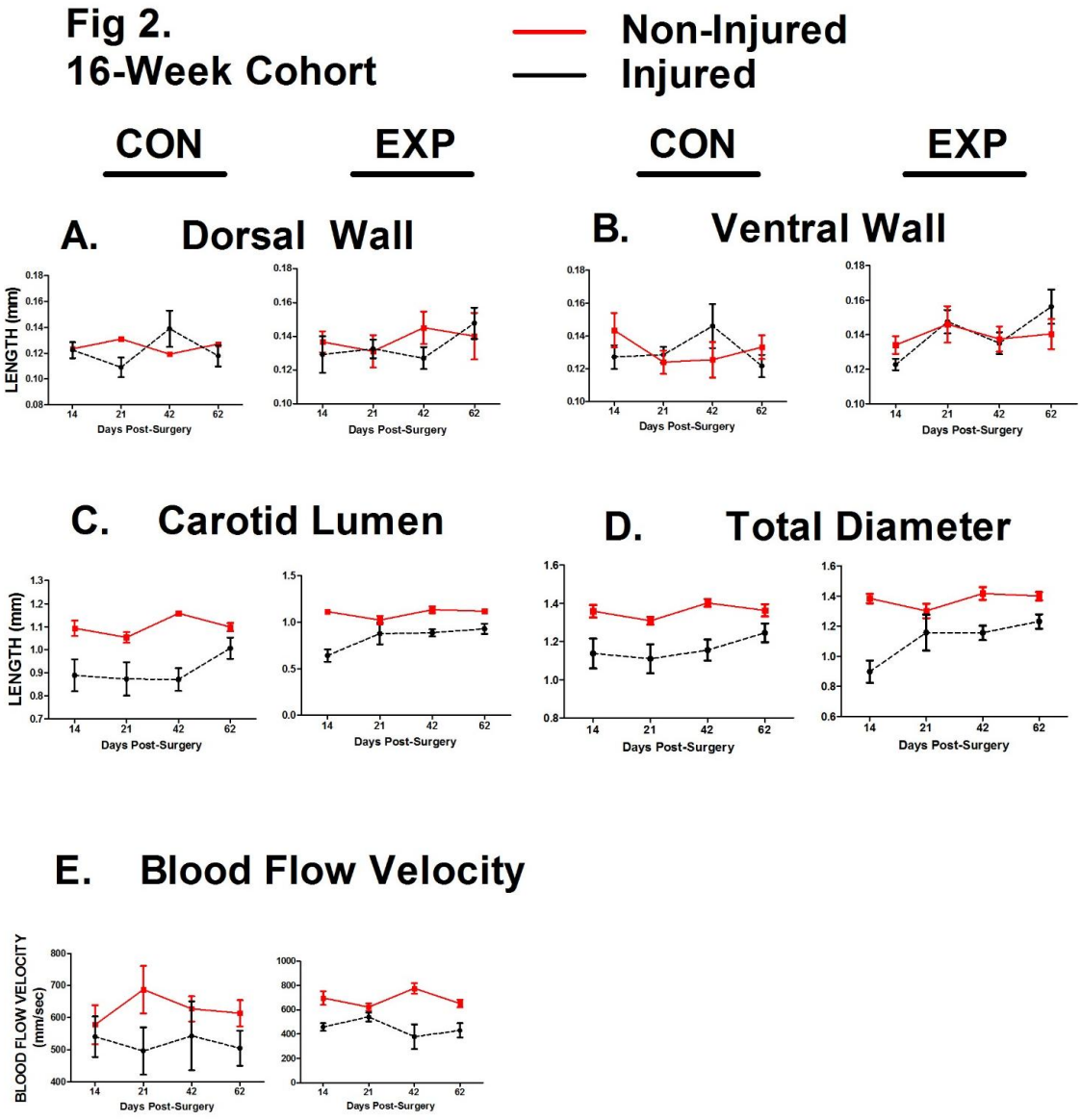

Figure 2 – Injured and non-injured carotid arteries of CON and EXP 16-week cohort rats were measured over time using ultrasound measurements. Injured carotid arteries of 16-week old CON and EXP rats were injured by balloon catheterization and visualized by ultrasound over 62 days. Dorsal and ventral intima media thickness, lumen and total diameters and blood flow velocity were measured in injured carotid arteries (black lines) and compared to the same parameters of their respective internal CON non-injured carotid arteries (red lines).

Neither the lumens nor the total vessel diameters of the carotid arteries of any animals changed significantly over time (Fig 1C,1D). However, we observed a significant difference between injured parameters verses the non-injured internal CONs. Comparison of blood flow velocities of the injured carotid in the CON rats (Fig 1E, injured panel) decreased over time while the blood flow velocity in the injured carotid arteries of the EXP rats (Fig 1E, non-injured panel) remained stable; an indication of occlusion in the injured carotid.

16-week cohort – (Figure 2)

Intima media thickness measurements (Fig 2A, B) were not significantly different between injured and non-injured carotid arteries of both CON and EXP rats. However, injured carotid lumenal measurements (Fig 2C, black lines) were significantly ($P < 0.05$) smaller than non-injured lumenal measurements (Fig 2C, red lines) of the non-injured carotid arteries at all time points in both CON and EXP rats. The disparity between injured and non-injured lumenal spaces was greater at early time points and substantially decreased by Day 62, suggesting recovery of the injured carotid arteries. In contrast, blood flow velocity in the injured carotid arteries did not improve over time (Fig 2E).

Comparison of 8-week and 16-week Cohorts – (Figure 3)

As age progressed, there were significant differences in the injured carotid lumen (Fig 3A) and injured total diameter (Fig 3C) measurements between the 8-week (black lines) and 16-week (red lines) cohorts over time. In the 8-week cohorts of both CON and EXP, the injured lumens of the 8-week CON (Fig 3A, black line) and EXP (Fig 3A, black line) never approached the increased dimensions of the 16-week injured lumens (red lines). In contrast, the non-injured lumens (Fig 3B) and diameters (Fig 3D) of the 8-week rats reached that of 16-week rats quickly. Interestingly, there were significant differences in injured lumen diameters between 8-week (black lines) and 16-week (red lines) in both CON (injured panel) and EXP (non-injured) rats (Fig 3C). There were no significant differences between the blood flow velocities of the injured carotid arteries (Fig 3E) of the 8-week rats and that seen for the 16-week cohorts over time. However, in Fig 3F, the blood flow velocities of the non-injured carotid of the 8-week rats (black lines) were greater than the blood flow velocities of the 16-week rats (red lines) in both CON and EXP rats. These data suggested that the 16-week non-injured carotid arteries compensated for their injured damaged carotid arteries, whereas the injured carotid arteries of 8 week and 16 week rats were affected by age.

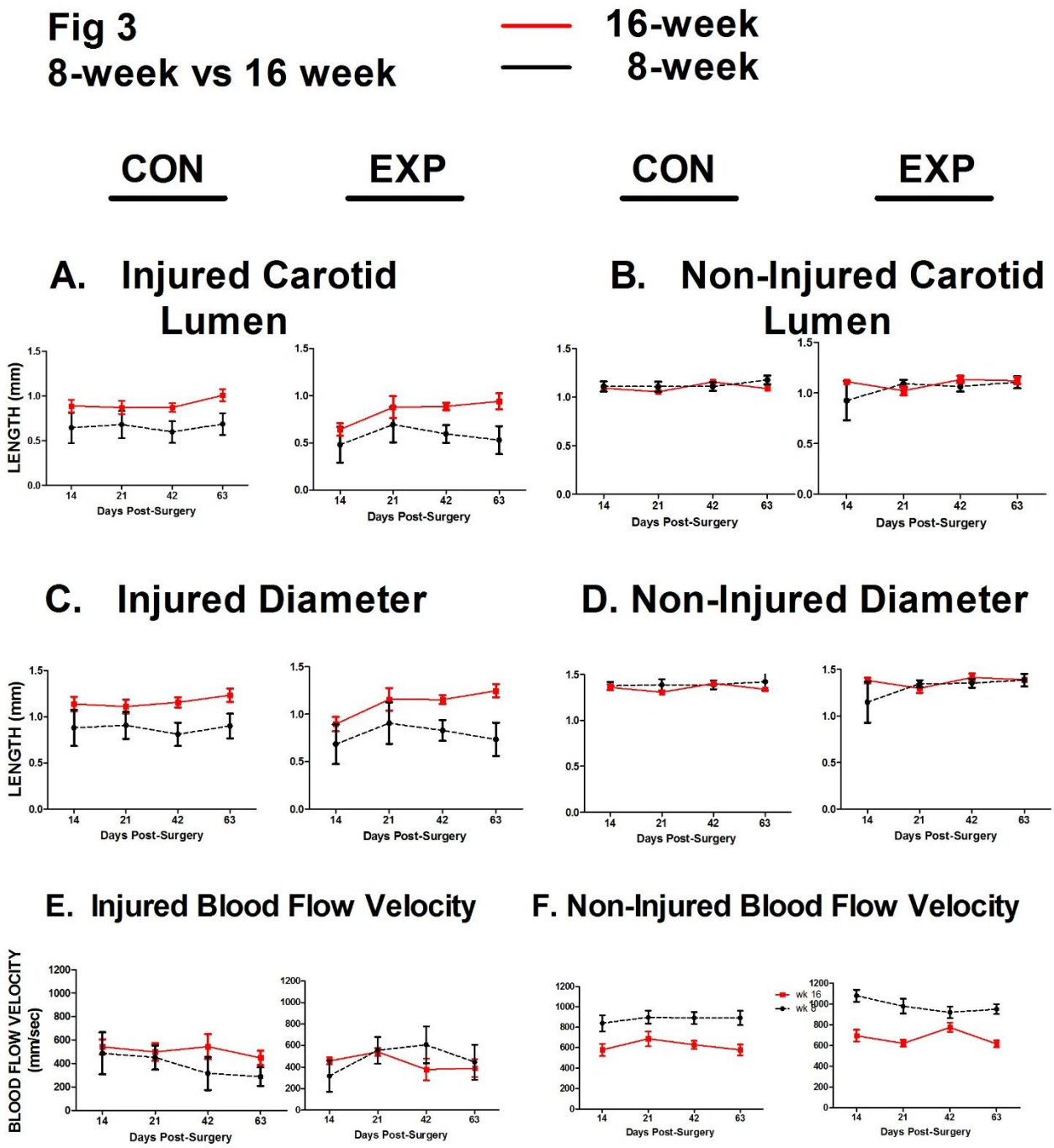

Figure 3 – Comparison of ultrasound measurements of carotid parameters of CON and EXP 8-week and 16-week cohort rats over time. Ultrasound measurements of carotid parameters in 16-week (red lines) and 8-week (black lines) rat cohorts were compared in CON and EXP rats.

CONFOCAL MICROSCOPY –

We wanted to correlate our ultrasound findings with vascular markers of inflammation. To this end, we utilized immunofluorescent confocal microscopy on explanted injured and non-injured carotid arteries from CON and EXP rats of both age cohorts and stained for VCAM-1 and ICAM-1 (Fig 4). Here we show

representative micrographs of explanted carotid arteries of rats from the different age groups and different phenotypes, noting the amounts of VCAM-1 and ICAM-1 on the endothelial cells and their respective lumens.

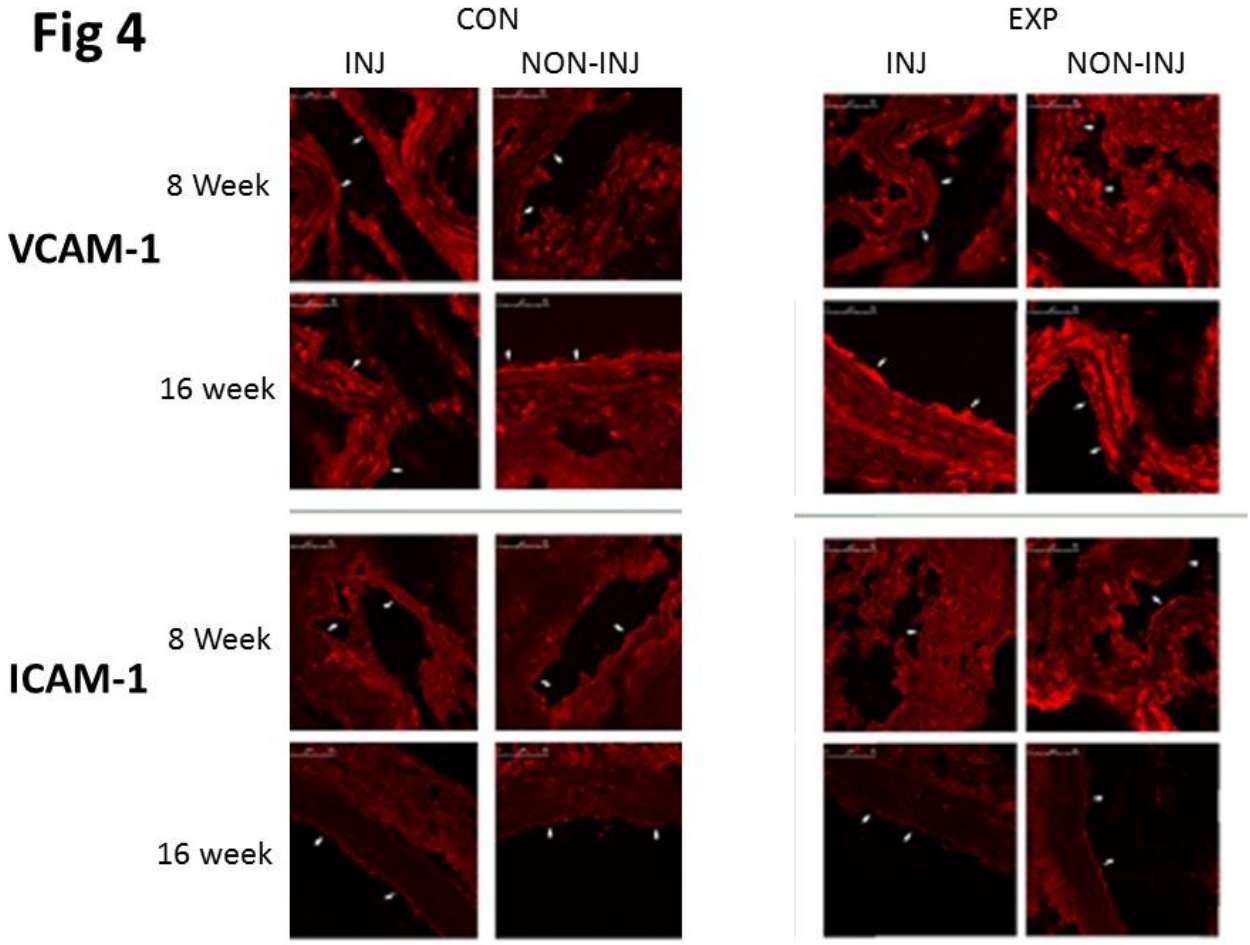

Figure 4 – Immunofluorescent microscopy of CON injured and non-injured carotid arteries versus EXP injured and non-injured carotid arteries of 8-week and 16-week old rats. Injured and non-injured carotid arteries from CON and EXP rats were explanted and frozen in OCT. Ten micron sections were prepared as described in METHODS and confocal microscopy was used to determine the amounts of VCAM-1 and ICAM-1 in the carotid tissue as well as lumen and intima parameters. Bar = 50 micrometers. White arrows indicate endothelial layer of carotid arteries.

There were six ways we compared neo-intima and VCAM-1/ICAM-1 expression among the different groups of carotid arteries: (1) CON 8-week injured carotid vs CON 8-week non-injured carotid, (2) CON 8-week injured carotid to EXP 8-week injured carotid, (3) EXP 8-week injured to EXP 8-week non-injured carotid arteries (4) CON 16-week injured carotid vs CON 16-week non-injured carotid, (5) CON 16-week injured carotid to EXP 16-week injured carotid, (6) EXP 16-week injured carotid to EXP 16-week non-injured carotid.

Observations: (1) CON 8-week injured carotid vs CON 8-week non-injured carotid: we see a slightly thicker neo-intima in the

injured carotid with increased incidence of VCAM-1. There was significantly more ICAM-1 expression in the injured carotid arteries of both CON and EXP 8-week old rats compared to their respective non-injured carotid counterpart. (2) CON 8-week injured carotid to EXP 8-week injured carotid: at this age, there appears to be no significant differences in intima size or VCAM-1/ICAM-1 expression between CON and EXP phenotypes. (3) EXP 8-week injured carotid to EXP 8-week non-injured carotid: there appears to be more VCAM-1 in the non-injured carotid, whereas ICAM-1 is much more highly expressed in the injured carotid. (4) CON 16-week injured carotid vs CON 16-week non-injured carotid: at this age, we see a smaller lumen in injured carotid than in the non-injured carotid and more VCAM-1 and ICAM-1 in injured as compared to the non-injured. (5) CON 16-week injured carotid to EXP 16-week injured carotid: significant increase in VCAM-1 and neo-intima in injured EXP carotid arteries versus CON carotid arteries and greater ICAM-1 expression in the EXP 16-week injured carotid arteries versus CON 16-week injured carotid arteries. (6) EXP 16-week injured carotid to EXP 16-week non-injured carotid: there is less lumen and more neo-intima in the injured carotid arteries versus the non-injured carotid arteries and more VCAM-1 and ICAM-1 in the EXP 16 week injured carotid arteries than that seen in the 16 week EXP non-injured carotid arteries.

DISCUSSION:

Obesity, hyperinsulinemia (HI), physical injury and age are important factors in the health and pathogenesis of the vasculature. In this study, we show that age and obesity contribute to the atherosclerotic phenotype.

In this study, age-matched CON and EXP male Zucker Diabetic Fatty (ZDF) rats were subjected to a balloon catheter carotid endarterectomy at 8 and 16 weeks of age in their left carotid arteries. We hypothesized that carotid endarterectomy recovery would be attenuated by age. We used ultrasound technology to determine the following parameters in injured and non-injured carotid arteries: (1) total vessel diameter, (2) lumen diameter, (3) dorsal and ventral intima media thickness and (4) blood flow velocities. Additionally, we employed immunofluorescent confocal microscopy to determine changes in (1) neointima size, (2) vessel lumen size and (3) VCAM-1 and ICAM-1 expression in the cross-sectional areas of the endothelial layers of injured and non-injured carotid arteries of CON and EXP 8-week and 16-week old cohorts.

In this report, we show that age and obesity exacerbated the effects of endarterectomy on a vascular tissue and blunt the reversion of injured carotid arteries to normal morphology and blood flow capacity. In the 8-week cohort rats all the non-injured carotid parameters (Fig 1, red lines) were greater than the injured carotid parameters (Fig 1, black lines), which was expected due to the induced injury in the injured carotid. There did not appear to be recovery of the carotid damage over the observed period either in the CON or EXP rats as noted by the red and black lines not converging. This suggests that the hyperinsulinemia contributed to slower recovery of these tissues. Hempe *et al*. [7] have shown that Zucker Fatty Diabetic rats demonstrated hyperinsulinemia up to 8 weeks of age with subsequent hyperinsulinemia levels rapidly declining at later ages. Additionally, Rask-Madison *et al*. [8] observed that hyperinsulinemic mice exhibited accelerated atherosclerosis, impaired vasodilation and increased adhesion of mononuclear cells attached to the endothelium as compared to mice with normal insulin concentrations. We hypothesized that high insulin levels present at the time of 8-week catheteri-

zation contributed to the slower recovery process caused by catheterization.

The results seen in the 16-week old cohort (Fig 2) were different. The injured right dorsal and ventral walls exhibited no differences in size in either CON or EXP groups. Lumen diameters, total diameters and blood flow velocities exhibited differences, but there was a trend towards parity at later ages. The lumen size (Fig 2C), total carotid diameter (Fig 2D) and blood flow velocities (Fig 2E) were significantly different in the non-injured carotid (red lines) as compared to the injured carotid arteries (black lines) in both CON rats (injured panel) and EXP rats (non-injured panel). However, we observed injured and non-injured carotid parameters approach parity, presumably due to age progression [16].

The hypothesis that hyperinsulinemia inhibits arterial recovery is still unclear. We illustrated in this study this possibility when we compared lumenal (Fig 3A) and total diameters (Fig 3C) of injured carotid arteries of 8-week (black lines) and 16-week (red lines) rats. Not only was the damage more extensive in the 8-week cohort (as demonstrated by reduced parameters), but the 8-week rats showed little or no recovery of their injured carotid arteries, whereas those same parameters in the 16-week old rats exhibited definite changes towards normalcy.

Interestingly, the blood flow velocities in the injured carotid arteries of 8-week CON and EXP rats (Fig 3E) were similar to that seen for 16-week cohort rats. In contrast, the blood flow velocities in the non-injured carotid arteries of 8-week CON and EXP rats (Fig 3F, black lines) were greater than that measured in the 16-week non-injured carotid arteries (Fig 3F, red lines). These results suggest that in both CON and EXP groups the blood flow velocity in the 16-week age group was slower in the non-injured carotid because it may not have had to compensate for the lack of flow through the injured carotid as it did in the 8-week old rats. Since we did not quantify intima hyperplasia measurements, we are cautious about these remarks.

In addition to ultrasound measurements of injured and non-injured carotid arteries of 8-week and 16-week rats, we also measured the prevalence of VCAM-1 and ICAM-1 in injured catheterized and non-injured CON carotid explants of CON and EXP rats using immunofluorescence microscopy. Carotid neo-intima growth is a surrogate marker of CVD and thus is a rationale for this for the present study. We wanted to see if there was a correlation between changes in the physical parameters of these carotid arteries and the expression of VCAM-1 and ICAM-1 on the endothelial cells of these vessels. We hypothesized that hyperinsulinemia would cause an increase in cell adhesion molecule expression on injured and obese carotid endothelial cells as compared to non-injured and CON vessels. We were interested in VCAM-1 and ICAM-1 expression because these two adhesion molecules are important in the process of monocyte binding to endothelial cells. Subsequent migration of monocytes across the endothelial layer can lead to the commencement or exacerbation of atherosclerosis [9]. We found evidence that increased pathophysiological parameters of carotid arteries, as measured by ultrasound technology, correlated with increased expression of VCAM-1 and ICAM-1. Additionally, our microscopy corroborated decreased lumen sizes of carotid arteries due to age and obesity. Thus, we postulate that hyperinsulinemia and age are strong predictors of increased expression of VCAM-1 and ICAM-1, decreased lumen and diameter size and other parameters of arterial inflammation and atherosclerosis.

The Zucker Diabetic Fatty rat is an excellent model to study the effects of

diabetes and cardiovascular complications [7]. Insulin resistance is considered a precursor to prediabetes and cardiovascular disease [10]. According to our results, it appears that atherosclerosis [11] and carotid artery anomalies can begin early in age [12].

Several pertinent studies have been performed in the past that show a relationship between cardiovascular disease (CVD), atherosclerosis, hyperinsulinemia and obesity. Wasniewska *et al*., demonstrated that hyperinsulinemia increased intima media thickness [13]. Leite *et al*., indicated that overweight and obese adolescent children exhibit carotid intima-media thickness [14]. Others have found that early atherogenesis occurs in obese adolescents [15] and the presence of Type-2 Diabetes Mellitus (T2DM) is a predictor of increased carotid intima-media measurements [16]. More research is necessary to determine the exact predictors and effectors of atherosclerosis. We are currently performing *in vitro* and *in vivo* experiments investigating the molecular mechanisms associated with vascular atherosclerosis and inflammation.

CONCLUSIONS:

The 16-week EXP rat cohort demonstrated some arterial recovery as shown by increased lumenal and total carotid diameters. These improved parameters were observed in both CON and EXP animals. We speculate that since 16-week old Zucker Diabetic Fatty rats have relatively normal physiologic levels as compared to the hyperinsulinemic conditions at 8-weeks, the damage to the injured carotid arteries by catheterization at 16 weeks was not as extensive as that seen at 8 weeks and some recovery was observed, especially at later time points. The observation that age contributes to cardiovascular damage suggests that hyperinsulinemia may contribute to the maladaptive response of the vascular endothelium, leading to atherosclerosis.

LIST OF ABBREVIATIONS –

ATH-EMB	Artery Endarterectomy
BSA	Bovine Serum Albumin
CVD	Cardiovascular Disease
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intercellular Adhesion Molecule-1
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffered Saline
USA	United States of America
VCAM-1	Vascular Cell Adhesion Molecule-1

DECLARATIONS –

ETHICS APPROVAL AND CONSENT TO PARTICIPATE – the Institutional Animal Care and Use Committee (ACORP# 1502R), the Subcommittee on Research Safety (S-16079E) and Research and Development Committee (April 13, 2016) have approved this research.

CONSENT FOR PUBLICATION – both authors give their consent for publication

AVAILABILITY OF DATA AND MATERIAL – All data and materials associated with this manuscript are located in the laboratory of Marc Goalstone, and are available for review by request.

COMPETING INTERESTS – There are no competing interests with regard to this research and manuscript

AUTHOR'S CONTRIBUTIONS – Both Drs. Pott and Goalstone contributed equally towards the preparation and science of this manuscript.

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