Evaluation of an Organic Mineral Complex on the Development of Cardiovascular Disease Risk Following a 10-week High-Fat Diet

by

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ABSTRACT

According to the World Health Organization, obesity has nearly tripled since 1975 and forty-one million children under the age of 5 are overweight or obese (World Health Organization, 2018). Exercise is a potential intervention to prevent obesity-induced cardiovascular complications as exercise training has been shown to aid nitric oxide (NO) production as well as preserving endothelial function in obese mice (Silva et al., 2016). A soil-derived organic mineral compound (OMC) has been shown to lower blood sugar in diabetic mice (Deneau et al., 2011). Prior research has shown that, while OMC did not prevent high fat diet (HFD)-induced increases in body fat in male Sprague-Dawley rats, it was effective at preventing HFD-induced impaired vasodilation (M. S. Crawford et al., 2019). Six-weeks of HFD has been shown to impair vasodilation through oxidative-stress mediated scavenging of NO as well as upregulation of inflammatory pathways including inducible nitric oxide synthase (iNOS) and cyclooxygenase (Karen L. Sweazea et al., 2010). Therefore, the aim of the present study was to determine whether OMC alters protein expression of iNOS and endothelial NOS (eNOS) in the vasculature of rats fed a control or HFD with and without OMC supplementation. Six-week old male Sprague-Dawley rats were fed either a standard chow diet (CHOW) or a HFD composed of 60% kcal from fat for 10 weeks. The rats were administered OMC at doses of 0 mg/mL (control), 0.6 mg/mL, or 3.0 mg/mL added to their drinking water. Following euthanasia with sodium pentobarbital (200 mg/kg, i.p.), mesenteric arteries and the surrounding perivascular adipose tissue were isolated and prepared for Western Blot analyses. Mesenteric arteries from HFD rats had more uncoupled eNOS ($p = 0.006$) and iNOS protein expression ($p = 0.027$) than rats fed the control diet. OMC was not effective

at preventing the uncoupling of eNOS or increase in iNOS induced by HFD. Perivascular adipose tissue (PVAT) showed no significant difference in iNOS protein expression between diet or OMC treatment groups. These findings suggest that OMC is not likely working through the iNOS or eNOS pathways to improve vasodilation in these rats, but rather, appears to be working through another mechanism.

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CHAPTER 1

INTRODUCTION

According to the World Health Organization, obesity has nearly tripled since 1975 and in 2016, 41 million children under the age of 5 were overweight or obese (World Health Organization, 2018). Obesity is known for chronic low-grade inflammation and is well-correlated with metabolic syndrome, hypertension, and diabetes (Fenton et al., 2009). Foods high in saturated fat primarily come from animal sources of food such as red meat, poultry, and full-fat dairy products (Mayo Clinic, 2019). Saturated fats are known to lower high-density lipoprotein (HDL) "good" cholesterol and raise low-density lipoprotein (LDL) "bad" cholesterol and increase your risk of cardiovascular disease (Te Morenga & Montez, 2017). High LDL levels contribute to dyslipidemia and the development of liver steatosis (Gabbia et al., 2019). Previous studies from our lab have shown a 6-week high fat diet (HFD) produces liver steatosis and increased plasma alanine aminotransferase (ALT) in rats, which suggests high saturated fat intake quickly results in liver damage (M. Crawford et al., 2019).

Exercise is a potential intervention to prevent obesity-induced cardiovascular complications as exercise training has been shown to aid nitric oxide (NO) production as well as preserving endothelial function in obese mice (Silva et al., 2016). In healthy arteries, NO is produced endogenously mainly by the protein endothelial nitric oxide synthase (eNOS) (Mu et al., 2019). As shown in **Figure 1**, when stimulated by acetylcholine (Ach), eNOS synthesizes L-Citrulline and NO, from L-Arginine. After release, NO diffuses to vascular smooth muscle cells to induce relaxation by binding to soluble guanylyl cyclase (sGC) which increases the concentrations of 3',5'-cyclic

guanosine monophosphate (cGMP) and, in turn, effects protein kinase G (PKG) resulting in relaxation (Calabrese et al., 2007; Zheng et al., 2016). Endothelium-dependent vasodilation becomes impaired in animals and humans that consume HFD (Van Guilder et al., 2006). In fact, six weeks of high fat intake results in impaired NO-mediated vasodilation in young male Sprague-Dawley rats (Karen L. Sweazea et al., 2010). This impairment is attributed to diet-induced high blood sugar and increases in body fat and inflammation as well as oxidative stress-mediated scavenging of NO by superoxide (K. L. Sweazea & Walker, 2011; Karen L. Sweazea et al., 2010). Reduced bioavailability of NO results in hypertension.

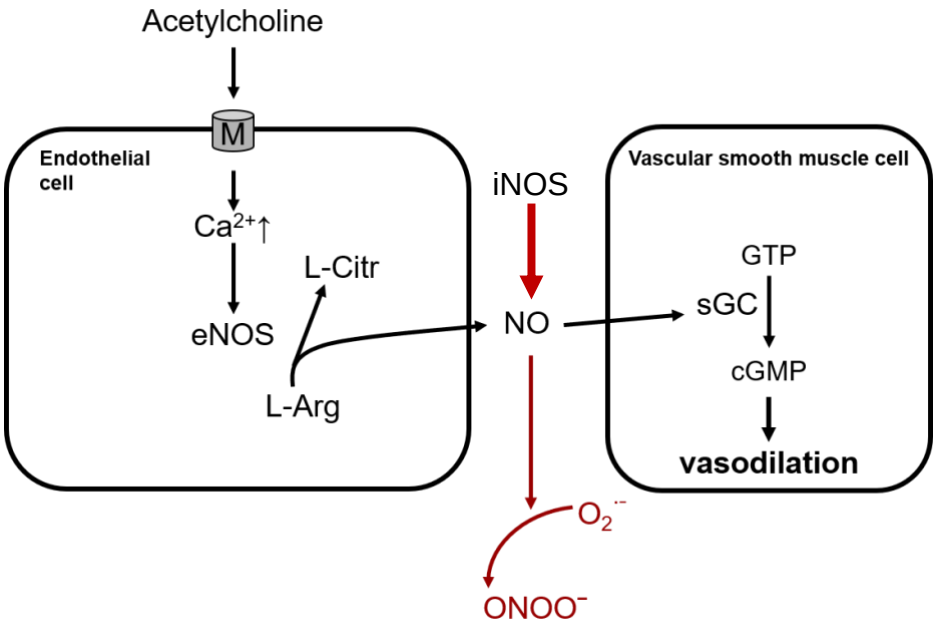

Figure 1: Nitric Oxide as A Vasodilator. Acetylcholine binds to the muscarinic receptor in the membrane of endothelial cells. The resulting increase in intracellular calcium is a stimulus for endothelial nitric oxide synthase (eNOS), which is responsible for converting L-arginine (L-Arg) to L-citrulline (L-Citr) and producing NO. NO diffuses to the vascular smooth muscle cells causing vasodilation through activation of soluble guanylyl cyclase (sGC). In the presence of superoxide ($O_2^{\cdot -}$), NO bioavailability is reduced when converted to Peroxynitrite ($ONOO^-$). Inducible NOS (iNOS) activates overproduction of NO during inflammatory states. Perivascular adipose tissue that surrounds blood vessels is a source of iNOS.

Consequently, inducible NOS (iNOS) has been shown to cause an increase in inflammation and overproduction of NO, which can combine with superoxide to create peroxynitrite ($ONOO^-$) (Fig. 1; Natarajan et al., 2018). $ONOO^-$ production directly stimulates inflammation through tumor necrosis factor – alpha (TNF-α) and nuclear factor-kappa β (NF-$\kappa\beta$) pathways often showing an increase in inflammatory cytokines, and later chemokines to induce widespread inflammation (Mark et al., 2001; Szabó, 2003). A 6-week HFD in adolescent rats showed an increase of NF-$\kappa\beta$ in the gut (M. Crawford et al., 2019) but 10-week HFD studies in rats have shown no significant increases in the gut or mesenteric TNF-α or NK-$\kappa\beta$ levels (M. S. Crawford et al., 2017).

Perivascular adipose tissue (PVAT) surrounds the vasculature and organs in mammals and is shown to regulate vascular tone (Hildebrand et al., 2018). PVAT is also an indicator of obesity and regulates smooth muscle cell migration and proliferation (Miao & Li, 2012). The purpose of this study was to observe PVAT as a tissue which secretes vasoactive substances such as NO, adiponectin, angiotensin II and superoxide, and its effect on the underlying endothelial tissue (Watts et al., 2020). These vasoactive substances are necessary for normal vascular function, but when imbalanced can cause chronic local or systemic low-grade inflammation, insulin resistance and type 2 diabetes (Guilherme et al., 2008).

Deneau et al. showed that a soil-derived mineral compound lowers blood sugar in diabetic mice (Deneau et al., 2011). A similar soil-derived compound, OMC, was used in our lab which prevented the development of high blood sugar in rats fed a HFD for 10 weeks, compared to controls fed a chow diet (M. S. Crawford et al., 2019). Furthermore, although OMC did not prevent HFD-induced increases in body fat, it was effective at

preventing HFD-induced impaired vasodilation in the rats (M. S. Crawford et al., 2017). A HFD intake in rats for 6 weeks impairs vasodilation through oxidative-stress mediated scavenging of NO, upregulation of inflammatory pathways including iNOS and cyclooxygenase-2 (COX2) (Karen L. Sweazea et al., 2010). Preliminary metabolomic analyses of OMC show that it has very little antioxidant activity, especially when considering the low ORAC scores found in Table 2. As acetylcholine-mediated vasodilation occurs primarily through eNOS in small resistance arteries from rats (Fig. 1), OMC may prevent impaired vasodilation by mitigating HF-mediated impairment of eNOS signaling or by reducing iNOS protein expression.

CHAPTER 2

AIM / HYPOTHESES

To determine whether OMC alters protein expression of iNOS and eNOS in the vasculature of rats fed a control or HFD with and without OMC supplementation.

HYPOTHESES

1. There will be an increase in the vascular inflammatory marker iNOS, and a decrease in eNOS in mesenteric arteries and PVAT isolated from the HFD rats, as compared to the control diet fed rats.
2. OMC will prevent inflammation, as evidenced by reduced iNOS and restored eNOS levels in the mesenteric artery and surrounding PVAT tissue from animals fed a HFD compared to control diet fed rats.

CHAPTER 3

METHODS

Animal Model

Six-week-old male Sprague-Dawley rats (157.5 ± 1.32g body mass; n = 42), purchased from Envigo (formerly Harlad Teklad) were used. Females were not included because they produce large amounts of estrogen which provides cardioprotective effects, thus preventing some of the vascular impairments of a HFD (Mendelsohn & Karas, 1999; Turgeon et al., 2004). Rats were divided into two dietary groups, as shown in **Table 1**: standard chow maintenance diet (5% kcal from fat; Teklad Global 2018, Indianapolis, IN) or a 60% kcal high fat diet (Cat. No. D12492; Research Diets Inc., New Brunswick, NJ) for 10 weeks. Rats were further divided and treated with OMC in their drinking water throughout the diet at one of the following doses: vehicle (0 mg/mL) (chow n=10, HFD n=8), 0.6mg/mL (chow n=6, HFD n=6), or 3.0 mg/mL(chow n=6, HFD n=6). The OMC supplement is a key ingredient in a proprietary nutritional supplement marketed by Isagenix International, LLC, a sponsor of the parent study (M. S. Crawford et al., 2019). The OMC supplemented water and diets were replaced every 2-3 days to prevent spoiling. At the end of the 10-week study, all rats were euthanized with sodium pentobarbital (200mg/kg i.p.) and the mesenteric artery and the surrounding PVAT were isolated and frozen at -80°C until analyses. Rats were singly housed and maintained on a 12:12 hour light: dark cycle and provided access to water and food ad libitum. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee, protocol number 17-1563R, which is included in the appendix.

Table 1: 10-week Rat Chow vs HF Diet Ingredients

	Protein (kcal)	Carbohydrates (kcal)	Lipids (kcal)
Chow	**24%**	**58%**	**18%**
Chow Ingredient (Plant based)	Wheat – 36% Corn – 34% Soy protein – 26%	Wheat – 54% Corn – 40% Soybean meal – 6%	Soybean – 60% Wheat/Corn – 40%
HFD	**20%**	**20%**	**60%**
HFD Ingredients (Animal products)	Casein Lactic 30 mesh	Corn – 63% Sucrose – 37%	Lard – 91% Soybean Oil – 9%

OMC supplement

The organic mineral complex (OMC) used in this study is a proprietary nutritional ingredient by the study sponsor, Isagenix International, LLC, (Gilbert AZ). This complex natural ingredient is obtained in the raw form from several mineral mines in North America. OMC is extracted, isolated, and manufactured by Mineral Biosciences, LLC (Goodyear, AZ) and is self-affirmed Generally Recognized as Safe per the Food and Drug Administration definitions (FDA, 2019). OMC is an ancient plant and soil-derived material, which undergoes significant proprietary isolation techniques to yield a soil-based blend containing over 50 minerals along with a high concentration of fulvic acid. Further chemical and biological analysis are detailed in **Table 2**.

Table 2: Physical, Chemical, and Functional Characteristics of OMC

Component Measured	Concentration or Value	Analytical Methodology or Source
Total Minerals	142391 ppm	ICP
Calcium	49610 ppm	ICP
Sulfur	28040 ppm	ICP
Potassium	15420 ppm	ICP
Sodium	14990 ppm	ICP
Magnesium	12630 ppm	ICP
Nitrate (NO_3^-)	1230 ppm	Univ. Wisconsin Soil and Forage Analysis Laboratory
Fulvic Acids	14.9%	Lamar et al., 2014 [42]
Humic Acids	<0.1%	Lamar et al., 2014 [42]
Protein	23 mg/g	CLG-PRO4 determination by combustion
Nucleic Acids	ND	DAPI (4',6-Diamidino-2-phenylindole)-staining
Total Polyphenols	0.24%	Folin-Ciocalteu
ORAC Score a-Hydrophilic	24.92	Brunswick Laboratories
ORAC Score a-Hydrophobic	5.44	Brunswick Laboratories

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Morphometrics at 10 weeks

Morphometric data are taken from a previous study from our lab and are shown in **Table 3** (M. S. Crawford et al., 2019). Rat body mass was measured weekly to identify if the chow vs HFD intake was causing any physical changes, especially in conjunction with the OMC supplement. Epididymal fat pad mass, abdominal circumference (immediately anterior to the hindleg), and nasoanal length were measured with a flexible tape measure

at the conclusion of the 10-week trial. Blood samples were collected after euthanasia by cardiac puncture, and the blood plasma was isolated and snap frozen at -80℃. To address overall animal adiposity, the epididymal fat pad was extracted from each rat (Karen L. Sweazea et al., 2010).

Table 3: Morphometrics at 10 Weeks (From the (M. S. Crawford et al., 2019) study; with permission.)

	Control	0.6 mg/ml OMC	3.0 mg/ml OMC
Body Mass (g)			
Chow	369 ± 9.33 (10)	377 ± 8.10 (6)	383 ± 14.8 (6)
HFD	**411 ± 9.84 (8)#**	**425 ± 16.8 (6)#**	417 ± 12.7 (6)
Epididymal fat pad mass (g)[a]			
Chow	3.70 ± 0.16 (10)	4.14 ± 0.24 (6)	3.89 ± 0.41 (6)
HFD	**6.09 ± 0.32 (8)#**	**7.44 ± 1.09 (6)#**	**6.67 ± 0.65 (6)#**
Epididymal fat pad mass (% body mass)			
Chow	1.00 ± 0.03 (10)	1.10 ± 0.07 (6)	1.01 ± 0.08 (6)
HFD	**1.48 ± 0.05 (8)#**	**1.73 ± 0.20 (6)#**	**1.59 ± 0.12 (6)#**
Abdominal circumference (cm)			
Chow	16.8 ± 0.28 (10)	17.5 ± 0.17 (6)	17.3 ± 0.20 (6)
HFD	**17.9 ± 0.25 (8)#**	**18.9 ± 0.62 (6)#**	**18.3 ± 0.24 (6)#**
Naso-anal length (cm)			
Chow	22.3 ± 0.15 (10)	22.1 ± 0.14 (6)	22.5 ± 0.24 (6)
HFD	**23.1 ± 0.14 (8)#**	**23.1 ± 0.20 (6)#**	**23.2 ± 0.30 (6)#**

Table 3: Data expressed as mean ± SEM (n). Data analyzed by two-way ANOVA. #p<0.02 vs respective chow treated animal. Data was log transformed prior to statistical analyses to approximate normality.

Western Blot Analyses

Protein expression was determined for each sample first then data were normalized to β-actin followed by the chow control treatment for each western blot. The validated primary antibodies for total eNOS (1:2,500; Cat. 610296; BD Transduction Laboratories, San Jose, CA), iNOS (1:1,000; Cat. 610431, BD Transduction Laboratories), and β-actin

(1:10,000; Cat. Ab8227; Abcam, Cambridge, MA) were purchased through Fisher Scientific (Waltham, MA) & Abcam.

Western blot analyses were modeled after a previous study published by our laboratory (M. S. Crawford et al., 2019; K. L. Sweazea & Walker, 2011). Mesenteric arteries were isolated and snap frozen on dry ice. Frozen arteries were homogenized in a cold tissue protein extraction reagent (T-PER; Cat. #78510, Thermo Fisher Scientific, Waltham, MA) containing HALT Protease Phosphatase Inhibitor Cocktail (Cat. #78440, Thermo Fisher Scientific) prepared according to the manufacturer's protocol. Tissues were further homogenized in 2 mL microcentrifuge tubes containing 3.0 mm high impact zirconium beads (Cat. #D1032-30; Benchmark Scientific; Edison, NJ) using a BeadBug homogenizer (Benchmark Scientific). Samples were centrifuged at 400 rpm for 3 min. PVAT tissue was homogenized on ice for 3 minutes using a 2 mL DWK Life Sciences Tenbroek handheld ground glass homogenizer (Cat. #08-414-18; Fisher Scientific) using the same protease phosphatase inhibitor cocktail. The homogenates were centrifuged at 14,000 rpm for 10 minutes at 4°C and supernatant was analyzed using the Bradford Method (Bio-Rad, Hercules, CA). 25µg total protein per lane was applied to 7.5% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) alongside a molecular marker, Kaleidescope (Cat. #1610375, Bio-Rad Laboratories Hercules, CA) and was separated by molecular weight by electrophoresis for 35 minutes at 200V, then 5 minutes at 205V. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) and blocked overnight in 5% Carnation nonfat dry milk to reduce nonspecific binding. For eNOS protein detection, membranes were washed with TTBS then exposed to eNOS mouse monoclonal antibody (1:2,500; Cat. 610296; BD

Transduction Laboratories, San Jose, CA) on a rocker at 4°C. For iNOS protein detection, membranes were washed with TTBS then exposed to iNOS mouse monoclonal antibody (1:1,000; Cat. 610431, BD Transduction Laboratories) incubated on a rocker for 4 hours at room temperature. Both membranes were incubated overnight with an anti-rabbit β-Actin antibody (1:10,000; Cat. Ab8227; Abcam, Cambridge, MA) which was used as a loading control, and purchased through Fisher Scientific (Waltham, MA). Membranes received 5 - 5 minute washes in TTBS. Secondary antibodies, anti-mouse for eNOS (1:5,000) and iNOS (1:2,000) and anti-rabbit (1:5,000) horseradish peroxidase-conjugated secondary antibodies (Cat.# PI-2000 and PI-1000; Vector Laboratories, Burlingame, CA) at room temperature for 1 hour. The membranes were washed 3 times in TTBS, followed by 3 washes in TBS. The membranes were exposed to enhanced chemiluminescence substrate, ECL (Thermo Scientific, Rockford, IL) for 1 minute. Proteins of interest were visualized by exposure to x-ray film (Kodak X-OMAT, Thermo Fisher Scientific, Pittsburg, PA). The X-Ray films were developed then analyzed using NIH ImageJ software.

Statistical Analyses

Data was expressed as mean ± standard error of the mean (SEM) and was analyzed by Two-way ANOVA with diet and OMC dose as factors. Where significant effects were found, Tukey posthoc analyses was used to assess significant differences between and within OMC groups. P-value ≤ 0.05 was accepted as statistically significant. SigmaPlot version 14.0 (Systat software, San Jose, CA) was used to analyze all results.

CHAPTER 4

RESULTS

Rats fed the high fat diet gained significantly more weight than their comparative chow fed control rats. Further, epididymal fat pad mass, waist circumference, and nasoanal length were all significantly increased in HFD rats as compared to the chow controls, however tail length was not different between groups. These metrics, which indicate obesity, were compared using Lee's Index of Obesity, showed that the rats were actually overweight, not obese (Novelli et al., 2007). No morphometric variables were affected by the OMC treatments.

By utilizing Western Blot methods, the mesenteric arteries showed a significant increase in uncoupled eNOS protein expression between the control and OMC treatment groups with the 0.0 mg/mL ($p = 0.018$) and 3.0 mg/mL ($p = 0.031$) doses as identified in **Figure 2**. Mesenteric artery iNOS protein expression was significantly increased between the high fat and chow diet ($p = 0.027$), but OMC did not play a role in this difference, see **Figure 3**. Finally, iNOS expression in mesenteric PVAT was not significantly different between treatments ($p = 0.086$), or within groups ($p = 0.787$), as identified in **Figure 4**.

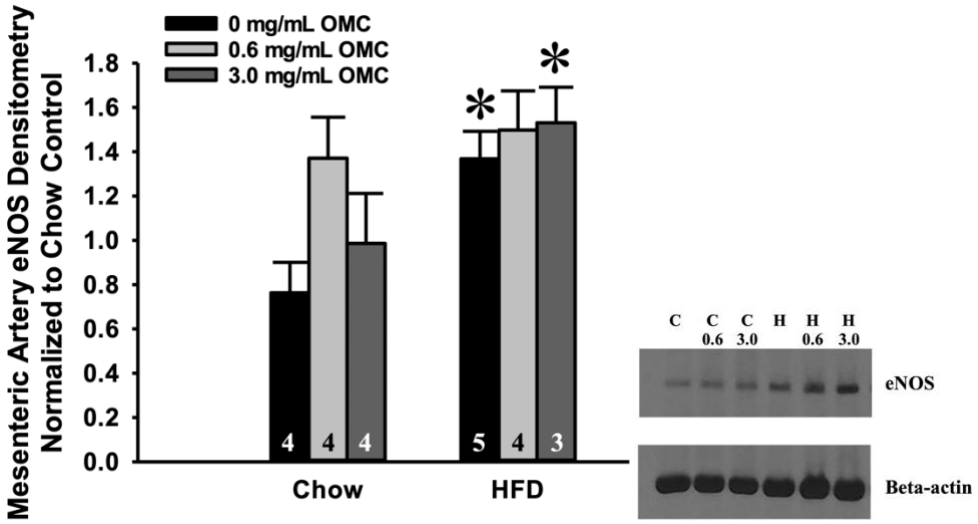

Figure 2: Western Blot Analyses of eNOS Protein Expression In Mesenteric Arteries Isolated From Chow and HFD-Fed Animals Treated With OMC. Densitometry of eNOS monomer (140 kDa) protein expression. All densitometry values were normalized to the β-actin loading control and expressed as a ratio of Chow control values. Data shown as mean $\pm$ SEM. Numbers on the graphs represent sample sizes (n). ***p<0.05 vs respective Chow-fed animals.**

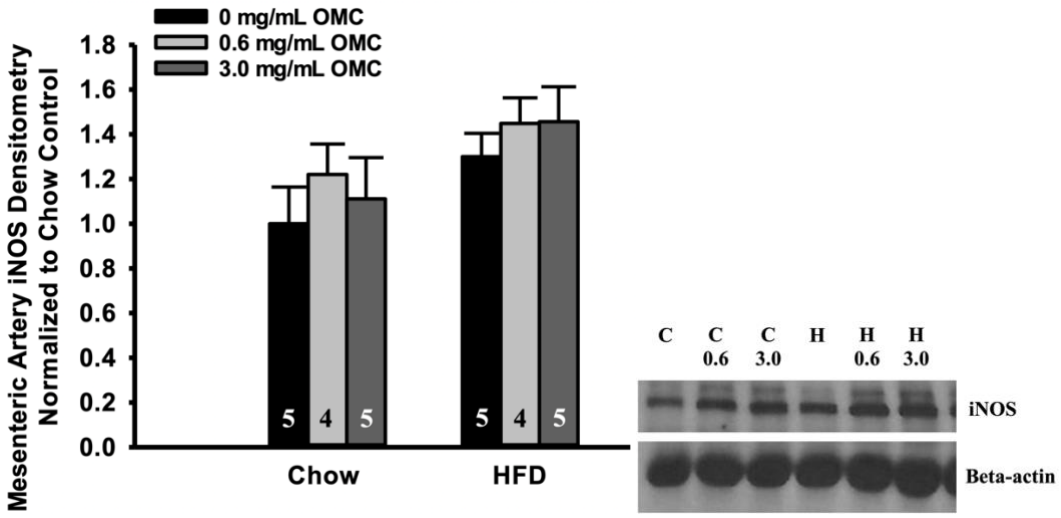

Figure 3: Western blot analyses of iNOS protein expression in mesenteric arteries isolated from Chow and HFD-fed animals treated with OMC. Densitometry of iNOS (130 kDa) protein expression. All densitometry values were normalized to the β-actin loading control and expressed as a ratio of Chow control values. Data shown as mean $\pm$ SEM. Numbers on the graphs represent sample sizes (n).

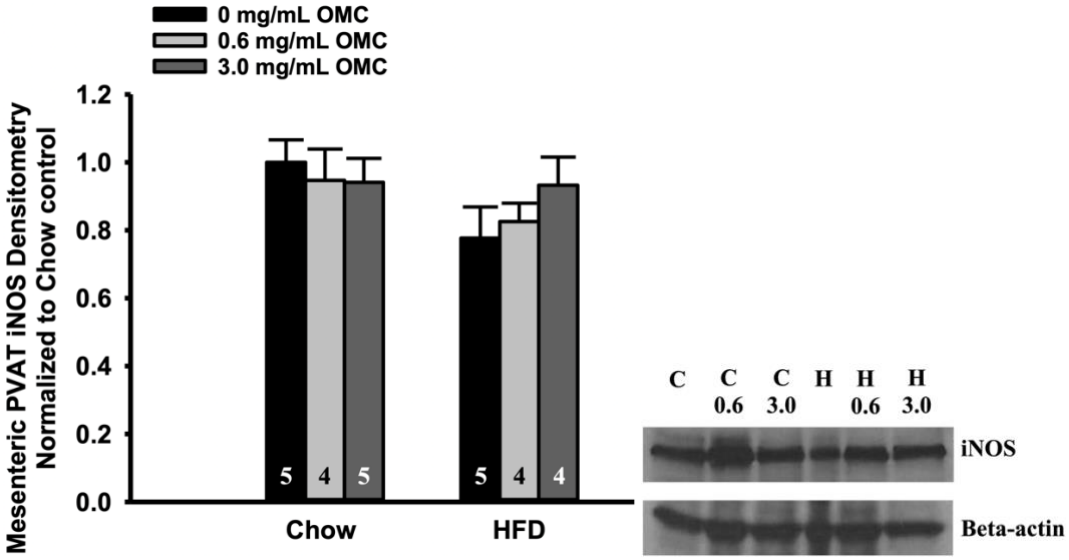

Figure 4: Western blot analyses of iNOS protein expression in mesenteric PVAT isolated from Chow and HFD-fed animals treated with OMC.

Densitometry of iNOS (130 kDa) protein expression. All densitometry values were normalized to the β-actin loading control and expressed as a ratio of Chow control values. Data shown as mean $\pm$ SEM. Numbers on the graphs represent sample sizes (n).

CHAPTER 5

DISCUSSION

The purpose of this study was to determine whether varying concentrations of OMC supplementation alters iNOS and eNOS protein expression in the vasculature of rats fed a 10-week chow or HFD. Consistent with our hypothesis, there was a significant increase in iNOS and uncoupled (i.e. inactive) eNOS protein expression shown without OMC supplementation in the mesenteric arteries of HFD rats compared to chow fed rats. OMC did not prevent these effects of HFD.

As we predicted, we observed a significant increase in uncoupled eNOS in the mesenteric artery. Uncoupled eNOS is the inactive form of eNOS which resides in the cytosol instead of as an active eNOS which is 'coupled' with a G-Protein receptor Kinase found in the endothelial membrane (Liu et al., 2014). An accumulation of uncoupled eNOS becomes especially dangerous under inflammatory conditions where an imbalanced ratio of coupled vs uncoupled eNOS would result in an increased in eNOS derived superoxide and cellular damage (Sullivan Jennifer C. & Pollock Jennifer S., 2006). This excess of uncoupled eNOS in the presence of superoxide may contribute to further damage from the free radical compound, $ONOO^-$ (Natarajan et al., 2018).

The iNOS expression in the mesenteric artery is also increased in the HFD which may have been caused by a cascade of cytokines from $ONOO^-$ (Mark et al., 2001). Meanwhile, the PVAT is not showing a significant difference in iNOS expression. Although, iNOS protein expression in the mesenteric PVAT showed a trend between the chow and HFD with a p-value of 0.0872. This may be a consequence of the rats not spending enough time on the HFD. With increased time, it is predicted that the cytokines

observed would recruit chemokines which would induce widespread inflammation (Szabó, 2003).

Critical features of metabolic syndrome are associated with endotoxemia, endothelial dysfunction, hyperglycemia, and insulin resistance (Hsu & Wen, 2002; McMurray et al., 2016), which may proceed increased production of inflammatory cytokines and oxidative stress (Mark et al., 2001; Szabó, 2003). These findings suggest that once this oxidative stress begins in the rat, it may further impact endothelium-dependent vasodilation by increasing vascular levels of superoxide while at the same time reducing the availability of endogenous NO in the mesenteric artery as shown in prior studies from our lab (K. L. Sweazea & Walker, 2011; Karen L. Sweazea et al., 2010). This supports the notion that uncoupled eNOS may have produced superoxide, instead of NO, which ultimately led to a decreased availability of NO when combined with O_{2}^{-} to form $ONOO^{-}$ (Rahangdale et al., 2009).

The findings from this study serve as a possible explanation why OMC, when given as a preventative supplement, is not showing immediate effects on reducing iNOS and increasing coupled eNOS concentrations in the mesenteric artery and PVAT. Instead of acting as an antioxidant, like previously thought due to the high percentage of fulvic acid in OMC, it is likely a source of exogenous NO (Linsha Ma & Linsha Ma, 2017; Winkler & Ghosh, 2018). As shown in the metabolomic analysis of OMC in **Table 2**, OMC has a high concentration of nitrates (NO_{3}^{-}), and when consumed as a dietary supplement, NO3- has been shown to be absorbed systemically, reduced to nitrite (NO_{2}^{-}) by bacterial nitrate reductase in the salivary glands, and again reduced and oxidized in the stomach to NO (Rajendran et al., 2019). This process does not involve the endogenous

eNOS or iNOS pathways and may offset the $ONOO^-$ consumption of NO by serving as an exogenous NO donor. In summary, supplemented NO_3^- may explain the mechanism by which OMC was observed in a previous study to prevent HFD induced impaired vasodilation (M. S. Crawford et al., 2019; K. L. Sweazea & Walker, 2011).

Limitations:

- This study had a small sample sizes (3-5 rats/group), it may have been underpowered to detect a significant difference in iNOS protein expression in the PVAT. With a larger sample size, greater significance may be found within and between groups.
- During this 10-week study the rats became overweight, not obese, thus it is possible that a longer study may result in obesity and different outcomes than those observed in the present study.
- This study is intended to be a model for a human response to a similar diet and supplement. A stark limitation to this design is that the rat diet was not a good representation of the complex human diet. It is also unlikely that the rat model for metabolic syndrome (MetS) can adequately develop the range of MetS observed in humans.
- OMC was delivered with the start of the diet treatment and was thus seen as a preventative measure, not a restorative measure. Thus, it was not possible to measure the ability for OMC to restore vascular health.

CHAPTER 6

CONCLUSION

As hypothesized, we observed an increase in the vascular inflammatory marker iNOS, and an increase in uncoupled eNOS expression in mesenteric arteries isolated from the HFD rats, as compared to the control diet fed rats. Contrary to our hypothesis, we did not observe a significant effect of the HFD on iNOS protein expression in the PVAT. We also hypothesized that OMC would prevent inflammation, as evidenced by reduced iNOS and restored eNOS levels in the mesenteric artery and surrounding perivascular adipose tissue from animals fed a HFD compared to control diet fed rats. It was observed that neither eNOS nor iNOS protein expression were altered by OMC treatments in either the chow or HFD fed rats. It is important to note that OMC was delivered with the diet and is thus seen as a preventative intervention, not a restorative intervention.

Future studies should investigate the effectiveness of OMC as a restorative dietary mechanism to see if differences are observed in iNOS and eNOS expression levels when the rats are given OMC after already having been fed the HFD for an extended period of time. It would also be interesting to observe other inflammatory pathways. In this limited study we only looked at iNOS, but cyclooxygenase would be good pathway to investigate to see if OMC is increasing its expression. Lastly, a future study should investigate the presence of superoxide in the vasculature by staining tissues with dihydroethidium, DHE, which fluoresces in the presence of superoxide.

In conclusion, despite the apparent antioxidant effects of OMC seen in prior studies, this study suggests that OMC is not modifying eNOS or iNOS to prevent impaired vasodilation induced by a HFD. Instead we hypothesize that the combination of organic

acids, trace elements, nitrate and mineral composition of the supplement function collectively in this protection by a non-antioxidant mechanism. Future studies examining the effectiveness of OMC as a restorative dietary supplement would be needed to determine if it alters existing biology or aids in circulating NO. OMC is currently offered as a part of a nutritional supplement offered by Isagenix International, LLC, and it is unknown if this data observed in rats would accurately translate to the same results in humans.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 312
Tempe, Arizona 85287-6111
Phone: (480) 965-6788 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**17-1563R**
Protocol Title:	**Exploration of the metabolic and vascular protective effects of an organometallic complex (OMC)**
Principal Investigator:	**Karen Sweazea**
Date of Action:	**2/23/2017**

The animal protocol review was considered by the Committee and the following decisions were made:

The protocol was approved.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree.

Total # of Animals:	**60**	
Species:	**Rats**	**Pain Category: C**
Protocol Approval Period:	**2/23/2017 – 2/22/2020**	
Sponsor:	**Isagenix International LLC**	
ASU Proposal/Award #:	**FP00010367; FP00010473**	
Title:	**Exploration of the metabolic and vascular protective effects of an organometallic complex (OMC)**	

Signature: _____ for C. Miller
IACUC Chair or Designee

Date: 2/27/2017

Cc: IACUC Office
IACUC Chair