Effect of omega-3 high-fat diet on skeletal muscle protein degradation in glucocorticoid-induced muscle wasting

Katie Marie Brown

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EFFECT OF OMEGA-3 HIGH-FAT DIET ON SKELETAL MUSCLE PROTEIN DEGRADATION IN GLUCOCORTICOID-INDUCED MUSCLE WASTING

By

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ABSTRACT

Background: Omega-3 PUFAs elicit beneficial effects in several muscle atrophy conditions.

Objective: Determine if a high-fat diet rich in n-3 is protective of glucocorticoid-induced protein degradation. Methods: Male wild type C57BL/6 mice were randomized into two groups: n-6 (45% fat 177.5 g lard) and n-3 (45% fat 177.5 g Menhaden oil). After 4 weeks on their diets, groups were divided to receive either daily injections of dexamethasone (3 mg/kg/day) or sterile PBS, for 1 week while continuing diets. Results: Dexamethasone reduced gastrocnemius weight by 12% independently of diet. Protein degradation signaling was altered by dexamethasone with increased atrogin-1 expression, decreased phosphorylation of FOXO3a, increased phosphorylation of GSK-3β, and increased myostatin and MURF-1 gene expression. However, the negative effects of dexamethasone were not attenuated by an n-3 high-fat diet. Conclusion: These data support the detrimental effects of dexamethasone on muscle atrophy and report no benefit of an n-3 high-fat diet.
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LIST OF ABBREVIATIONS

AA – arachidonic acid
Akt – protein kinase B
cDNA – complementary DNA
dex – dexamethasone
DHA – docosahexaenoic acid
EPA – eicosapentaenoic acid
FoxO3a – forkhead box transcription factor O3a
eIF2B – eukaryotic transcription factor 2B
GC – glucocorticoid
GSK-3β – glycogen synthase kinase 3β
GR – glucocorticoid receptor
GRE – glucocorticoid response element
GTT – glucose tolerance test
HFD – high-fat diet
IGF-1 – insulin-like growth factor 1
KO – knockout
LPS – lipopolysaccharide
mRNA – messenger RNA
mTOR – mammalian target of rapamycin

MuRF1 – muscle RING finger 1

NF-kB – nuclear factor- kB

n-3 – omega-3

n-6 – omega-6

PBS – phosphate buffered saline

PI-3K – phosphoinositide 3-kinase

PUFA – polyunsaturated fatty acid

p70s6k – ribosomal protein S6 kinase

REDD-1 – regulated in development and DNA damage response 1

RNA – ribosomal nucleic acid

SDS – sodium dodecyl sulfate

S6K1 - ribosomal protein S6 kinase 1

TBST – tris buffered saline with tween

UPS – ubiquitin-proteasome system

4E-BP1- eukaryotic translation initiation factor 4E-binding protein 1
INTRODUCTION

Skeletal muscle is involved in various metabolic functions contributing to whole-body protein metabolism and energy expenditure (Wolfe, 2006). Protein turnover, the balance between muscle protein synthesis and protein breakdown, reflects and determines the amount of skeletal muscle mass in an individual (Sandri, 2013). Thus, maintaining muscle mass is crucial as an imbalance in protein turnover (e.g., muscle atrophy) is associated with increased morbidity and disease risk (Jeromson, Gallagher, Galloway, & Hamilton, 2015). Muscle atrophy is associated with many chronic diseases and pathological conditions such as obesity, prolonged fasting, cancer, sepsis, cachexia, and AIDS among others. In addition, treatment with synthetic glucocorticoids (GCs) also induces muscle atrophy (Fappi et al., 2014). Exogenous glucocorticoids, such as dexamethasone and prednisone, are used both acutely and chronically to treat a variety of inflammatory and autoimmune diseases (e.g., cancer, asthma, rheumatoid arthritis, starvation, sepsis, metabolic acidosis). While GCs are effective at combatting inflammation, they do so at the expense of skeletal muscle mass by increasing the rate of protein degradation and suppressing protein synthesis (Bodine et al., 2001; Braun & Marks, 2015; Fappi et al., 2014; Schakman, Kalista, Barbe, Loumaye, & Thissen, 2013). GCs have been shown to exacerbate metabolic derangements caused by a high-fat diet (HFD) including significant reductions in lean body mass (Auvinen, Coomans, Boon, Romijn, Biermasz, Meijer, Havekes, Smit, Rensen, & Pereira, 2013). Therefore, treatments to prevent glucocorticoid-induced muscle wasting are necessary to improve patient survival outcomes and quality of life.

The polyunsaturated fatty-acids (PUFAs) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have emerged as key nutrients augmenting skeletal muscle protein turnover (Gingras et al., 2007; Kamolrat & Gray, 2013; Khal & Tisdale, 2008; Liu et al., 2013; Murphy et al., 2011; Ryan et al., 2009; Smith et al., 2011, 2015; You, Park, Song, & Lee, 2010). PUFAs are essential nutrients involved in cell membrane structure,
membrane fluidity, cell signaling, and regulation of gene transcription and enzyme activity (Castillero, Martín, López-Menduiña, Villanúa, & López-Calderón, 2009; Jeromson et al., 2015; Lee, Lee, Kang, & Park, 2016; Murphy, Mourtzakis, & Mazurak, 2012). Omega-3 PUFAs also exhibit anti-inflammatory, anti-cachectic, anti-catabolic, and anabolic properties in the skeletal muscle (Beck, Smith, & Tisdale, 1991; Castillero et al., 2009; Magee, Pearson, & Allen, 2008; Murphy et al., 2011; Ryan et al., 2009; Smith et al., 2011; Whitehouse & Tisdale, 2001). While the PUFAs EPA and DHA promote an anti-inflammatory environment, the omega-6 PUFA arachidonic acid (AA, 20:4n-6) exerts a pro-inflammatory response (Robinson, Buchholz, & Mazurak, 2007; Ruxton, C. H. S., Reed, S. C., Simpson, M. J. A., & Millington, 2004). Of recent interest is the manipulation of the n-6/n-3 ratio, relative to the total amount of PUFAs, as key in affecting metabolic and physiological functioning. Consumption of a HFD or “Western” diet rich in saturated fatty-acids and linoleic (LA, 18:2n-6) PUFAs has been linked to adverse metabolic profiles such as alterations in fatty acid composition, acid-base balance, glycemic load, and nutrient metabolism (Buettner, R., Scholmerich, J., and Bollheimer, 2007; Cordain et al., 2005; Jeromson et al., 2015). While the ideal ratio of n-6/n-3 is 2:1, the typical ratio of the Western diet is approximately 20:1 n-6/n-3; this relatively high intake of n-6 to n-3 may exacerbate an already pro-inflammatory state in the context of disease (Jeromson et al., 2015; Robinson et al., 2007; Simopoulos, Leaf, & Salem, 1999). To date, no studies have examined the effects of n-3s on skeletal muscle preservation during both glucocorticoid use and consumption of a high-fat diet. This project seeks to elucidate differences in glucocorticoid-induced muscle atrophy when consuming either a diet rich in n-6 or n-3 PUFAs, using C57BL/6 male mice.

**Mechanisms of Skeletal Muscle Degradation**

Muscle atrophy is defined as the loss of muscle mass due to a reduction in density or muscle fiber size; this is due to either an increase in skeletal muscle protein breakdown and/or a
decrease in skeletal muscle protein synthesis (Fappi et al., 2014). Both catabolic conditions (e.g. cancer, sepsis, diabetes, and fasting among others) and inactivity induce muscle atrophy by activation of several signaling pathways (Bodine et al., 2001; Sandri, 2013). Two major degradation pathways are activated in these conditions: the autophagic-lysosomal system and the ubiquitin-proteasome system (UPS) (Bonaldo & Sandri, 2013; Fappi et al., 2014; Sandri, 2013). The regulation of autophagy in skeletal muscle has been well described (Bonaldo & Sandri, 2013). Defined, it is a homeostatic mechanism used for the disposal and recycling of proteins and organelles within the cell cytoplasm; this involves a series of steps including autophagosome formation, via cell signals, to deliver damaged proteins to the lysosome for degradation. In mammalian skeletal muscle, macroautophagy is initiated by activation of a “regulatory complex,” containing proteins Atg14, Vps15, Vps34, Beclin 1, and Ambra 1, which further induces LC3 recruitment to the autophagosome for degradation by lysosomal protease cathepsin L. (Bonaldo & Sandri, 2013; Sandri, 2013). Indeed, this process is feedback regulated; under nutrient sufficiency, growth/proliferation, and mTOR signaling directly, autophagy is inhibited; while, cellular stress and starvation upregulate the autophagy pathway via activation of autophagy-related genes. A key regulator in the autophagy process is transcription factor FoxO3, known to activate lysosomal-protein breakdown by upregulating LC3, Bnip3, Gabarap, ATg12, and VPS34 autophagy genes (Sandri, 2010). The UPS tags specific proteins for degradation by the 26S proteasome via enzymes (E1, E2, E3) in a series of steps deemed the ubiquitination process; once a protein is “ubiquitinated” or tagged, it is sent to the proteasome for degradation (Sandri, 2013). Indeed, these pathways exhibit crosstalk and modulate one another to induce muscle atrophy. Recent focus has been on the UPS as a therapeutic target since inhibition of autophagy has dystrophic effects on muscle due to its homeostatic nature (Bilodeau, Coyne, & Wing, 2016; Bonaldo & Sandri, 2013).
Muscle Atrophy F-Box (MAFbx/atrogen-1) and muscle RING finger 1 (MuRF1) are two E3 ubiquitin ligases that are muscle-specific and upregulated during muscle atrophy. Indeed, MuRF1 and atrogen-1 knockout (KO) mice are resistant to muscle atrophy induced by several catabolic conditions. MuRF1 KO mice, but not atrogen-1 KO mice, are resistant to dexamethasone-induced muscle atrophy; whereas in fasting conditions, atrogen-1 knockdown prevents muscle wasting. However, mice lacking both genes are resistant to denervation-induced muscle wasting. Thus, these E3 ligases are deemed key regulators of muscle atrophy, yet their exact mechanisms are not fully known (Baehr, Furlow, & Bodine, 2011; Bodine et al., 2001; Bonaldo & Sandri, 2013). MuRF1 and atrogen-1 are regulated by several anabolic and catabolic signaling pathways including Insulin/IGF-1, Myostatin-SMAD2/3, glucocorticoid, and cytokine signaling (Bonaldo & Sandri, 2013). Additionally, a variety of transcription factors are involved in the regulation of muscle atrophy; forkhead box transcription factor (FoxO), negatively regulated by the Insulin/Akt pathway, and nuclear factor-κB (NF-kB), regulated by inflammatory cytokines, have been found to modulate MuRF1 and atrogen-1 in several models of atrophy (Khal & Tisdale, 2008; Sandri, 2013).

**Glucocorticoids and Protein Turnover**

Endogenous glucocorticoids (i.e. cortisol) are steroid hormones released by the adrenal glands in response to stressful conditions; they exert anti-inflammatory and immunosuppressive actions as well. During a hypermetabolic state, GCs induce proteolysis to increase free amino acids as substrates for hepatic gluconeogenesis to sustain metabolic demands. This physiological response is both normal and beneficial acutely; however, in pathological conditions such as cancer cachexia, starvation, and sepsis among others, elevated circulating levels of endogenous GCs elicit harmful effects on muscle mass and function. Despite its negative side effects, exogenous GCs (e.g. dexamethasone, prednisone, and hydrocortisone) are one of the most
prescribed steroid therapies. Given both acutely and chronically, GCs are used for treatment of a variety of inflammatory diseases further exacerbating inflammatory-induced muscle atrophy (Bodine & Furlow, 2015; Braun & Marks, 2015; Fappi et al., 2014; Schakman et al., 2013).

**Signaling pathways in GC-induced muscle atrophy.** It is well known that GCs regulate protein metabolism in skeletal muscle and promote a catabolic environment. GC-induced muscle atrophy is characterized by a decrease in protein synthesis and increase in protein degradation. GCs exert their detrimental effects mainly on fast twitch glycolytic fibers causing a reduction in both fiber cross-sectional area and decreased myofibrillar protein content (Bodine & Furlow, 2015; Braun & Marks, 2015; Schakman et al., 2013). It is well established that the glucocorticoid receptor (GR) is required for binding GCs to activate signals to induce muscle atrophy. Briefly, GCs bind to the GR allowing translocation to the nucleus where they bind to glucocorticoid response element (GREs) in the promotor regions of target genes (Yoon, 2017). Mechanistically, GCs have been reported to alter both gene transcription and the insulin signaling pathway. Studies have shown GCs to act on the IGF-1/PI-3K/Akt/mTOR pathway to suppress protein synthesis, as well as the UPS and autophagic-lysosomal system to promote protein degradation (Braun & Marks, 2015; Fappi et al., 2014). Additionally, GCs alter skeletal muscle production of two growth factors, namely by downregulating Insulin-like Growth Factor 1 (IGF-1), an anabolic growth factor, and upregulating Myostatin, a negative regulator of muscle growth (Gilson et al., 2007; Schakman et al., 2013; Wang, Jiao, Zhao, Wang, & Lin, 2016).

The inhibitory effect of protein synthesis results from several mechanisms. First, GCs inhibit the transport of amino acids into the cell thus limiting an anabolic response; and secondly, GCs inhibit the action of IGF-1, insulin, and amino acids on 4E-BP1 and S6K1 phosphorylation-key proteins in the initiation of mRNA translation. 4E-BP1 and S6K1 are downstream targets of
mTOR phosphorylation; thus, GCs suppress protein synthesis mainly by inhibiting mTORC1 signaling (Bodine & Furlow, 2015; Schakman et al., 2013). One study has assessed the effects of GCs on mTOR phosphorylation in skeletal muscle both in vivo and in vitro. Dexamethasone treatment (2 mg/kg for 4d) with a 0.68% alanine diet significantly inhibited mTOR and p70S6K phosphorylation compared to the control (alanine diet alone) during a refeeding state (Wang, Jia, Xiao, Jiao, & Lin, 2015). A recent study has attributed GCs repression of mTOR signaling to enhanced transcription of regulated in development and DNA damage response 1 (REDD1), a downstream target of the glucocorticoid receptor in skeletal muscle (Wang, Kubica, Ellisen, Jefferson, & Kimball, 2006). Moreover, REDD1 has been identified as a target gene of the GR in skeletal muscle (Shimizu et al., 2011). Lastly, recent studies have investigated glycogen synthase kinase 3β (GSK3β) as a factor in GC suppression on protein synthesis. GSK3β is a downstream target of the IGF-1/Akt pathway, known to inhibit protein synthesis by signaling to eukaryotic transcription factor 2B (eIF2B) (Schakman et al., 2013). Studies have shown that GSK3β inhibition augments the IGF-1 dependent hypertrophic effect on skeletal muscle, increases cell hypertrophy, and contributes to myogenic differentiation (van der Velden et al., 2006; Vyas, Spangenburg, Abraha, Childs, & Booth, 2002). Furthermore, GC-induced muscle atrophy has been blunted by GSK3β inhibitors in several studies, suggesting GCs depend on this protein to induce its catabolic effects (Evenson, Fareed, Menconi, Mitchell, & Hasselgren, 2005; Fang et al., 2005). Although this mechanism is yet elucidated, recent studies have shown GSK3β inactivation to be associated with UPS inhibition, specifically regulating the expression of muscle-specific E3 ligases, atrogin-1 and MuRF1 (Verhees et al., 2011).

It is mainly through the proteolytic systems that GCs mediate their catabolic actions by upregulating the expression of atrogenes MuRF-1, atrogin-1, and transcription factor FoxO1/3a (Bodine & Furlow, 2015; Sato et al., 2017; Schakman et al., 2013). Activation of these genes is
associated with an increased rate of proteolytic actions in the proteasome and increased
ubiquitination of proteins (Bodine & Furlow, 2015; Schakman et al., 2013). MuRF-1 and
atrogin-1 have been found to respond differently in GC treatment. One study reported that
MuRF-1 knockout mice were resistant to dexamethasone-induced muscle atrophy by maintaining
muscle mass and fiber cross sectional area; while atrogin-1 deletion exhibited no protective
effect against GC-induced muscle atrophy (Baehr et al., 2011; Bodine et al., 2001). These
findings confirm that MuRF-1 is necessary for GCs to induce muscle atrophy. GCs regulate
expression of atrogenes via transcription factors, namely FoxO (Lecker et al., 2004; Qin et al.,
2014). Qin et al. and colleagues attributed this increase in FoxO expression to GREs found in the
FoxO1 gene (Qin et al., 2014). The MuRF-1 gene also contains a GRE that responds to GC
administration, whereas no GRE has been found in atrogin-1 (Braun & Marks, 2015; Wada et al.,
2011). This evidence possibly explains Baer et al.’s findings that atrogin-1 deletion did not
attenuate muscle loss in response to GC treatment. Moreover, activation of FoxO transcription
factors cause rapid protein degradation by upregulating transcription of several components of
the ubiquitin proteasome and autophagic systems: MuRF-1, atrogin-1, and REDD1 (Braun &
Marks, 2015; Fappi et al., 2014). It seems evident that the GRE and FoxO transcription factors
work synergistically on the E3 ligases to induce proteolysis; however, more in vivo studies are
necessary to further elucidate the mechanisms of glucocorticoid-induced muscle atrophy. See
Figure 1 for a schematic diagram of pathways regulating protein synthesis and degradation.

**Omega-3 PUFAs and Skeletal Muscle Preservation**

PUFAs, are essential dietary components, as they are not endogenously produced by
humans. Other than a source of energy, these long-chain fatty acids are the structural components
of cell membranes, act as signaling molecules, and regulate enzyme activity, transcription of
genes, and membrane fluidity (Castillero et al., 2009; Jeromson et al., 2015; Lee et al., 2016;
Murphy et al., 2012). EPA, DHA, and AA are all precursors of eicosanoids and docosanoids. They regulate inflammatory and immune responses, with AA having a pro-inflammatory effect and EPA and DHA exerting anti-inflammatory responses (Robinson et al., 2007; Ruxton, C. H. S., Reed, S. C., Simpson, M. J. A., & Millington, 2004). There are several proposed mechanisms by which n-3s preserve lean muscle mass. In addition to their anti-inflammatory response, n-3s exert anti-catabolic and anabolic effects. Although n-3s anti-inflammatory response is well characterized, the mechanism behind n-3 PUFAs preventing muscle wasting and maintaining muscle mass is less understood (Castillero et al., 2009; Liu et al., 2013; Magee et al., 2008). The anti-catabolic response is thought to be mediated by the activation of Akt/FOXO, and suppression of the NF-κB and UPS pathways; while the anabolic response is associated with upregulation of the mTOR-p70s6k signaling pathway (Gingras et al., 2007; Kamolrat & Gray, 2013; Liu et al., 2013; Y. Wang, Lin, Zheng, Zhang, & Huang, 2013; Whitehouse, Smith, Drake, & Tisdale, 2001).

**Attenuation of Protein Degradation.** Several studies have examined the effect of n-3s on skeletal muscle in multiple models of atrophy including sepsis, arthritis, starvation, cancer, cachexia, and glucocorticoid use (Beck et al., 1991; Castillero et al., 2009; Fappi et al., 2014; Khal & Tisdale, 2008; Liu et al., 2013; Whitehouse & Tisdale, 2001). In weaning piglets given a lipopolysaccharide (LPS) challenge, fish-oil supplementation attenuated LPS-induced muscle atrophy. This attenuation was accompanied with increased phosphorylation of Akt and FoxO1, and decreased expression of atrogin-1 and MuRF-1 genes (Liu et al., 2013). Similarly, a study investigated EPA’s effect on protein degradation in murine myotubes given LPS as a model of sepsis. EPA administered pre-sepsis completely attenuated skeletal muscle degradation by downregulating expression and activity of the ubiquitin proteasome system, namely the α- and β-subunits of the 20S proteasome (Khal & Tisdale, 2008). You et al. found that supplemental
fish-oil prior to and during hindlimb immobilization alleviated soleus muscle atrophy via activation of Akt/p70 S6 and suppression of E3 ubiquitin ligases when compared to corn-oil (control) (You et al., 2010). In arthritis-induced muscle wasting, EPA supplementation decreased atrogin-1 and MuRF1 gene expression as well as attenuated the decreases in gastrocnemius weight when compared to coconut oil (control) (Castillero et al., 2009). The effect of EPA on muscle protein degradation and the activation of the UPS have also been investigated in acutely fasted mice. Pre-treatment of mice with EPA prior to a 24h fast significantly reduced proteolysis in the soleus muscle, as well as decreased both the α- and β-subunits of the 20S proteasome (Whitehouse & Tisdale, 2001). Additionally, several studies have investigated n-3’s anti-catabolic effect in cancer and cachectic models. In both human and experimental animals, fish-oil and EPA supplementation have been shown to prevent cancer-induced reductions in body weight and skeletal muscle mass (Beck et al., 1991; Murphy et al., 2011; Whitehouse et al., 2001). Treatment of mice bearing the cachexia-inducing (MAC16) tumor with EPA attenuated protein degradation via inhibition of the UPS; functional proteasome activity was suppressed, together with decreased expression of α-subunits of the 20S proteasome (Whitehouse et al., 2001). These findings confirm that n-3 supplementation primarily inhibits activation of the UPS to attenuate protein degradation in numerous atrophic conditions. Although the evidence builds a strong case for omega-3’s protecting skeletal muscle mass in GC-induced wasting, a study by Fappi et al found contradicting results; the study investigated the effect of n-3 supplementation on GC-induced muscle atrophy and found that n-3 supplementation (0.1g/kg/day) for 40 days did not attenuate dexamethasone-induced muscle atrophy in rats due to reduced myogenin levels, increased atrogin-1 expression, and atrophy of type 1 and 2A fibers (Fappi et al., 2014). Although, the dose used in this study may not have been effective as many studies have used a much higher dose of 2.5g/kg or greater and observed beneficial effects (Beck et al., 1991;
Whitehouse et al., 2001; Whitehouse & Tisdale, 2001). Based on the current literature, we hypothesized that a diet rich in omega-3 PUFAs, in the form of fish oil, would protect lean muscle mass from the atrophic effects of GC usage. Specifically, we expected to see n-3s preserve skeletal muscle by inhibiting protein degradation.

**Study Aim**

To determine if a high fat diet rich in n-3 is protective of GC-induced protein degradation.

**METHODS**

*Animals and Experimental Design*

All experimental and housing protocols were approved by the Institutional Animal Care and Use Committee of the University of Memphis.

C57BL/6 male mice, 7 weeks of age, were purchased from Envigo. All animals were kept on a 12:12-h light-dark cycle and had ad libitum access to food and water during the course of the study. After 3 days of acclimation, animals underwent baseline testing including a glucose tolerance test (GTT) and MRI after being fasted for 5 h. Mice were then randomized into two groups initially to receive either a high-fat diet rich in omega-6 (n-6, 45% fat (177.5 g lard), 35% carbohydrate, and 20% protein, n-6:n-3 PUFA, 13:1), n=16, or a high-fat diet rich in omega-3 (n-3, 45% fat (177.5 g Menhaden oil), 35% carbohydrate, and 20% protein, n-6:n-3 PUFA, 1:3, diet composition listed in Table 1), n=16. After 4 weeks (wk) on their respective diets, both groups were divided with half of the mice receiving either a subcutaneous injection of dexamethasone (3 mg/kg body weight) or sterile PBS while continuing their current diet throughout the 5th and last week—45% n-6 + dexamethasone (n-6 + dex) n=8 and 45% n-3 + dexamethasone (n-3 + dex) n=8. This dosage is similar to what has been previously done in other studies to induce muscle wasting (Fappi et al., 2014; Gilson et al., 2007). We closely monitored daily body weight, food
intake, and grooming in all mice for the duration of the experiment. Remaining food (from previous day consumption) and body weight were measured 3 times a week during the first four weeks and daily following dexamethasone injections. MRI testing was performed on mice at 8, 12, and 13 weeks of age for assessment of body composition and muscular wasting. At the end of the study animals were fasted for 5h prior to harvesting tissue. Tissue collection was completed with mice anesthetized by isoflurane and euthanized by cervical dislocation.

**Blood Draw**

Blood was collected on all animals at 8, 12, and 13 weeks of age via the facial vein. Approximately 50-100ul of blood was collected into an EDTA containing tube for plasma collection.

**Glucose Tolerance Test (GTT)**

At 8, 12, and 13 weeks of age, mice were injected with glucose intraperitoneally (2 g/kg; 20% glucose solution) following a 5 hour fast. Blood samples (12-15uL) were collected from the tip of the tail vein immediately before injection and at 15, 30, 60, 90, and 120 min post-injection. Blood glucose concentration was measured in whole blood (~2.5 uL) using the Bayer Contour glucometer (Bayer HealthCare LLC, Mishawaka, IN). This protocol has been done previously in cachectic animals without adverse events.

**MRI Test**

At 8, 12, and 13 weeks of age, mice underwent MRI testing to determine body composition of fat mass and fat-free mass (ECO MRI-100, Houston, TX).

**Non-Survival Surgery**

At the end of the study (14 weeks of age), all animals were fasted for 5 hours prior to harvesting tissue. Puromycin (0.04µmol/g) was injected intraperitoneally 30min before tissue collection to measure protein synthesis rates. Tissue collection was completed with the mouse
anesthetized by isoflurane (2-5%). Mice were euthanized by cervical dislocation while anesthetized. Hindlimb skeletal muscles (soleus, plantaris, gastrocnemius, tibialis anterior, extensor digitorum longus (EDL)), epididymal fat pad, heart, and spleen were excised and snap frozen in liquid nitrogen for further analysis. Prior to being frozen the gastrocnemius was divided into red and white portions representing portions that are high in oxidative (red) fibers and glycolytic (white) fibers, respectively. Tibias were also removed and weighed as a correction factor for body size.

*Western Blot Analysis*

Western blot analysis was performed on harvested muscle tissue to determine differences in protein expression levels. Portions of the white gastrocnemius muscle were homogenized in Mueller buffer and protein concentration measured using the Bradford method (Bradford, 1976). Homogenates were loaded on 10% SDS-polyacrylamide gels, proteins were separated, and transferred overnight to polyvinylidene difluoride membranes. Ponceau staining was used to visually confirm gel transfer and equal loading. Membranes were blocked Tris-buffered saline with 0.1% Tween 20 (TBST) and 5% milk for 1 h at room temperature. Primary antibodies for phosphorylated (P)-FOXO3a (Millipore, Temecula, CA), FOXO3a, P-GSκ-3β, and GSκ-3β (Cell Signaling) were incubated at a ratio of 1:2,000 for 24 h in 5% TBST milk at -4 °C. Secondary conjugated antibodies were used at a ratio of 1:5,000 and were incubated for 1-2 h in 5% TBST milk at room temperature. Enhanced chemiluminescence (Geneessee Scientific, San Diego, CA) was used to visualize the antibody-antigen interactions and developed using a Chemidoc system (Biorad, Hercules, CA). Blots were analyzed by measuring the integrated optical density of each band using ImageJ software. All Western blots were normalized to the non-phosphorylated control.
**RNA Isolation and qPCR**

The following genes were analyzed for expression: atrogin-1, MURF-1, REDD1, and myostatin. To isolate RNA from mouse white gastrocnemius muscle, tissue was homogenized in 3-5 mL RNA STAT-60. Total RNA was extracted from STAT-60 solution by the addition of chloroform:isoamyl alcohol (24:1). Extracted RNA was dissolved in water, reprecipitated using sodium acetate and isopropanol, washed with 75% ethanol, and quantified using a Nanodrop (ThermoFisher Scientific, Waltham, MA). For qPCR measurement of RNA transcripts, 1 µg of RNA was reverse transcribed to cDNA. The cDNA was mixed with forward and reverse primers for the intended gene target and ABsolute Blue SYBR Green qPCR master mix. The 2−ΔΔCT method was used to determine changes in gene expression between treatment groups. See Table 2 for primer sequences.

**Statistical Analysis**

All data are represented as means ± SE. A two-way ANOVA was used to determine the effects of diet and dexamethasone treatment using GraphPad Prism 8. Bonferroni post hoc analysis was used to examine interactions. Significance was set at p ≤ 0.05. Effect size was calculated using Cohen’s D.

**RESULTS**

**Muscle Mass**

Gastrocnemius muscles were measured at the time of sacrifice and mean weights of each group are presented in Figure 2. Dexamethasone significantly decreased gastrocnemius weight by 12% in mice fed either diet, p=0.0089. These data suggest that dexamethasone suppresses muscle mass independently of n-3 supplementation.
Protein Degradation

To determine the effect of an omega-3 rich diet on glucocorticoid-induced muscle atrophy, markers of protein degradation were measured. Phosphorylation of FOXO3a, a marker of both proteasomal degradation and autophagic degradation, and GSK-3β, a factor in GC suppression on protein synthesis, were measured via western blot. The phosphorylation of FOXO3a was not significantly altered by diet or dexamethasone; however, there was an effect of dexamethasone (Cohen’s d=1.42) to decrease phosphorylation of FOXO3a compared to control (Fig. 3A). Although the phosphorylation of GSK-3β was not significantly (p=0.19) altered by diet or dexamethasone, dexamethasone (Cohen’s d=1.42) increased phosphorylation of GSK-3β (Fig. 3B).

We next examined gene expression of REDD-1, myostatin, MURF-1 and atrogin-1 normalized to GAPDH. There was no difference in REDD-1 expression with diet or dexamethasone treatment (Fig. 4A). Myostatin, a negative regulator of muscle mass, was not significantly altered by diet or dexamethasone; however, there was a large effect of dexamethasone (Cohen’s d=2.1) to upregulate myostatin expression (Fig. 4B). As markers of the proteasomal degradation pathway, the E3 ligases MURF-1 and atrogin-1 were measured. There was no significant effect of either diet or dexamethasone (p=0.15) on MURF-1 expression. Although, there was a large effect of dexamethasone (Cohen’s d=6.47) to increase MURF-1 expression regardless of diet (Fig. 4C). Atrogin-1 expression was significantly higher (p=0.0035) with dexamethasone in mice fed either diet when compared to the control (Fig. 4D). These data support dexamethasone induction of muscle atrophy via upregulation of the ubiquitin pathway independently of high fat diet composition.
DISCUSSION

Supplementation with omega-3 PUFAs has well-known benefits to skeletal muscle protein turnover. In various models of muscle atrophy (i.e. sepsis, arthritis, starvation, cancer, and cachexia), studies investigating n-3s have shown that either supplementing with n-3s or altering the n-6/n-3 ratio maintains or increases protein synthesis and inhibits muscle atrophy. Exogenous glucocorticoids are given as a drug-therapy in a variety of muscle wasting and inflammatory conditions; however, chronic GC usage is linked to increased muscle atrophy. Recent studies investigating both n-3 supplementation and GC-induced muscle atrophy have found conflicting results compared to the aforementioned benefits seen in other atrophy conditions (Fappi et al., 2014; Fappi et al., 2019). Additionally, studies have shown that a HFD contributes to muscle wasting by altering protein synthesis and increasing markers of UPS degradation (Abrigo et al., 2016; Sishi et al., 2011). Altering the n-6/n-3 ratio could alleviate the deleterious effects of consuming a high n-6 to n-3 diet. However, little is known of the effects of n-3s on glucocorticoid-induced muscle atrophy while consuming a high-fat diet. Therefore, we sought to induce muscle atrophy by dexamethasone and observe the effect of an omega-3 HFD on markers of protein degradation. We observed that dexamethasone administration increased muscle atrophy by significantly reducing muscle weight and upregulating several atrogenes, however a HFD rich in n-3s did not attenuate these alterations.

Several studies have found n-3 PUFAs to have a protective, anabolic, and anti-catabolic effect on skeletal muscle mass in healthy and diseased conditions (Gingras et al., 2007; Murphy et al., 2011; Ryan et al., 2009; Smith, Gordon I; Atherton, Philip; Reeds, Dominic N; Mohammed, Selma B.; Rankin, Debbie; Rennie, Michael J.; Mittendorfer, 2012; Smith et al., 2011, 2015). In contrast, Fappi et al. investigated the effect of n-3s on glucocorticoid-induced muscle atrophy and reported that while dexamethasone decreased gastrocnemius muscle weight,
concomitant n-3 supplementation did not alter these reductions (Fappi et al., 2014). Our results are consistent with Fappi et al.; indeed, dexamethasone significantly reduced gastrocnemius mass, but a HFD rich in n-3s did not attenuate muscle loss (Fig 2). These findings were not surprising as it is well established that glucocorticoid-induced atrophy is fiber-type specific, namely affecting glycolytic fibers like the gastrocnemius (Bodine & Furlow, 2015). Additionally, a HFD rich in saturated fat and omega-6 PUFAs has been shown to reduce muscle mass by decreasing myofibrillar proteins via overactivation of the UPS, myonuclear apoptosis, and oxidative stress (Bhatt, O’Doherty, Reider, Dedousis, & Dube, 2005; Sishi et al., 2011). We speculate that n-3 supplementation alone is not enough to negate the effects of both glucocorticoids and a HFD acting on the UPS and other cell signaling mechanisms to induce protein degradation. More research is necessary to elucidate the mechanistic effect of different HFD compositions in conjunction with glucocorticoids on muscle mass.

Protein turnover is determined by the ratio of protein synthesis to protein degradation processes. In conditions of muscle wasting, protein degradation is increased while synthesis is suppressed. The UPS and autophagic systems are activated in catabolism and work synergistically to promote degradation. In any condition inducing muscle atrophy, atrogenes are upregulated in these pathways; further, atrogenes MURF-1 and atrogin-1 are modulated by glucocorticoids, Insulin/IGF-1, and myostatin-SMAD2/3 pathways, and FOXO and NF-kb transcription factors. Although a HFD is one factor involved in disrupting protein turnover processes, PUFAs have been shown to positively affect Akt/FOXO signaling. Studies have shown that in some muscle wasting conditions, n-3 supplementation increases phosphorylation of FOXO and decreases atrogin-1 and MURF-1 expression (Castillero et al., 2009; Liu et al., 2013; You et al., 2010). In contrast, glucocorticoids induce muscle atrophy by activating FOXO to upregulate transcription of MURF-1 and atrogin-1. In our study, there was an effect of
dexamethasone (Cohen’s $d=1.42$) to decrease phosphorylation of FOXO3a (Fig 3A), regardless of diet. While MURF-1 expression was not significantly altered by dexamethasone ($p=0.15$) or diet, atrogin-1 expression was increased in both groups receiving dexamethasone (Fig 4C-D). Other studies have shown n-3 supplementation to aggravate dexamethasone-induced atrophy by further increasing activation of atrogin-1 and MURF-1 compared to dexamethasone alone (Fappi et al., 2014; Fappi et al., 2019); however, the current study found no differences in mice fed a HFD high in n-3s versus a HFD high in n-6s with concomitant dexamethasone administration.

Additionally, glucocorticoids alter growth factors IGF-1 and myostatin to affect downstream signaling of protein degradation processes; studies show that IGF-1 is downregulated by GC administration whereas myostatin is upregulated (Gilson et al., 2007; Schakman et al., 2013; R. Wang et al., 2016). In our study, although not significant, there was a large effect of dexamethasone (Cohen’s $d=2.1$) to upregulate myostatin expression (Fig. 4B), however a HFD in n-3s did not attenuate this effect. Downstream of the IGF-1/Akt pathway, GSK-3β is activated by GCs to further inhibit protein synthesis. The current study found an effect of dexamethasone (Cohen’s $d=1.42$) to increase phosphorylation of GSK-3β with no attenuation by a HFD high in n-3s (Fig. 3B). In contrast, when assessing n-3 supplementation in conjunction with dexamethasone administration, Fappi et al. showed no change in phosphorylation of GSK-3β compared to the control (Fappi et al., 2014). Yet other studies report that GSK-3β is necessary for muscle atrophy as it regulates MURF-1 and atrogin-1 expression induced by dexamethasone (Verhees et al., 2011). Determining the role of GSK-3β in GC-induced muscle atrophy with a HFD warrants further investigation.

REDD-1 is a known target gene of the GR in skeletal muscle (Shimizu et al., 2011; Wang et al., 2006); moreover, REDD-1 deletion has been reported to prevent dexamethasone-induced muscle atrophy as evidenced by sustained mTOR activity and protein synthesis (Britto et al.,
A study by Wang et al. found that dexamethasone enhanced REDD-1 expression in rat skeletal muscle via activation of its downstream targets Tuberin and Rheb; this activation further suppressed downstream mTOR signaling to inhibit protein synthesis (Wang et al., 2006). In our study, REDD-1 expression was not altered by diet or dexamethasone (Fig. 4A). Recent evidence suggests a homeostatic role for REDD-1 as it is required for proper functioning of mitochondrial capacity and insulin sensitivity, both of which influence protein turnover (Horak et al., 2010; Williamson et al., 2014). A study by Frost et al. corroborates the homeostatic role of REDD-1; it showed that IGF-1, a potent anabolic stimulator, increased REDD-1 gene and protein expression with greater protein synthesis in myotubes expressing REDD-1 (Frost, Huber, Pruznak, & Lang, 2009). Although not significant, our results show that REDD-1 expression was greater in the groups receiving PBS compared to dexamethasone; this suggests a homeostatic role for REDD-1 and that dexamethasone induces muscle atrophy independently of REDD-1 activation.

**CONCLUSION**

To our knowledge, we are the first to investigate the effects of omega-3 supplementation in a HFD on GC-induced muscle atrophy. We have shown that dexamethasone administration reduces gastrocnemius weight, however a HFD rich in n-3s does not elicit any protective effects on muscle mass. With dexamethasone, there was an effect to increase protein degradation regardless of HFD type. There was an effect of dexamethasone to decrease phosphorylation of FOXO3a, however a HFD rich in n-3s did not attenuate this. Dexamethasone administration significantly increased atrogin-1 expression regardless of HFD type. There was a large effect of dexamethasone to increase MURF-1 gene expression, with no attenuation by a HFD rich in n-3s. Additionally, there was an effect of dexamethasone to upregulate myostatin, a negative regulator of muscle mass, but there were no differences between mice consuming an omega-6 HFD or an omega-3 HFD. However, there was no significant effect of dexamethasone or diet on REDD-1
gene expression. This study corroborates the potentially harmful effects of glucocorticoids on skeletal muscle. Because glucocorticoids are used as a therapeutic treatment for a variety of inflammatory, auto-immune, and cancerous conditions, any nutritional approach to negate its negative effects would be beneficial to the affected population. Although, our study shows that omega-3 supplementation in a HFD does not protect skeletal muscle from the detrimental effects of GC-induced atrophy.

LIMITATIONS AND FUTURE DIRECTIONS

One limitation to our study may be use of a rodent model; generally, a rat is more sensitive to GC-induced muscle atrophy compared to a mouse. Much smaller doses of dexamethasone (<0.5 mg/kg) will cause significant atrophy in a rat over 7-days versus a higher dose of 1-3 mg/kg needed to observe the same effects in a mouse (Bodine & Furlow, 2015). This may be the reason why Fappi et al. showed more robust effects of dexamethasone on markers of protein degradation than our study observed. Another limitation is that there is no standard dosage for dexamethasone among studies. We administered dexamethasone at 3 mg/kg over a 7-day period; perhaps given a longer duration, we would have observed greater effects. Future studies may consider looking at the effect of omega-3 supplementation in a HFD with other nutritional supplements to combat GC-induced muscle atrophy.
References


Appendix

TABLES AND FIGURES

Table 1. Composition of high-fat lard (n-6) and high-fat Menhaden oil (n-3) diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>n-6</th>
<th>n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lard</td>
<td>177.5</td>
<td>177.5</td>
</tr>
<tr>
<td>Menhaden Oil, ARBP-F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>202.5</td>
<td>202.5</td>
</tr>
<tr>
<td>Saturated (g)</td>
<td>60.2</td>
<td>59.8</td>
</tr>
<tr>
<td>Monounsaturated (g)</td>
<td>67.7</td>
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</tr>
<tr>
<td>Polyunsaturated (g)</td>
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</tr>
<tr>
<td>Saturated (%)</td>
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<td>31.5</td>
</tr>
<tr>
<td>Monounsaturated (%)</td>
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</tr>
<tr>
<td>Polyunsaturated (%)</td>
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<tr>
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<tr>
<td>C18, Stearic</td>
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<tr>
<td>C18:1, Oleic</td>
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<td>C18:2, Linoleic</td>
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<td>C20, Arachidic</td>
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<td>0.3</td>
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<td>C22:6, Docosahexaenoic, n3</td>
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<td>n6 (g)</td>
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<tr>
<td>n3 (g)</td>
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<td>n6/n3 ratio</td>
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Table 2. Gene primers for qPCR analysis.

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<th>fwd</th>
<th>rev</th>
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</thead>
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<tr>
<td>Myostatin</td>
<td>3'ACCCATGAAAAGACCGTACAAG5'</td>
<td>3'TCATCACAGTCAAGCCCCAAAG5'</td>
</tr>
<tr>
<td>REDD-1</td>
<td>3'TGGTGCCCACTTTTTCGGTG5'</td>
<td>3'GTCAGGGACTGGCTGTAACC5'</td>
</tr>
<tr>
<td>MuRF-1</td>
<td>3'ACCTGCTGGTGGAAACATC5'</td>
<td>3'AGGAGCAAGTGGCACCCTCA5'</td>
</tr>
<tr>
<td>Atrogin-1</td>
<td>3'GTGTTCAGCAGGCGGAAAG5'</td>
<td>3'TTGCCAGAGAACACGCTATG5'</td>
</tr>
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</table>
Figure 1. Schematic figure of pathways regulating protein synthesis and protein degradation.
Figure 2. Gastrocnemius mass in mice fed either a high fat diet in lard (n-6) or Menhaden oil (n-3) with dexamethasone injection. All data are presented as mean ± SEM. Significance was set at p<0.05. *signifies a main effect of dexamethasone.
Figure 3. Protein degradation signaling in gastrocnemius of mice fed either a high fat diet in lard (n-6) or Menhaden oil (n-3) with dexamethasone injection. A) Ratio of phosphorylated to total FOXO3a protein. B) Ratio of phosphorylated to total GSK-3β protein. C) Representative western blot images. All data are presented as mean ± SEM. Significance was set at p<0.05.
Figure 4. Protein degradation signaling in gastrocnemius of mice fed either a high fat diet in lard (n-6) or Menhaden oil (n-3) with dexamethasone injection. A) Relative fold change of REDD1 normalized to GAPDH. B) Relative fold change of Myostatin normalized to GAPDH. C) Relative fold change of MURF-1 normalized to GAPDH. D) Relative fold change of atrogin-1 normalized to GAPDH. All data are presented as mean ± SEM. Significance was set at p<0.05. *signifies a main effect of dexamethasone.
IACUC Protocol
FOR USE OF LIVE VERTEBRATES FOR RESEARCH, TEACHING OR DEMONSTRATION
UNIVERSITY OF MEMPHIS

Date submitted to Attending Veterinarian for pre-review: 8/30/2018

IACUC Protocol # 0830 Date Submitted to IACUC 10-2-2018

Dates Protocol will be in effect: from 10/25/18 to 10/24/21
(not to exceed three years including two yearly renewals)

Is this protocol related to an external grant or contract application? Yes ☐ No ☒

If yes, complete the following:

Agency: Date Submitted

Grant #

University account for Animal Care Facility per diem charge:

If the protocol is not related to an external grant or contract application, complete the following:

University account for Animal Care Facility per diem charge: 210655

Project Title: (If project relates to a grant or contract application, give that title; if multiple protocols relate to one grant, give unique titles for each protocol; if the project is related to a class, give the course name and number):

Effect of Omega-3 supplementation in high-fat diet on skeletal muscle protein turnover in glucocorticoid-induced muscle wasting.

I. Personnel

Investigator/Instructor: Melissa Puppa

Department: School of Health Studies

Academic Rank: Assistant professor

Campus phone: 901-678-3419 Emergency phone: 443-414-1378

Attending Veterinarian: Karyl Buddington
List all individuals that will handle animals using this protocol, their affiliation, and their level of expertise (e.g. relevant qualifications). If the protocol applies to a class then so specify.

**Melissa Puppa - PI**
Has 8+ years of experience working with mice including breeding, exercise training/testing, injections, surgery, dietary interventions, gavage, GTT, electroporation, retro-orbital eye bleed, and dissection/necropsy.

**Katie Brown**
- 1+ year of animal experience including animal handling, cervical dislocation, and CO2 asphyxiation, exercise testing, and dissection.

**Aaron Persinger**
- 2 years of animal experience including animal handling, cervical dislocation, and CO2 asphyxiation, exercise testing, and dissection.

**William Hawkins**
1+ year of animal experience including animal handling, cervical dislocation, and CO2 asphyxiation, exercise testing, and dissection.

**Marie van der Merwe**
- Has 10+ years working with rodents including breeding, injections, dietary interventions, GTT, cheek bleed, and dissection/necropsy.

If additional personnel become involved in handling animals used in this protocol, it is the responsibility of the principal investigator to notify the Animal Care Facility in writing before they start.

Has the investigator/instructor and all personnel listed above received the appropriate vaccinations (tetanus, rabies)?

Yes ☒ No ☐ Not Applicable ☐

Is it necessary for personnel listed on this protocol to be tested for TB?

Yes ☐ No ☒

If you have questions about the kind of vaccination or about TB, call the Animal Care Facility at 678 2359.

All U of M personnel involved in this protocol must complete the animal care and use training program before animals can be procured or before experiments/teaching or demonstration. In submitting this protocol, I, as Principal Investigator/Instructor accept the responsibility for compliance with this requirement.

In addition, the Principal Investigator/Instructor must be willing to provide appropriate supervision for all persons working on this protocol. In the case of a class, the Instructor must be responsible for training any students in classes involved prior to using animals.

II. Project Description

A. Summary (Enter a brief description below of your project, using lay terminology):

Skeletal muscle mass is involved in numerous metabolic functions, with key roles in whole-body protein metabolism and energy expenditure. Therefore, maintenance of muscle mass is key as an imbalance in protein turnover, such as muscle atrophy, is associated with increased morbidity.
and mortality. Muscle atrophy underlies many chronic diseases and pathological conditions such as obesity, prolonged fasting, cancer, sepsis, cachexia, AIDS, and among others, glucocorticoid treatment. Treatments to prevent muscle wasting are necessary to improve patient survival outcomes and quality of life. Omega-3 polyunsaturated acids (PUFAs), specifically EPA/DHA, have been found to exert anti-catabolic, anti-cachectic, anti-inflammatory, and anabolic properties within skeletal muscle. Because the Westernized diet, high in fat (45%), is prevalent throughout the population and contributes to obesity and other metabolic perturbations, we find it pertinent to include in this study. We propose a diet high in Omega-3s (45% fat) compared to a Western diet (45% fat) to preserve lean muscle mass in glucocorticoid-induced skeletal muscle wasting.

B. Describe IN DETAIL the procedures you will follow. Include accompanying documentation and reference to previously published work in the box below. Provide a complete bibliographic citation and describe any variations from the published technique. The bibliography may be included in the box below or appended to this protocol.

**Animal Procedures:** Male wild type C57BL/6 mice will be randomized into two groups initially: 45% lard/Western diet (HFL) n=16 or 45% omega-3 rich diet (n-3) n=16. After 4 weeks on their respective diets, both groups will further divide in half to receive either dexamethasone or sterile PBS while continuing their current diet throughout the 5th and last week – 45% lard/Western diet + dexamethasone (HFL + dex) n=8 and 45% omega-3 rich diet + dexamethasone (n-3 + dex) n=8.

All mice will be purchased at 7-8 weeks of age and allowed to acclimate for a minimum of 3 days prior to baseline testing. At baseline animals will undergo a glucose tolerance test (GTT) and MRI after being fasted for 5h. Following baseline testing, animals will be placed on their respective diets (HFL or n-3) for 5 weeks. Food consumption and body weight will be measured 3 times/week and daily during the 5th week. At 12 weeks of age, mice will repeat the GTT and MRI testing completed at baseline and the next day animals will receive either a subcutaneous injection of dexamethasone (3 mg/kg body weight) or sterile PBS. This dosage is similar to what has been previously done in other studies to induce muscle wasting\textsuperscript{1,2}. Animals will be monitored daily for the remainder of the study including food consumption and weight. At 13 weeks of age, mice will undergo a GTT and MRI testing. At the end of the study animals will be fasted for 5h prior to harvesting tissue. Tissue collection will be completed with the mouse anesthetized with isoflurane. Mice will be euthanized by heart removal while anesthetized. Cervical dislocation will be used as a secondary measure.

**Animal Monitoring:** We will closely monitor body weight, food intake, and grooming in all mice for the duration of the experiment. Mice and food consumed will be weighed 3 times a week and then daily following dexamethasone injection. In addition, we will conduct a MRI on mice at 8 wk, 12 wk, and 13 wk of age for assessment of body composition and muscular wasting. We will monitor for any acute signs of sickness and body weight loss. Mice exhibiting any of these signs will be removed from the study and euthanized by carbon dioxide asphyxiating.

**Dexamethasone injections:** Animals will be injected subcutaneously with dexamethasone (3 mg/kg/day) or sterile PBS after 4 weeks on their respective diets (12 wk of age). Weights will be taken and dosages calculated daily to meet body weight criteria.

**Blood Withdrawals:** Will be conducted on all animals at 8 weeks of age, 12 weeks of age, and at sacrifice. The facial vein, which does not require the use of anesthesia, will be the primary means of blood collection. The facial vein will be punctured with a very quick prick with a needle. Approximately 50-100ul of blood will be drawn into a capillary tube and used for analysis. Animals will be injected with 500ul of sterile saline to protect them from dehydration following blood withdraw.
An alternative to facial vein blood withdraw is to use the lateral saphenous vein in the leg. This method does not require anesthesia. The hind leg will be gently immobilized in the extended position and punctured with a quick prick with a needle. Approximately 50-100ul of blood will be drawn into a capillary tube and used for analysis.

A second alternative is to use retro-orbital blood draw. This will only be used if we are not able to collect blood from the facial or saphenous vein, as it is a more invasive method. Animals will be lightly anesthetized by isoflurane inhalation (2-5%/1L O2/min) and waste gases will be scavenged using carbon filters that will be weight after use and disposed of and replaced after increase of 50g is observed. Approximately 50-100ul of blood will be drawn with a capillary tube and used for analysis. Animals will be injected with 500ul of sterile saline to protect them from dehydration following blood withdraw. If the retro-orbital method is used for blood draw and an incident occurs such as the eye dislocates, or the eye becomes damaged or infected, euthanasia will be used immediately.

No more than one blood draw will be conducted on any single day.

**Glucose tolerance test:** Following a 5 hour fast at 8 wk of age, 12 wk of age, and 13 wk of age, mice will be injected with glucose intraperitoneally (2 g/kg; 20% glucose solution). Blood samples (12-15uL) will be collected from the tip of the tail vein or from the facial vein immediately before injection and at 15, 30, 60, 90, and 120min post-injection. Blood glucose concentration will be measured in whole blood (~2.5 uL) using the Bayer Contour glucometer. This protocol has been done previously in cachectic animals without adverse events.

**MRI:** Following a 5 hour fast at 8 wk of age, 12 wk of age, and 13 wk of age, mice will undergo MRI testing.

**Non-Survival Surgery:** At the end of the study (14 week of age), all animals will fasted for 5 hours prior to harvesting tissue. Puromycin (0.04μmol/g) will be injected intraperitonally 30min before tissue collection to measure protein synthesis rates. Tissue collection will be completed with the mouse anesthetized with isoflurane (2-5%). Mice will be euthanized by removal of the heart while anesthetized. Cervical dislocation will be used as a secondary means of euthanasia. These methods will be used at the end of non-survival surgery since the excision of muscle to measure muscle protein synthesis requires the mouse to be alive as lack of oxygen to the tissue will interfere with basal protein synthesis rates. We will also be collecting the heart for future analysis. Hindlimb skeletal muscles (soleus, plantaris, gastrocnemious, tibialis anterior, EDL), epididimal fat pad, heart, and spleen will be excised and snap frozen in liquid nitrogen for further analysis. Tibias will also be removed as a correction factor for body size. Non-survival surgery is required for the excision of muscle to measure muscle protein synthesis as lack of oxygen to the tissue will interfere with basal protein synthesis rates.

C. Rationale for Involving Animals and the Appropriateness of Species and Number Used.

Indicate (here) briefly the short and/or long-term benefits (to humans and/or other animals) of this use of animals for research, teaching or demonstration. Provide rational for and the number of animals to be used. In addition, state briefly why living animals are required for this study, rather than some alternative model.

Skeletal muscle atrophy underlies many chronic diseases and pathological conditions with obesity and glucocorticoid treatment being among them. Obesity and skeletal muscle atrophy are both associated with increased morbidity and mortality. Understanding the potential for a dietary intervention that can aid in the prevention of muscle loss is beneficial for patient quality of life. Based on previously published data and for statistical significance measures, a sample size of 8/group (for a total of 32 male mice) is needed to detect changes in skeletal muscle mass with dexamethasone induced muscle wasting. Groups will consist of male wild type C57BL/6 mice fed either a 45% lard/Western diet (HFL) n=16, or a 45% omega-3 diet (n-3) n=16. Mice will then be divided into two more groups to receive dexamethasone injections: lard/Western diet plus dexamethasone (HFL + dex) n=8, and omega-3 diet plus dexamethasone (n-3 + dex) n=8.

D. Do the procedures described in B above, have the potential to inflict more than momentary pain or distress (this does not include pain caused by injections or other minor procedures)?

Yes ☐ No ☒

I have considered alternatives to procedures that might cause more than momentary or slight pain/distress, and I have not found such alternatives. As such, I have used one or more of the following methods and sources to search for such alternatives: (check below each method used)

☐ Agricola Data Base ☒ Medline Data Base ☐ CAB Abstracts
☐ TOXLINE ☐ BIOSIS ☐ Lab. Animal Sci. Journal
☐ Lab. Animals Journal ☐ Lab Animal ☐ Animal Welfare Info Center
☐ ATLA (Alternatives to Laboratory Animal Journal) ☐ Quick Biblio. Series
☐ Lab Animal Welfare Bibliography (QL55L27311988) ☐ "Benchmarks"

☑ "Alternatives to Animal Use in Research, Testing and Education"

☐ Current Contents
☐ CARL

☐ Direct contact with colleagues (if selected, you MUST document this below)
List search words for the literature search:

- Omega-3, protein turnover, skeletal muscle, fish-oil, obesity, Western diet, muscle wasting, glucose tolerance test. Key words used were search alone and in combination with cachexia using "and"

What is the length of time that the literature search covers?

1985-present

### III. Animal Use

A. List all animal species to be used (example below).

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<thead>
<tr>
<th>Species</th>
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<th>Age</th>
<th>Sex</th>
<th>Weight</th>
<th>Where Housed (Bldg./Rm#)</th>
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<tbody>
<tr>
<td>e.g. Hooded Wistar rats</td>
<td>45</td>
<td>2 months</td>
<td>male</td>
<td>250-350 gm</td>
<td>Psychology Bld./422I</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>32</td>
<td>2 months</td>
<td>Male</td>
<td>20-30 g</td>
<td>Life Science</td>
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</tbody>
</table>

1Individuals using ectotherms need to only approximate numbers.
2Individuals using fish or other ectotherms need not answer this question.

Is any species threatened or endangered? Yes ☐ No ☒

B. Source of animals

☒ Commercial vendor (Source______Envigo_______)

☐ Bred at The University of Memphis

☐ Captured from wild. Identify method of capture:

☐ Transferred from another study (IACUC Protocol Number )

☐ Donated (Source )

☐ Tennessee Wildlife Resources Agency

Is the supplier a USDA approved source? Yes ☒ No ☐

If not, explain why:

☐ Animals are already in residence at U of M
C. Will surgery be conducted on animals?

Yes ☒ No ☐

If yes, complete this section:

☒ Non Recovery Surgery ☐ Recovery Surgery
☐ Multiple Survival Surgery (if the latter is checked, complete section F)

Surgeon(s) (Name/Job/Title/Academic Rank) Location of Surgery (Bldg. & Room #)

Melissa Puppa, PhD/ will train students and supervise non-recovery surgery/ PI /Assistant Professor
Katie Brown/ Will assist and conduct with non-survival surgery/Graduate Student
Aaron Persinger/ Will assist and conduct with non-survival surgery/Graduate Student
William Hawkins/ Will assist and conduct with non-survival surgery/ Student Volunteer
Marie van der Merwe/ will train students and supervise non-recovery surgery/ CoI /Assistant Professor

D. Will Anesthetic(s), Analgesic(s), or Tranquilizing agents be administered?

Yes ☒ No ☐

If yes, complete this section (example below).

<table>
<thead>
<tr>
<th>Species &amp; Sex</th>
<th>Agent</th>
<th>Dose</th>
<th>Route</th>
<th>Performed by (Name/Title/Academic Rank)</th>
</tr>
</thead>
</table>
| Male C57BL/6 mice | Isoflurane | 2-5%      |       | Melissa Puppa/PI/ PhD
|               |            |           |       | Katie Brown/Graduate student/BS
|               |            |           |       | Marie van der Merwe/CoI/PhD
|               |            |           |       | Aaron Persinger/ Graduate student/ BS
|               |            |           |       | William Hawkins/ Volunteer/ BS |

E. Will euthanasia be carried out?

Yes ☒ No ☐

If yes, complete this section (example below).

<table>
<thead>
<tr>
<th>Species &amp; Sex</th>
<th>Agent</th>
<th>Dose</th>
<th>Route</th>
<th>Performed by (Name/Title/Academic Rank)</th>
</tr>
</thead>
</table>
| Male C57BL/6 mice | Cardiac removal |         |       | Melissa Puppa/PI/ PhD
|               |             |         |       | Marie van der Merwe/CoI/PhD
|               |             |         |       | Katie Brown/Graduate student/BS |
If no, describe disposition of animal(s) at conclusion of this study in box below.

F. Will special housing, conditioning, diets or other conditions be required? Yes ☒ No ☐

If yes, please explain in box below.

Special diets will be used for a high fat (45%) “Western” diet or Omega-3 intake. Mice will be randomized to either the Western diet or Omega-3 rich diet. Diets will be purchased from Research Diets.

G. Will animals be removed from the U of M campus at any time? Yes ☐ No ☒

If yes, please indicate to where and for how long in box below.

H. If they are to be housed for more than 24 hours outside approved facilities at U of M, provide a scientific justification in box below.

IV. Toxic and Hazardous Substances

A. Check off any of the following below that will be used in these experiments?

☐ Infectious agents (Fill out a, b)
☐ Radioisotopes (Fill out a, b, e)
☐ Toxic chemicals or carcinogens (Fill out a, b)
☐ Recombinant DNA (Fill out a)
☐ Experimental drugs (Fill out a)
☐ Malignant cells or hybridomas (Fill out a, c)
☐ Adjuvants (Fill out a)
☒ Controlled substances (Fill out a, d, e)

For each checked off category, answer the questions indicated below:

a. Identify the substance(s) and completely describe their use, including how will be injected or given to the animal(s):

Daily dexamethasone injections will be given subcutaneously for 7 days (3 mg/kg body weight) to induce muscle wasting.

Isoflurane will be given as the primary anesthetic during non-survival surgery.

b. Describe all procedures necessary for personnel and animal safety including biohazardous waste, carcass disposal and cage decontamination:

All personnel will wear standard PPE including a laboratory coat and gloves before handling animals or animal cages. If gloves become compromised in any way (rips or holes) they will be replaced immediately. All animal carcasses will be disposed of in a plastic bag and placed in the freezer for later incineration. All cages will be decontaminated in the University of Memphis Vivarium, Life Science Building.

c. If transplantable tumors or hybridoma cells are to be injected into the animals, have the tissues/cells been tested for inadvertent contamination by viruses or mycoplasma? Yes ☐ No ☐

If yes, what was the result (indicate in box below).


d. In the box below, provide a complete list of these substances, and if their use is not explicitly explained in the materials already provided, explain their use and role in the research.

Provide DEA license # covering the use of these substances:

To whom (or what entity) is the license issued?
e. Provide Radioisotope License Number:

To whom is the license issued?

V. Categories of Animal Experimentation Based Upon Level of Manipulation and Pain:
   (check off each category that is applicable to this application)

☐ A. Animals will be involved in teaching, research, experiments or tests involving no pain, distress, or use of pain-relieving drugs.

☐ B. Animals will be subject to mild stress only (e.g., food or water deprivation of less than 24 hours for use in behavioral studies such as operant conditioning; physical restraint for less than 30 minutes), and will not be subject to surgery, painful stimuli, or any of the other conditions described below. Procedures described in this protocol have the potential to inflict no more than momentary or slight pain or distress on the animal(s)----that is, no pain in excess of that caused by injections or other minor procedures such as blood sampling.

☒ C. Animals will have minor procedures performed, blood sampling, etc. while anesthetized.

☐ D. Live animals will be humanely killed without any treatments, manipulations, etc. but will be used to obtain tissue, cells, sera, etc.

☒ E. Live animals will have significant manipulations, surgery, etc. performed while anesthetized. The animals will be humanely killed at experiment termination without regaining consciousness.

☒ F. Live animals will receive a painful stimulus of short duration without anesthesia (behavior experiments with flight or avoidance reactions--e.g., shock/reward) resulting in a short-term traumatic response. Other examples in this category are, blood sampling, injections of adjuvants, or drugs, etc.

☐ G. Live animals will have significant manipulations performed, such as surgery, while anesthetized and allowed to recover. Such procedures cause post-anesthetic pain/discomfort resulting from the experiment protocol (e.g., chronic catheters. surgical wounds, implants) which cause a minimum of pain and/or distress. Also included are mild toxic drugs or chemicals, tumor implants (including hybridomas). tethered animals, short-termed physically restrained animals (up to 1 hour), mother/infant separations.

☐ H. Live animals will have significant manipulations or severe discomfort, etc. without benefit of anesthesia, analgesics or tranquilizers. Examples to be included in this category are: toxicity testing, radiation sickness, irritants, burns, trauma, biologic toxins, virulence challenge, prolonged: restrictions of food or water intake, cold exposure, physical restraint or drug addiction. All use of paralytic agents (curare-like
drugs) must be included in this category. Describe any abnormal environmental conditions that may be imposed. Describe and justify the use of any physical restrain devices employed longer than 1 hour.

VI. Justifications for Category G Studies and Deviations from Standard Techniques

Describe in the box below any steps to be taken to monitor potential or overt pain and/or distress during the course of this study and how such pain or distress will be alleviated. Be as detailed as necessary to justify your procedure.

Mice will be randomized into either a high-fat Western diet or Omega-3 rich diet and be on their respective diets for 4 weeks. Each group will then randomly be further divided to receive daily dexamethasone injections subcutaneously for 7 days (3 mg/kg body weight) to induce muscle wasting. Mice will be monitored regularly to ensure the health of the animal. At the end of the study all animals will be anesthetized by isoflurane. We will ensure complete anesthetization by checking pedal reflex, and by using tail pinch. If mice are not fully anesthetized, we will wait until they exhibit no response to the stimuli before we begin the surgery. The animal will be euthanized by heart removal and cervical dislocation as a secondary measure. Hindlimb skeletal muscles (soleus, plantaris, gastrocnemious, tibialis anterior, EDL), and epididimal fat pad will be excised and snap frozen in liquid nitrogen for further analysis. Non-survival surgery is required for the excision of muscle to measure myofibrilar protein synthesis as lack of oxygen to the tissue caused by death will interfere with basal protein synthesis rates.

VII. Certifications
(By submitting this protocol, I am acknowledging that I comply with the certifications included in Section VII.) (check one)

☒ Animal Use for Research. I certify that the above statements are true and the protocol stands as the original or is essentially the same as found in the grant application or program/project. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not purchase animals nor proceed with animal experimentation until approval by the IACUC is granted.

☐ Animal Use for Teaching/Demonstration. I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file in the IACUC office. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not proceed with animal experimentation until approval by the IACUC is granted.
Estimate the cost of maintaining animals used in this protocol based on current per diem charge at University of Memphis.

Please specify cost per unit of time:  $0.35/cage/day (10 cages) = $3.50/day* 40 days

Specify anticipated total costs for project duration:  $140 cage costs + $900 animals ($25/mouse)

As supervisor of this project it is required that you inform your department chair concerning any animal per diem costs related to this project that are to be paid by the department.

By submitting this protocol, the Principal Investigator/Course Director indicates that the following have been considered:

1. Alternatives to use of animals.
2. Reduction of pain and stress in animals to the lowest level possible.
3. The proper needs of the animals with respect to housing and care.
4. The lowest number of animals used that will give the appropriate experimental results.
5. Use of the most primitive species that will give the appropriate experimental results.
6. Proper training of all personnel in the care and handling of the species used and in the procedures called for in this protocol before beginning the experiment/teaching or demonstration.
7. That this protocol is not an unnecessary repeat of results already in the literature or in the case of teaching/demonstrations, results that can be demonstrated using models or video material.

Principal Investigator/Course Director (Type Name)  Melissa Puppa

e-mail address  mpuppa@memphis.edu

Date  8-30-2018

Federal Law requires that members of the IACUC be given adequate time to read and review protocols including any changes or revisions in them.

Pre-review of protocols by the Attending Veterinarian is required before submission to the IACUC. New protocols or modifications or renewals to protocols must be submitted to the IACUC Chair by the 1st business day of the month to be considered for review during that month. Incomplete protocols will be returned to the principal investigator.

E-mail the completed protocol to the IACUC Chair, Dr. Amy de Jongh Curry, adejongh@memphis.edu

February, 2015
IACUC PROTOCOL ACTION FORM

To: Melissa Puppa
From: Institutional Animal Care and Use Committee
Subject: Animal Research Protocol
Date: October 26, 2018

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. 830:

**0830 Effect of Omega-3 supplementation in high-fat diet on skeletal muscle protein turnover in glucocorticoid-induced muscle wasting**

- Your protocol is approved for the following period:
  - From: October 25, 2018
  - To: October 24, 2021

- Your protocol is not approved for the following reasons (see attached memo).

- Your protocol is renewed without changes for the following period:
  - From: 
  - To: 

- Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated for the following period:
  - From: 
  - To: 

- Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated 

Amy L. de Jongh Curry, PhD, Chair of the IACUC
Dr. Karyl Buddington, University Veterinarian and Director of the Animal Care Facility