EXCESSIVE ETHANOL INTAKE IN MICE DOES NOT IMPAIR RECOVERY OF TORQUE FOLLOWING REPEATED BOUTS OF ECCENTRIC CONTRACTIONS

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Samantha E. Moser

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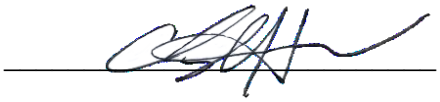
This thesis has been approved by

The Honors Tutorial College and the Department of Applied Health Science & Wellness

Dr. Cory Baumann

Assistant Professor, Biomedical Sciences

Thesis Adviser



Dr. Cheryl A. Howe

Associate Professor, Exercise Physiology

Director of Studies, Translational Health

_____

Dr. Donal Skinner

Dean, Honors Tutorial College

Table of Contents

Page

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Abstract

MOSER, SAMANTHA E., B.S., May 2022, Honors Tutorial College

Excessive Ethanol Intake in Mice Does Not Impair Recovery of Torque Following Repeated Bouts of Eccentric Contractions

Director of Thesis: Cory W. Baumann

Purpose: Between 40-60% of chronic alcoholics develop alcoholic myopathy, skeletal muscle atrophy and weakness due to excessive ethanol (EtOH) intake. To date, most studies have examined the mechanisms of chronic alcoholic myopathy under basal or unstressed conditions despite physical stress being a normal occurrence in a physiological setting. Therefore, there were two goals of this study: determine whether recovery of torque is impaired following repetitive bouts of physical stress in skeletal muscle developing alcoholic myopathy (Experiment #1) and, in skeletal muscle with alcoholic myopathy (Experiment #2).

Methods: Twenty C57BL/6 male and female mice were randomly assigned to receive either 20% EtOH in their drinking water or 100% water (n=10 per group, 5 per sex). Anterior crural muscles were subjected to repeated bouts of physical stress using in vivo eccentric contractions, with tetanic isometric torque being measured immediately pre- and post-injury. A total of ten bouts were completed with 14 days between bouts 1-5 (Experiment #1) and 6-10 (Experiment #2), and 12 weeks between bouts 5 and 6.

Results: Mice consuming EtOH had BACs up to 270 mg/dL. In Experiment #1, five bouts of 150 eccentric contractions did not reduce recovery of torque, regardless of sex or

EtOH treatment ($p \geq 0.173$). Similarly, in Experiment #2, pre-injury torques did not differ from day 14 values regardless of sex or treatment ($p \geq 0.322$). However, there was a group effect in female mice for bouts 1 and 5 during Experiment #2, with female EtOH mice being weaker than controls ($p \leq 0.002$).

Conclusions: Chronic alcoholic myopathy in a mouse model does not affect the muscle's ability to recovery from tetanic isometric torque after repeated bouts of eccentric contractions. This suggests that EtOH may not be as detrimental to skeletal muscle recovery as once predicted.

Chapter 1: Introduction

Background

Excessive alcohol use is the third most preventable cause of death and the leading cause of premature mortality in the United States (Stahre et al., 2014). Alcohol abuse has been known to cause over 60 types of diseases and injuries and can affect at least 200 others (Rocco et al., 2014). Almost every organ and tissue in the body is affected by chronic levels of alcohol abuse, including the liver, pancreas, brain, heart, and skeletal muscle (Mukherjee, 2013; Rachdaoui & Sarkar, 2013; Rocco et al., 2014; Tasnim et al., 2020). It also negatively affects various body systems including the endocrine, digestive, immune, and vascular systems (Rachdaoui & Sarkar, 2017). These disturbances can result in stress intolerance, reproductive dysfunction, thyroid problems, immune abnormalities, infectious diseases, cancer, bone diseases, and psychological and behavioral disorders (Rachdaoui & Sarkar, 2013, 2017).

Some of earliest recognized changes produced by excessive alcohol consumption are the histological, biochemical, and physiological alterations observed in striated muscle (Lang et al., 2005). In fact, 40-60% of chronic alcoholics develop alcoholic myopathy, a disorder defined as loss of muscle strength and size that results from excessive alcohol intake (Fernandez-Solà et al., 2007; Preedy et al., 2001; Urbano-Márquez & Fernández-Solà, 2004). Chronic alcoholic myopathy is often characterized by proximal muscle weakness, specifically in the pectoral and pelvic girdle regions, that occurs over years of sustained drinking (Preedy et al., 2001; Simon et al., 2017). Several reports have documented reductions in muscle mass (atrophy) up to 20% and strength

loss up to 35% due to chronic alcohol abuse (Estruch et al., 1998; Lang et al., 2005; Martin et al., 1985; Urbano-Márquez & Fernández-Solà, 2004). Subsequently, alcoholic-related weakness manifests as defects in function, as measured by walking capacity and physical activity levels (Vancampfort et al., 2019).

Weakness may be the result of reduced neuromuscular excitation, skeletal muscle quantity (i.e., size) and/or muscle quality (i.e., contraction efficiency) (Steiner & Lang, 2015a). Chronic alcoholic myopathy primarily leads to atrophy of type II muscle fibers as evidenced by loss of type II fiber cross sectional area (Kimball & Lang, 2018; Lang et al., 2001, 2005; Slavin et al., 1983; Steiner & Lang, 2015a). Reductions in fiber cross-sectional area or size are observed due to disturbances in protein synthesis and degradation as a result of alcohol-induced alterations in anabolic and catabolic signaling (Kimball & Lang, 2018; Simon et al., 2017; Steiner & Lang, 2015a). Moreover, this type of weakness may be a consequence of reduced neuromuscular excitation due to peripheral neuropathy (Agelink et al., 1998; Julian et al., 2019a) or reductions in muscle quality via dysfunction in calcium transport and cross-bridge formation (Edmonds et al., 1987; Simon et al., 2017; Urbano-Márquez & Fernández-Solà, 2004). It is generally accepted that alcohol-induced weakness is due to multiple factors across several sites, however, the precise contributions of each remain to be fully delineated.

Mechanisms causing weakness associated with chronic alcoholic myopathy have primarily been revealed in resting or unstressed skeletal muscle. Although these studies provide pivotal information about the pathophysiology of chronic alcoholic myopathy, details of how excessive alcohol intake influences physically stressed muscle function

has become an area of great interest. For instance, several studies have induced physical stress in human skeletal muscle via eccentric contractions to assess how alcohol intake impacts recovery from injury (Barnes et al., 2010a, 2010b; P. Clarkson & Reichsman, 1990; Levitt et al., 2017; McLeay et al., 2017; Parr et al., 2014). These studies initially posited that because excessive alcohol consumption impacts several aspects related to protein synthesis pathways, recovery from eccentric contractions would be blunted if alcohol was consumed concurrently with or after the injury. However, despite alcohol's documented negative effects on protein homeostasis in unstressed muscle, markers of damage does not appear to be exacerbated when consuming alcohol acutely after eccentric contractions (Barnes et al., 2010a, 2010b; Clarkson & Reichsman, 1990; Levitt et al., 2017; McLeay et al., 2017; Parr et al., 2014). Several caveats to these findings are denoted, including that acute alcohol intake after a single injury may not reflect physiological conditions and that detrimental effects of chronic alcohol may only be apparent after cumulative injuries (Laki´cevi´c, 2019; Simon et al., n.d.). This study bridges that gap in the literature to determine how chronic usage of alcohol affects muscle function and recovery from multiple injury bouts.

Purpose

The purpose of this study is twofold. First, investigate whether recovery of strength is impaired following repetitive bouts of physical stress in C57BL/6J male and female mice that are developing chronic alcoholic myopathy. Second, determine if recovery of strength is impaired following repetitive bouts of physical stress once mice have developed chronic alcoholic myopathy. Based on previous research that reported

proteins synthesis pathways are blunted after excessive alcohol intake, we hypothesized that mice would have an impaired ability to recover from repetitive bouts of physical stress in the process of developing chronic alcoholic myopathy, which would be further impaired once they developed chronic alcoholic myopathy.

Specific Aims and Hypotheses

Aims

Aim 1: Determine if alcohol reduces muscle recovery from repetitive stress as mice are developing alcoholic myopathy.

Aim 2: Determine if repetitive stress exacerbates weakness in a mouse model of chronic alcoholic myopathy.

Hypotheses

H01: Muscle recovery from injuries will be blunted in mice consuming chronic amounts of alcohol.

H02: Alcohol myopathic mice will be weaker at baseline and have an impaired ability to recover from injuries.

Chapter 2: Literature Review

Impact of Alcoholism

Alcohol use has been a major problem for decades and continues to be a health issue within society today. Recent data from a 2019 National Survey on Drug Use and Health (NSDUH) reported that 54.9% of adults consumed alcohol within the past month. Of that percentage, 25.8% were engaged in binge drinking and 6.3% were engaged in heavy chronic alcohol use. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), binge drinking is defined as a pattern of drinking that brings blood alcohol concentration (BAC) to 0.08 g/dl or higher. This is usually around 5 or more drinks for men or 4 or more for women in about 2 hours. Heavy alcohol use (i.e., chronic drinking) is defined as consuming 4 or more drinks on any day or more than 14 drinks per week for men and more than 3 drinks on any day or more than 7 drinks per week for women (*Drinking Levels Defined | National Institute on Alcohol Abuse and Alcoholism (NIAAA)*, n.d.). Adults are not the only people who are consuming alcohol (Niaaa, n.d.). According to the same NSDUH survey mentioned above, 14.5 million people ages 12 and older had developed alcohol use disorder (AUD), defined as a medical condition characterized by an impaired ability to stop or control alcohol use despite adverse social, occupational, or health consequences (Niaaa, n.d.; *Understanding Alcohol Use Disorder | National Institute on Alcohol Abuse and Alcoholism (NIAAA)*, n.d.). Of that 14.5 million, 414,00 were adolescents ages 12-17 (Niaaa, n.d.). Even more worrisome is that these numbers are on the rise, as the percentage of people with AUD increased over 22% from 2003 to 2010 (Grant et al., 2017).

Excessive alcohol use is the third most preventable cause of death in the United States and the leading cause of premature mortality (Stahre et al., 2014). Reasons for this increased mortality rate include accidental injury, liver cirrhosis, cancers, violence, and many others (Rao & Andrade, 2016). Although injuries may not directly result in death, if severe or repetitive, these injuries can reduce health span and quality of life. Injuries often result from falling, peripheral neuropathy, and vehicle accidents (Dekeyser et al., 2013). In fact, 1 in 10 deaths in working-age adults are associated with excessive alcohol consumption (Stahre et al., 2014). Due to the abundance of people consuming alcohol becoming hospitalized and dying prematurely, the economic effect has been on the rise as well. In 2010, the economic cost of excessive alcohol consumption was $249 billion (Sacks et al., 2015).

Although the prevalence of individuals with AUD is on the rise, AUD has been shown to be higher in men than women (Agabio et al., 2017). In fact, despite women drinking less than men, they are thought to be more vulnerable to the effects of alcohol. When compared to men, women develop AUD quicker, have an increased alteration of sex hormones, and are at risk for more health problems including alcohol-related liver disease, cardiomyopathy, and breast cancer (Agabio et al., 2017; Erol & Karpyak, 2015). The rise in high-risk drinking, regardless of sex, will continue to promote negative health effects among the population. This is evident by the 47% increase in alcohol-related emergencies and deaths between the years 2006 to 2014 (Niaaa, n.d.). Alcohol has detrimental effects on many established “alcohol-sensitive” organs (e.g., liver, kidneys, pancreas), but also has direct and indirect effects on many other tissues and cell types

such as skeletal fibers/muscle. Harmful consumption of alcohol can result in muscle damage, muscle weakness, and atrophy (Preedy et al., 2003; Simon et al., n.d.; Urbano-Márquez & Fernández-Solà, 2004). In fact, 40-60% of chronic alcoholics develop alcoholic myopathy, that is, atrophy, and weakness of skeletal muscle (Fernandez-Solà et al., 2007; Urbano-Márquez & Fernández-Solà, 2004).

Alcoholic Myopathy

Alcoholic myopathy is loss of muscle strength and size that results from acute or chronic alcohol consumption (Preedy et al., 2001). Acute alcoholic myopathy occurs when someone has a severe alcoholic binge that results in BAC at or above 0.08 g/dL and is characterized by weakness, tenderness, pain, and inflammation of muscles. It can often be seen with elevated serum creatine kinase or myoglobin and rhabdomyolysis (Simon et al., n.d.). These effects are short lived as they resolve after 1 to 2 weeks of abstinence. Acute alcoholic myopathy is not as common as it only affects approximately 20 out of 100,000 people or 1% of alcoholics. To the contrary, chronic alcoholic myopathy is much more common, affecting approximately 2,000 per 100,000 people or 50% of alcoholics (Preedy et al., 2003; Simon et al., n.d.). Chronic alcoholic myopathy is also associated with greater health implications and mortality compared to acute myopathy (Simon et al., n.d.). Due to these facts, the focus of this research project will be on chronic alcoholic myopathy.

Chronic alcoholic myopathy is characterized by proximal muscle weakness, specifically in the pectoral and pelvic girdle regions, that occurs over years of regular drinking (Preedy et al., 2001; Simon et al., n.d.). When comparing non-chronic alcoholics

to chronic alcoholics (people who have reported a daily alcohol (i.e., ethanol (EtOH)) consumption of >100 g for at least 4 years), subjects who consume alcohol can have up to 35% strength loss due to alcohol abuse (Red Line in Figure 1) (Estruch et al., 1998). One standard drink is defined as any beverage containing 0.6 fl oz or 14 grams of pure alcohol. Therefore, intake of 100 g of EtOH is equivalent to approximately 7 drinks (*What Is A Standard Drink? | National Institute on Alcohol Abuse and Alcoholism (NIAAA)*, n.d.). One of the main factors in chronic alcoholic myopathy is the atrophy of type II muscle fibers (see sections below for information on muscle type) (Slavin et al., 1983). This loss of muscle size and strength also appears to be sex dependent. Females tend to develop muscle atrophy quicker than males. It takes females 5-7 years of chronic alcohol intoxication to have over 40% type II muscle atrophy, which would take 10 or more years for the same percentage of atrophy to occur in males (Nemirovskaya et al., 2015; Shenkman et al., 2019; Slavin et al., 1983). Muscle atrophy from chronic alcohol consumption has been suggested to be directly and indirectly linked to the toxic effects of alcohol. These toxic effects include, but are not limited to: neuropathy, malnutrition, vitamin deficiencies, hormonal alterations, liver dysfunction, and muscle dysfunction (Preedy et al., 2001). Abstaining from alcohol consumption results in improved muscle function but levels of muscular strength do not always fully recover. As seen in Figure 1 (Blue Line), after 5 years of abstinence, subjects who used to be chronic alcoholics still presented signs of weakness (Estruch et al., 1998).

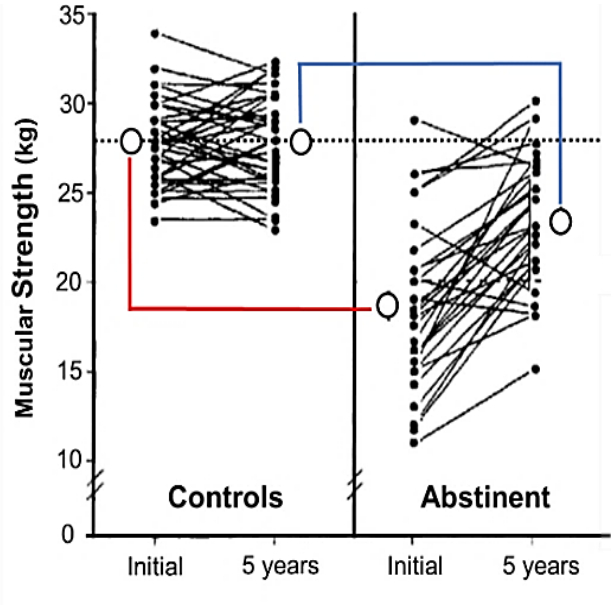

Figure 1. Deltoid muscular strength in control (no alcohol) and abstinent subjects. Abstinent subjects were chronic alcoholics at "initial" and followed-up after 5 years of abstinence. Red lines depict strength loss (35% deficit) due to alcohol abuse. Blue lines depict strength is lower (20% deficit) than controls even after 5 years of abstinence (Figure from Estruch et al. (1998)).

Along with the human studies, animal studies have also demonstrated strength loss occurs due to chronic alcohol consumption. A recent report administered either alcohol-free Lieber-DeCarli rodent liquid diet (product #F1259SP; Bio-Serv) or an alcohol-containing liquid diet (product #1258SP; Bio-Serv) to young male mice for a 14-16 week duration (Crowell et al., 2019). The alcohol group underwent a ramping protocol over six days that increased the alcohol content of their diet from 8.8 to 30%. Utilizing an *in vitro* model of muscle physiology, these researchers tested maximal isometric force in mice after chronic consumption of alcohol compared to control mice (Crowell et al., 2019). As seen in Figure 2, chronic alcohol ingestion decreased maximum tetanic tension in the extensor digitorum longus muscle by 16%, indicating skeletal muscle weakness (Crowell et al., 2019).

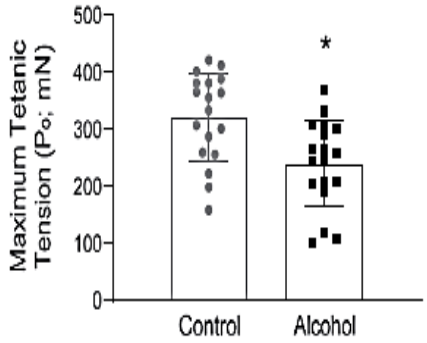

Figure 2. Differences in maximum isometric force in mice that consume alcohol vs controls (Figure from Crowell et al. (2019)).

Skeletal Muscle Form and Function

Skeletal muscle functions to produce force, power, and movement. It can also play a role in the storage of amino acids and carbohydrates as well as maintenance of core body temperature. Skeletal muscle makes up essentially 40% of total body weight and contains 50-75% of all the proteins in the body with approximately 30-50% of whole-body protein turnover. Skeletal muscle doesn't consist entirely of protein. In fact, protein only makes up 20% of skeletal muscle with 75% being water and the other 5% consisting of salts, minerals, fats, and carbohydrates. However, most of the muscle mass is a result of protein synthesis and protein degradation which can be affected by nutrition, hormones, exercise, disease, injury, alcohol, and other factors (Frontera & Ochala, 2015).

There are multiple components that make up muscle. Each individual muscle is surrounded by connective tissue called epimysium. Within a muscle there are hundreds of muscle fibers bundled into fascicles that are surrounded by the perimysium. Each muscle fiber is composed of thousands of myofibrils made up of billions of myofilaments. The single muscle fiber is wrapped in a fine layer of connective tissue called the endomysium.

Myofilaments, when assembled in an orderly pattern, form sarcomeres. Sarcomeres contain thick filaments, called myosin, and thin filaments, called actin, and are the basic contractile units of skeletal muscle (Frontera & Ochala, 2015). Alongside these sarcomeres are transverse-tubules (T-tubules), sarcoplasmic reticulum (SR), and mitochondria. The SR regulates the storage, release, and re-uptake of calcium.

Calcium is important for muscle contraction as it is an essential part of excitation-contraction coupling. For a muscle to contract, actin and myosin must interact. This starts by an action potential traveling down the afferent nerve to the sarcolemma. The action potential is then conducted down to T-tubules where it activates voltage gated dihydropyridine receptors on the SR that open and cause calcium to flow in. The influx of calcium causes the opening of ryanodine receptors and allows calcium to enter the sarcoplasm. This calcium will then bind with troponin which will move tropomyosin off the myosin binding sites on the actin filaments. With the active site now open, the myosin head will bind to actin, called coupling, and a cross bridge is formed allowing the muscle to contract and produce force (Rebbeck et al., 2014).

Along with calcium release, there are multiple factors that can affect the force produced by a muscle. The size of the muscle, also known to be an indicator of muscle quantity, is one major indicator of muscle force. The nervous system, muscle architecture, space between myofilaments, fiber type and number of cross bridges formed can also affect how much force a muscle can generate (Frontera & Ochala, 2015). Fibers can be classified into different categories based on color of muscle fibers, contractile properties in response to electrical stimulation, speed of shortening, degree of fatigability,

metabolic pathways, calcium handling, and protein expression. The main three types of fibers are type I, type IIa, and type IIx. Type I is known for being slow, oxidative, and fatigue resistant. Type IIa is fast, oxidative, and has intermediate metabolic properties. Type IIx is known for being the fastest, glycolytic, and highly fatigable (Frontera & Ochala, 2015). No matter the type of muscle fiber, each can be affected differently depending on the type of action the muscle is performing.

There are three different types of skeletal muscle actions: isometric, concentric, and eccentric. Isometric actions are characterized by force being generated with no movement in the joint. Concentric muscle actions are when the muscle shortens as force is produced. Eccentric contractions are when the force produced by the muscle is less than the outside force causing the muscle to lengthen. If unaccustomed to or done in excess, eccentric contractions (or exercise) will cause muscle damage (Gordon L. Warren et al., 2001). However damaging, muscle fibers contains heat shock proteins, regenerative pathways, and satellite cells responsible for muscle growth, repair and/or regeneration (Macaluso & Myburgh, 2012).

Direct Effects of Ethanol on Skeletal Muscle

There are many different proteins that make up a muscle, and the interaction and abundance between/of these proteins dictate skeletal muscle strength. Skeletal muscle strength is therefore comprised of two factors: skeletal muscle quantity and quality. Here, a general overview of how alcohol impacts these two factors is outlined.

Muscle Quantity

General Overview of Protein Synthesis

Many studies have shown that chronic alcohol consumption will lead to atrophy and reduced cross-sectional area of type II muscle fibers. Reduced cross-sectional area is related to impaired protein synthesis because of a translation initiation inefficiency, meaning that tRNA, 40S and 60S ribosomal subunits are not assembled properly leading to lack of protein synthesis (Kimball & Lang, 2018; Steiner & Lang, 2015a). Mechanistic target of rapamycin complex 1 (mTORC1) is a critical protein in the process of protein synthesis, especially in translation initiation. During initiation, the 40S ribosomal subunit interacts and binds to methionyl-tRNAi (met-tRNAi) which will create the 43S pre-initiation complex. Eukaryotic initiation factor 4F (EIF4F) and initiation factors eIF4A, eIF4E, and eIF6G bind to the 43-pre-initiation complex that attaches to mRNA at the 5' cap to form 48S pre-initiation complex. mTORC1 binds by phosphorylating eukaryotic initiation factor 4E binding protein-1 (4E-BP1) that frees eIF4E to bind to eIF4G (Kimball & Lang, 2018). mTORC1 also phosphorylates ribosomal protein S6 kinase (S6K1) (Shah et al., 2000; Steiner & Lang, 2015a). The activation of S6K1 phosphorylates other proteins that subsequently cause initiation and protein synthesis.

Protein Synthesis Under Alcoholic Conditions

The total amount of mTORC1 protein in skeletal muscle is not affected by the consumption of acute or chronic alcohol ingestion. However, the protein-to-protein interaction of mTORC1 is impaired causing translational inefficiency (Steiner & Lang, 2015a). Chronic alcohol consumption decreases phosphorylation of mTORC1 as well as suppresses phosphorylation of ribosomal protein S6. It does this initially by decreasing phosphorylation of 4E-BP1, meaning it will stay bound to eIF4E. Without the capability

for eIF4E to be free, it cannot bind to eIF4G and thus prevents the eIF4F complex from becoming active (Table 1) (Lang et al., 2001). As a result, the mRNA will not be able to bind to the 43S pre-initiation complex and translation is disrupted (Lang et al., 2005; Shah et al., 2000). In a similar way, acute alcohol consumption also results in decreased protein synthesis in a dose- and time-dependent manor. Within a few hours after large doses of alcohol intake, decreases in muscle protein synthesis, translational efficiency, and mTORC1 signaling can already be observed as shown in Table 1 (Lang et al., 2001). With acute alcohol consumption, these results can last for up to 24 hours, though are reversible (Lang et al., 2005). When rats with acute alcoholic myopathy abstained from alcohol for just 3 days, mTORC1 and all other values related to translation initiation returned to normal. However, depending on the dosage, it may take weeks of abstinence to fully reverse. With chronic alcoholic myopathy, changes with protein synthesis are usually not seen until at least 2 months or even up to 12 months of alcohol misuse (Peters et al., 1985).

	Skeletal muscle	
	Acute	Chronic
eIF2α total	↔	↔
eIf2α (P)	↔	↔
eIF2Bε content	↔	↔
EIF2B activity	↔	↓
4E-BP1·elF4E	↑	↑
eIF4G·elF4E	↓	↓
4E-BP1 γ-form	↑	↑
eIF4E total	↔	↔
eIF4E (P)	↔	↔

Table 1. Changes in components of eIF2 and eIF4E systems in straited muscle from rats either after acute alcohol intoxication (2.5 hours) or alcohol complete diets for 14-16 weeks (Figure from Lang et al. (2001)).

Insulin-like growth factor-1 (IGF-1) is a hormone that acts alongside growth hormone for tissue development and growth (Lang et al., 2001). It plays a direct role in increasing protein synthesis in skeletal muscle and activating the mTORC1 pathway. As a result, if IGF-1 decreases in the plasma and in the muscle, protein synthesis will also decrease (Steiner & Lang, 2015a). Multiple studies have shown that chronic alcohol consumption decreases the amount of total IGF-1 in the blood, liver, and skeletal muscle (Lang et al., 1998, 2001). Through the reduction of IGF-1 in the blood and the decrease activation of mTORC1, the eIF4E complex remains nonactivated and results in reductions in protein synthesis.

In overview, alcohol consumption can also affect muscle quantity (i.e., size) through reducing cross-sectional area of type II muscle fibers (Kimball & Lang, 2018).

This muscle atrophy is likely linked to reductions in protein synthesis mediated through mTORC1 signaling. That is, less synthesis would result in reduced contents of actin and myosin filaments. It is recognized that larger fiber cross-sectional area has a direct relationship with a greater capacity to generate force.

Muscle Quality

Skeletal muscle quality can generally be defined as contraction efficiency, or the ability of muscle proteins to work collectively to result in a muscle contraction (Baumann, Kwak, et al., 2016). Of particular emphasis is the ability of the muscle to regulate calcium. As previously stated, calcium is integral in muscle contraction, specifically in allowing the coupling of actin and myosin. Following a muscle contraction, the SR calcium transport ATPase (SERCA) pumps calcium back into the SR causing muscle relaxation (Kuo & Ehrlich, 2015). Chronic alcohol ingestion has been shown to decrease the activation and contractility of actin and myosin through alterations of calcium transport from the SR. Alcohol consumption has also been reported to slow SR calcium release and reuptake, and reduce myofibrillar calcium sensitivity (Edmonds et al., 1987; Martyn & Munsat, 1980). Less coupling of actin and myosin reduces the quality of the muscle because fewer cross bridges will be formed resulting in less force production, regardless of muscle size or quantity (Rubin et al., 1976).

Indirect Effects of Ethanol on Skeletal Muscle

Alcoholics not only consume chronic amounts of EtOH but are also likely to have a poor diet, low levels of physical activity, issues sleeping, and many additional bad habits (e.g., smoking) (Simon et al., n.d.). These confounding factors can also impact

muscle quantity and quality and exacerbate the effects that EtOH has on skeletal muscle form and function. Here, we briefly highlight how malnutrition and vitamin D deficiency in alcoholics also induce weakness.

Malnutrition

Alcoholics are often prone to being malnourished (Nicolás et al., 2003). Between 5-40% of alcoholics are malnourished due to lack of proper amounts of protein, energy, or both. Some reasons for this malnourishment include deficient food intake, liver cirrhosis, malabsorption, and the poor nutritive value of alcohol (Nicolás et al., 1993). Although alcohol provides 7 kcals/g of energy, it carries no nutritive value due to its interference with absorption, transport, and utilization of essential nutrients (Patek, 1979). In a study done by Nicolás et al. (2003), energy and protein malnutrition affected skeletal muscle in chronic alcoholics. Out of 146 chronic alcoholics, 61 complained of muscle discomfort, ~70 complained of subjective shoulder and pelvis weakness, and 6 complained of general muscle weakness and severe myalgia. These display a dose dependent effect as the more EtOH the individuals drank, the more severe their symptoms became. Independent of EtOH consumption, the data also showed that malnourished alcoholics had significantly less muscle strength and muscle efficiency compared to well-nourished alcoholics. This suggests that energy and protein malnutrition exacerbate muscle weakness in chronic alcoholics (Nicolás et al., 2003). Some studies may even argue that the strength loss may be due to malnutrition in chronic alcoholics, rather than EtOH (Martyn & Munsat, 1980).

Vitamin D

Vitamin D is important in regulating and increasing muscle contraction (Polly & Tan, 2014). González et al. (2010) investigated how muscle fiber area relates to serum 1,25 (OH)2 Vitamin D levels in murine models with alcoholic myopathy. Their results showed a direct relationship between serum 1,25 (OH)2 Vitamin D levels and type IIa fiber size (González-Reimers et al., 2010). Overall, general malnourishment or lower Vitamin D levels may result in protein imbalance and lead to muscle atrophy and weakness. Despite these findings, animal studies have shown that nutritional factors are not essential for the development of skeletal muscle myopathy (Kimball & Lang, 2018); meaning chronic alcohol intake may be directly causing myopathy.

Impact of Ethanol on Damaged Skeletal Muscle

Muscle function is not only impacted by the direct and indirect effects of EtOH, but also how skeletal muscle copes with stress. That is, weakness is a product of chronic EtOH consumption and physiological stress. One such physiological stress is repetitive muscle contractions, specifically the high forces that eccentric contractions placed on skeletal muscle. Stress results in damage to the muscle fibers; however, muscle is plastic and subsequently recovers (under healthy conditions). If EtOH increases damage and blunts recovery, then muscle of chronic alcoholics will be weaker from the combined effects of EtOH directly and skeletal muscle repair may be impacted. Here, we highlight human studies that assess the effects on EtOH consumption and stress in the form of eccentric contraction-induced injury.

Although not always noticeable, an integral part of muscle functioning is to produce eccentric contractions. Throughout everyday life, muscles are eccentrically

contracting to support body weight against gravity and take part in normal movements such as walking, jogging, and regaining balance. Eccentric actions also absorb shock during descents or decelerations and store elastic recoil energy to help prepare for concentric actions. Eccentric contractions, when performed in excess, cause contraction-induced injury to skeletal muscle resulting in inflammation, necrosis, and degeneration due to physical damage to muscle proteins (Clarkson & Tremblay, 1988; Nosaka & Clarkson, 1995; Warren et al., 2001). These eccentric muscle actions result in functional changes including temporary muscle soreness, swelling, and weakness. Blood biomarkers of eccentric contraction-induced injury are often assessed by plasma creatine kinase (CK) and plasma myoglobin levels because of muscle membrane damage (Sorichter et al., 1999). Multiple factors are known to alter recovery from contraction-induced injury, but one modifiable life-style factor thought to delay the recovery process is alcohol. It is posited that alcohol consumption blunts the recovery process due to decrements in proteins turnover via catabolism through elevated oxidative stress and anabolism through mTORC1 inhibition (see *Protein Synthesis Under Alcoholic Condition*s) (Lang et al., 2001).

Multiple studies have looked at acute alcohol consumption immediately following the completion of eccentric exercises. Despite alcohol's documented negative effects on the muscle's biochemistry (e.g., mTORC1, IGF-1), consuming alcohol acutely after performing repeated eccentric contractions did not impact soreness, strength, or plasma CK when compared to controls (subjects that did not consume alcohol) (Barnes et al., 2010a, 2010b; Clarkson & Reichsman, 1990; Levitt et al., 2017; McLeay et al., 2017;

Parr et al., 2014). For instance, in a study by Levitt et al. (2017), thirteen recreationally resistance trained women performed 300 maximal single-leg eccentric leg extensions and then drank either 1.09 g/kg body mass EtOH or a placebo. After consumption, blood was taken and different biochemical markers were analyzed including CK. Consuming alcohol after the injury did not result in a difference in CK or muscle strength between the alcohol and control groups, indicating that muscle function was not impaired (Levitt et al., 2017). Although alcohol and controls groups were similar, Parr et al. (2014) reported mTORC1 phosphorylation and muscle protein synthesis were lower in the alcohol consuming subjects 2 and 8 hours post exercise when compared to controls (Parr et al., 2014). Overall, these studies suggest that acute alcohol consumption immediately following eccentric contractions does not affect markers of injury yet does result in biochemical changes within the muscle. It is possible that the biochemical changes would be additive overtime and eventually result in impaired recovery. With continued inhibition of mTORC1 and repeated stress, one would hypothesize that muscle function would be lost. As a result, it is worthwhile to investigate how the chronic effects of alcohol would impact muscle function following repeated injuries. Thus, the first aim of this proposal is to investigate how muscle recovers from repetitive bouts of physical stress as mice are developing alcoholic myopathy.

To our knowledge, only one study has investigated how chronic alcohol consumption affects the regeneration of damaged skeletal muscle (Dekeyser et al., 2013). In this study, C57BL/6 mice were given either 20% EtOH or water for 18-20 weeks to develop alcoholic myopathy. The mice were then injected with a 1.2% barium chloride

solution (i.e., a chemical-induced injury) into their tibialis anterior (TA) muscle. The muscles were removed, and muscle cross sectional area, inflammation and oxidative stress were assessed. Chronic alcohol consumption was reported to delay the normal regenerative response in skeletal muscle as measured by muscle cross-sectional area. Recovery of TA fiber area was reduced after the injury in alcohol fed mice as seen in Figures 3A and B. Also, total protein oxidative stress was higher in the alcohol group compared to the controls. One explanation of these differences could be that ciliary neurotrophic factor and amount of fibrosis were higher in alcohol fed mice. Results from this study would suggest that if chronic amounts of alcohol are consumed, recover from skeletal muscle injury will be blunted and may develop into persistent weakness, less flexibility and mobility, and increased the likelihood of re-injury (Dekeyser et al., 2013).

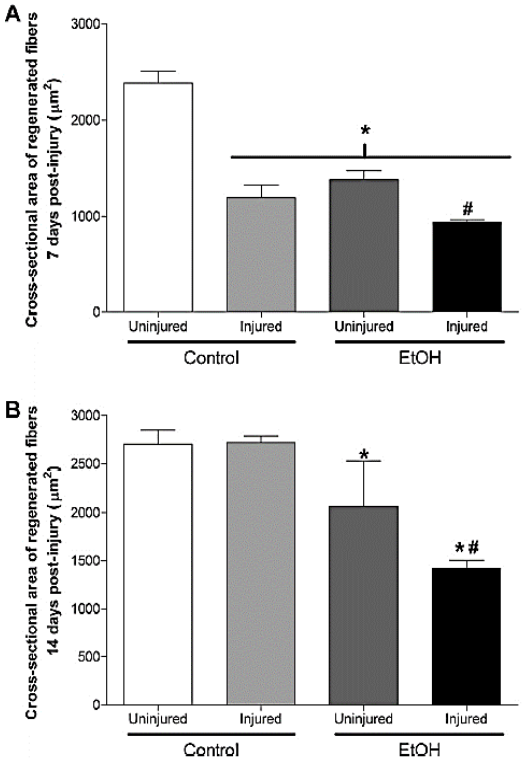

Figure 3. Recovery of TA fiber area 7 (A) and 14 (B) days after chemical muscle injury in alcohol fed and control fed mice (Figure from Dekeyser et al. (2013)).

Despite most research suggesting consuming alcohol immediately following eccentric exercise does not affect function, very little is known about how prior acute or chronic intake of alcohol affects muscle function from repetitive injuries (i.e., stressors). Importantly, in a physiological setting, the muscle is injured countless times, rather than a single isolated time. As a result, more research needs to be done on how chronic alcohol consumption, alcoholic myopathy, and stress affects muscle function. Thus, the second aim of this proposal is to investigate how muscle recovers from repetitive bouts of physical stress in mice with alcoholic myopathy.

Methodological Literature Review

Methods

To assess how alcohol effects recovery of peak torque following repetitive bouts of physical stress, mice were fed alcohol and subjected to *in vivo* eccentric contractions. Twenty male (n=10) and female (n=10) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) at 5 months of age. Following accumulation to the mouse facilities, mice underwent an EtOH ramping protocol of 5% EtOH for three days followed by 10% for 4 days and then 15% for 3 days. The mice were then provided with 20% EtOH for the remainder of the study. Aim one began two weeks into 20% EtOH consumption, at which time tetanic isometric torque of the left TA was measured followed by 150 eccentric contractions every 2 weeks for a total of 5 injuries.

Briefly, mice were first anesthetized in an induction chamber using 2% isoflurane mixed with oxygen at a flow rate of 1000 $mL \cdot min^{-1}$ (Baumann et al., 2020; Lowe et al.,

1995; Warren et al., 1999). The left leg was then shaved using hair trimmers and Nair and cleaned with betadine and EtOH. The anesthetized mouse was placed under a temperature-controlled platform set at 37-39 °C to maintain core body temperature. The left knee was clamped, and the left foot was secured to a "shoe" that was attached to the shaft of an Aurora Scientific 300B servomotor (Aurora, ON, Canada). Sterilized needles were inserted for percutaneous stimulation of the left common peroneal nerve (Baumann et al., 2020). Voltage was optimized using 5–15 isometric contractions (150 ms train of 0.2 ms pulses at 150 Hz) (Lowe et al., 1995). Once optimized, peak isometric torque was measured at 250 Hz.

The injury protocol began approximately 45-60 seconds later and consisted of 150 maximal eccentric contractions. During these eccentric contractions, the foot was passively dorsiflexed at 19.5° and then during stimulation, the foot was moved to 19.5° of plantarflexion at 2000°/s (Lowe et al., 1995). Five minutes after the completion of the last eccentric contraction, another 250 Hz isometric stimulation was completed. Each mouse was brought back every 2 weeks for a total of 5 eccentric injuries. After the fifth injury, mice were allowed to recover for two weeks, and peak isometric torque was re-assessed. At this point, experiment 1 ended and experiment 2 began.

Over the next three months, the mice continued to drink 20% EtOH without any injuries or assessments. By drinking without being tested, mice were able to develop chronic alcoholic myopathy. At the completion of three months, they were brought back and injured an additional 5 times, as done in experiment 1. Following the last bout of eccentric contractions, mice were given 14 days to recover and peak isometric torque was

measured. All mice were then euthanized by exsanguination following the cardiac punction in accordance with the Ohio University Animal Care and Use Committee. A cardiac puncture was performed to collect blood. This blood was centrifuged at 4°C for 10 min at 5000 RPM and the plasma was separated for storage at -80°C. AAM1 alcohol analyzer (Analox Instruments Ltd., Lunenburg, MA) was used to measure blood EtOH concentrations (BEC; mg/dl) (Yu et al., 2019).

Rationale of Methods

To demonstrate if alcohol influences recovery from skeletal muscle injury, mice consumed chronic amounts of EtOH and were subjected to repeated bouts of physical stress. Important aspects of this study include selection of mouse strain, inclusion of both sexes, variables of EtOH consumption, *in vivo* strength assessment, and method of injury induction. Here, each of these points are highlighted.

Selection of Mouse Strain

The C57BL/6 is one of the most commonly used mouse strains for basic laboratory research. The C57BL/6 strain is popular because it has a well-defined short life span, the major physiological systems are well-documented, the cost is conservative, and there are similarities with human aging. Moreover, it is already established that alcohol feeding regimens in this mouse strain result in similar blood alcohol concentrations as those observed in humans. For example, Crowell et al. (2019) obtained 11–12-week-old male C57BL/6 mice to determine if acute alcohol intoxication or chronic alcohol consumption decreased intrinsic contractile functioning of fast twitch skeletal muscle fibers. The mice were given an alcohol containing liquid diet with 30% alcohol

because it was shown to increase blood alcohol concentrations in this strain of mice to the level of chronic alcoholics. After assessing isometric and tetanic twitch force, their results confirmed with previous studies using this same strand of mice that alcohol reduced EDL mass and physiological cross sectional area in predominately type II fast-twitch skeletal muscles (Crowell et al., 2019). Dekeyser et al. (2013) also studied the skeletal muscle of C57BL/6 mice to assess how chronic alcohol consumption affects skeletal muscle regeneration following chemical-induced injury. These mice were approximately 8 weeks old and provided 20% EtOH in their water with regular food for 18-20 weeks. Based off of the reduced cross-sectional area of skeletal muscle fibers, increased fibrosis, and altered temporal expression of growth and fibrotic factors, chronic alcohol intake delays skeletal muscles normal regenerative response after injury (Dekeyser et al., 2013).

Inclusion of Both Sexes

This proposal was fundamentally directed towards understanding the synergistic effects of injury and chronic alcohol but also directly assessing sex as a biological variable. Indeed, although clinical manifestations of alcohol intoxication are observed in both sexes, observations suggest females are more susceptible than males, indicating sex as a biological variable in alcoholic myopathy. In fact, type II muscle fiber atrophy was over 40% in females after 5-7 years of alcohol intoxication, which in males, for the same percentage of atrophy, took almost 15 years (Nemirovskaya et al., 2015; Shenkman et al., 2019; Slavin et al., 1983). Therefore, we have set-up our experimental design to include both male and female mice.

Variables of EtOH Consumption

Our rationale for using a dosage of 20% EtOH is because it replicates blood alcohol contractions (BACs) reported in chronic alcoholics who consume at least 7 drinks/day (Collins & Neafsey, 2016; Mayo Clinic Laboratories, n.d.; Song et al., 2002) (Figure 4). A blood alcohol concentration above 30 mg/dl is an indicator for intoxication (Mayo Clinic Laboratories, n.d.). In a study by Song et al. (2002), C57BL/6 mice were given 20% EtOH in their water for 3-13 weeks. When blood alcohol was tested early in the morning after the mice had been drinking all night, blood alcohol levels were as high as 400 mg/dL and would lower throughout the day (Song et al., 2002). Moreover, this alcohol feeding regimen is also an accepted and appropriate laboratory model of alcohol-induced organ damage (D'Souza El-Guindy et al., 2010). These mice often presented with decreased spleen cellularity, increased activation of T cells, and decreased peripheral natural killer and Langerhans cell numbers. Natural killer and Langerhans cells are important for immune system response by determining the appropriate immune response and eliminating virally infected cells. When these cells do not function properly from alcohol, immune functioning may be negatively impacted, which can cause damage to the lungs, liver, and heart. We also note that because others have used this alcohol feeding regimen, this study will add or build on existing EtOH related literature.

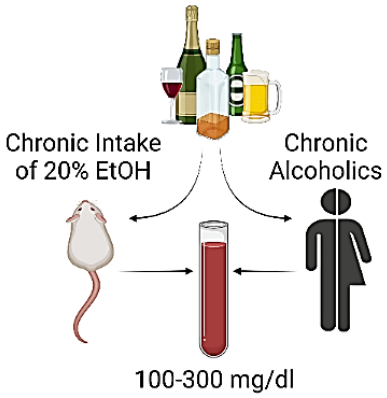

Figure 4. Schematic rationale for using this the highest dosage of 20% is that it has been shown to replicate blood alcohol contractions (BACs) reported in chronic alcoholic who consume 7+ drinks/day.

It is important to acknowledge that among chronic alcoholics, several factors beyond EtOH consumption may affect the individual's ability to respond to injury(ies). These factors include, but are not limited to, vitamin/mineral deficiency, poor diet, smoking and low physical activity levels (Simon et al., n.d.). Moreover, studies in humans often are unable to trace/track dosage and duration of consumption, making it difficult to precisely delineate when and how much alcohol impacts skeletal muscle. Here, we specifically isolated the direct effects of chronic alcohol consumption and markers of skeletal muscle injury while simultaneously tracking alcohol dosage and duration.

In Vivo Strength Assessment

Muscle strength in mice was measured *in vivo* using a custom physiology set-up to match those developed by Gordon Warren (Lowe et al., 1995) (Figure 5), that are currently being used at Georgia State University, University of Georgia, and University of Minnesota. *In vivo* was specifically used because it provides physiological relevant

results while keeping the muscles alive, maintains the mouse's blood supply, and does not require surgical isolation or neurovascular alterations (Lowe et al., 1995). This is because in the *in vivo* setup, the common peroneal nerve is stimulated using percutaneous needles that do not interfere with normal blood supply or any muscle connections. Moreover, this method is relatively non-invasive, and the same mouse can be repeatedly tested over time which allows for a repeated-measures, longitudinal study design. Longitudinal studies with repetitive testing on the same animals are ideal and powerful research designs (Baumann et al., 2020).

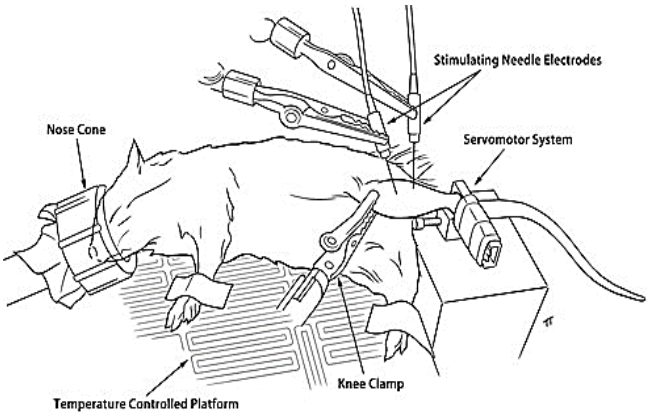

Figure 5. Graphic representation of an anesthetized mouse set up for in vivo contractility measurements of the anterior crural muscle using percutaneous needle electrode stimulation to activate the common peroneal nerve. Using this system and this specific muscle group and nerve, the same mouse can be repeatedly tested across time and treatment (Lowe et al. (1995)).

To accurately assess the effects of alcohol on muscle functioning, EtOH consumption and injury markers were repeatedly assessed in the same mice across time. By measuring alcohol consumption each week, bringing the same mice back to test every two weeks, and injuring them the same way each time, variability and bias between mice

is reduced. Moreover, torque values have been replicated using this *in vivo* set-up in the C57BL/6 mouse strain in various research laboratories across several universities (Baumann et al., 2016; Baumann et al., 2020). For example, Baumann et al. (2016) used an *in vivo* setup for C57BL/6 to determine whether mTORC1 is necessary for returning to normal muscular strength after injury (Baumann et al., 2016). Mice were given either saline or rapamycin (an inhibitor of mTORC1) and injured with 150 eccentric contractions. Prior to the injuries, pre-isometric torque values were taken. The maximal torque value before eccentric contractions for the mice injected with saline was 100.7 ± 3.6 $N \cdot mm \cdot kg^{-1}$ and the mice injected with rapamycin had a maximal torque of 105.0 ± 1.6 N mm/kg (Baumann et al., 2016). Similarly, Baumann et al. (2020) also used maximal isometric torque to assess muscle functioning after a bout of eccentric contraction-induced injury in *Mdx* mice (Baumann et al., 2020). The *Mdx* mouse is a dystrophin deficient mouse used to study Duchenne muscular dystrophy. In both the wildtype and *Mdx* mice, the pre-eccentric contraction isometric torque values were approximately 100 N mm/kg (Baumann et al., 2020). Despite the differences between mice, torque values preinjury were all roughly the same, indicating the repeatability of the *in vivo* setup in C57BL/6 mice.

Method of Injury Induction

A single bout of unaccustomed eccentric contractions can cause contraction-induced skeletal muscle injury (Lowe et al., 1995; Newham et al., 1987). In both humans and rodents, eccentric contraction-induced injury is characterized by the swelling of the muscle, infiltration of inflammatory cells, damage to myofibers, degradation of force-

bearing proteins, and release of muscle proteins into the blood (e.g. CK) (Clarkson & Hubal, 2002; Hyldahl & Hubal, 2014; Nosaka, 2008; Warren et al., 1993). Another common characteristic in humans is delayed onset muscle soreness (DOMS), pain felt in muscle that peaks 24–72 hours after eccentric contractions (Clarkson & Hubal, 2002; Newham et al., 1983; Nosaka, 2008). In addition to these markers of injury, eccentric contractions impair the force generating capacity of skeletal muscle, as measured by immediate and prolonged decrements in muscular strength (Clarkson & Hubal, 2002; Hyldahl & Hubal, 2014; Nosaka, 2008; Warren et al., 1993). The loss of strength is considered one of the most valid and reliable markers of eccentric contraction-induced injury (Warren et al., 1999). Both human and animal studies have shown a decrease in strength following a bout of eccentric contractions. Subjects who completed an eccentric biased downhill running protocol, stressing the quadriceps, had approximately 10-30% force loss immediately after exercise when compared to pre-exercise values. However, maximal force eccentric actions of the biceps usually results in 50-60% strength loss postexercise (Clarkson & Hubal, 2002; Hyldahl & Hubal, 2014). Similarly, mice that underwent twenty eccentric contractions had decrements in maximal isometric force of 42.6 ± 4.2% (Warren et al., 1993).

It is important to note that although eccentric contractions result in strength loss and injury, skeletal muscle is quite resilient. Therefore, the muscle can fully recover in the subsequent days to weeks depending on the injury model (Clarkson & Hubal, 2002; Lapointe et al., 2002; Nosaka, 2008; Nosaka et al., 2005). Even one bout of eccentric contractions is enough to induce adaptations within the muscle so that it is less vulnerable

to subsequent bouts of eccentric exercise, a phenomenon called repeated bout effect (Clarkson & Hubal, 2002; Lapointe et al., 2002; Nosaka et al., 2005). In a human study where participants performed two bouts of 12 maximal eccentric contractions of the elbow flexors, biochemical markers such as plasma CK and myoglobin as well as range of motion, arm circumference, and muscle soreness were assessed pre, immediately post, and 4 days after exercise. Results showed significantly smaller responses in all measures after the second bout of eccentric contraction-induced injuries compared to the first bout, indicating that the muscle did not endure as much damage and was able to recover faster (Nosaka et al., 2005).

Our laboratory therefore utilized eccentric contraction-induced injuries to test skeletal muscle recovery because it is a physiological measure that can be tracked via assessment of strength (e.g., *in vivo* torque). We and others have also documented mechanisms of strength loss, recovery and adaptivity in mice (Hyldahl & Hubal, 2014; McHugh, 2003; Warren et al., 2001).

Chapter 3: Manuscript

Excessive Ethanol Intake in Mice Does Not Impair Recovery of Torque Following Repeated Bouts of Eccentric Contractions

Samantha E. Moser[1], Austin M. Brown[1], Jeffrey S. Otis[2] & Cory W. Baumann[3,4]

[1] Honors Tutorial College, Ohio University, Athens, OH, USA

[2] Department of Kinesiology and Health, Georgia State University, GA, USA

[3] Ohio Musculoskeletal and Neurological Institute (OMNI), Ohio University, Athens, OH, USA

[4] Department of Biomedical Sciences, Ohio University, Athens, OH, USA

ABSTRACT

Purpose: Between 40-60% of chronic alcoholics develop alcoholic myopathy, skeletal muscle atrophy and weakness due to excessive ethanol (EtOH) intake. To date, most studies have examined the mechanisms of chronic alcoholic myopathy under basal or unstressed conditions despite physical stress being a normal occurrence in a physiological setting. Therefore, there were two goals of this study: determine whether recovery of torque is impaired following repetitive bouts of physical stress in skeletal muscle developing alcoholic myopathy (Experiment #1) and, in skeletal muscle with alcoholic myopathy (Experiment #2).

Methods: Twenty C57BL/6 male and female mice were randomly assigned to receive either 20% EtOH in their drinking water or 100% water (n=10 per group, 5 per sex). Anterior crural muscles were subjected to repeated bouts of physical stress using in vivo eccentric contractions, with tetanic isometric torque being measured immediately pre- and post-injury. A total of ten bouts were completed with 14 days between bouts 1-5 (Experiment #1) and 6-10 (Experiment #2), and 12 weeks between bouts 5 and 6.

Results: Mice consuming EtOH had BACs up to 270 mg/dL. In Experiment #1, five bouts of 150 eccentric contractions did not reduce recovery of torque, regardless of sex or EtOH treatment ($p \geq 0.173$). Similarly, in Experiment #2, pre-injury torques did not differ from day 14 values regardless of sex or treatment ($p \geq 0.322$). However, there was a group effect in female mice for bouts 1 and 5 during Experiment #2, with female EtOH mice being weaker than controls ($p \leq 0.002$).

Conclusions: Chronic alcoholic myopathy in a mouse model does not affect the muscle's ability to recovery from tetanic isometric torque after repeated bouts of eccentric contractions. This suggests that EtOH may not be as detrimental to skeletal muscle recovery as once predicted.

BACKGROUND

Essentially every organ and tissue in the body is affected by excessive alcohol consumption (Mukherjee, 2013; Rachdaoui & Sarkar, 2013; Rocco et al., 2014; Tasnim et al., 2020). Some of earliest recognized disturbances are the histological, biochemical, and physiological alterations observed in skeletal muscle (Lang et al., 2005). In fact, 40-60% of chronic alcoholics develop alcoholic myopathy, a disorder defined as loss of muscle strength and size that results from excessive alcohol intake (Fernandez-Solà et al., 2007; Preedy et al., 2001; Urbano-Márquez & Fernández-Solà, 2004). Chronic alcoholic myopathy is often characterized by proximal muscle weakness, specifically in the pectoral and pelvic girdle regions, that occurs over years of sustained drinking (Preedy et al., 2001; Simon et al., 2017). Indeed, several reports have documented reductions in muscle mass (atrophy) up to 20% and strength loss up to 35% due to chronic alcohol abuse (Estruch et al., 1998; Lang et al., 2005; Martin et al., 1985; Urbano-Márquez & Fernández-Solà, 2004). Subsequently, this alcohol-induced loss of strength or weakness is also observed as deficits in function, as measured by walking capacity and physical activity levels (Vancampfort et al., 2019).

Weakness may be the result of reduced neuromuscular excitation, skeletal muscle quantity (i.e., size) and/or muscle quality (i.e., contraction efficiency) (Barnes et al., 2012; Freilich et al., 1996; Steiner & Lang, 2015a). Chronic alcoholic myopathy primarily leads to atrophy of type II muscle fibers as evidenced by loss of fiber cross sectional area (Kimball & Lang, 2018; Lang et al., 2001, 2005; Slavin et al., 1983; Steiner & Lang, 2015a). Reductions in fiber cross-sectional area or size are observed due to disturbances in protein synthesis and degradation as a result of alcohol-induced alterations in anabolic and catabolic signaling (Kimball & Lang, 2018; Simon et al., 2017; Steiner & Lang, 2015a). Moreover, weakness after excessive alcohol intake may also be a consequence of loss of neuromuscular excitation due to peripheral neuropathy (Agelink et al., 1998; Julian et al., 2019b) or reductions in muscle quality via dysfunction in calcium transport and cross-bridge formation (Edmonds et al., 1987; Simon et al., 2017; Urbano-Márquez & Fernández-Solà, 2004). It is generally accepted that alcohol-induced weakness is due to multiple mechanisms across several sites, however, the precise contribution of each has yet to be fully established.

Mechanisms causing weakness due to chronic alcoholic myopathy have almost exclusively been documented in resting or unstressed skeletal muscle. Although these studies provide pivotal information about the pathophysiology of alcoholic myopathy, how excessive alcohol intake influences stressed muscle has become an area of great interest. For instance, several studies have induced physical stress in human skeletal muscle via eccentric contractions to determine if alcohol intake impacts recovery (Barnes et al., 2010a, 2010b; Clarkson & Reichsman, 1990; Levitt et al., 2017; McLeay et al.,

2017; Parr et al., 2014). These studies initially speculated that because excessive alcohol consumption impacts several aspects related to protein synthesis, recovery would be blunted if alcohol was consumed concurrently with or after the eccentric contractions. However, despite alcohol's documented negative effects on protein homeostasis in unstressed muscle, recovery did not appear to be blunted when individuals consumed alcohol acutely after eccentric contractions (Barnes et al., 2010a, 2010b; Clarkson & Reichsman, 1990; Levitt et al., 2017; McLeay et al., 2017; Parr et al., 2014). Nevertheless, several caveats were noted in these published findings, such as acute alcohol intake after a single bout may not reflect true physiological conditions and that the detrimental effects of chronic alcohol may only be apparent after cumulative, repeated bouts of eccentric contractions (Laki´cevi´c, 2019; Simon et al., 2017).

Therefore, the purpose of this study is twofold. First, investigate whether recovery of torque is impaired following repetitive bouts of physical stress in C57BL/6 male and female mice that are developing chronic alcoholic myopathy. Second, determine if recovery of torque is impaired following repetitive bouts of physical stress once mice have developed chronic alcoholic myopathy. Based on previous research that have specifically reported protein synthesis was blunted after excessive alcohol intake (Kimball & Lang, 2018; Simon et al., 2017; Steiner & Lang, 2015a), we hypothesized that recovery of torque from repeated bouts of stress would be impaired in mice as they develop chronic alcoholic myopathy, which would be further compromised once they developed chronic alcoholic myopathy.

METHODS

Ethical Approval and Animal Models

Twenty C57BL/6 mice (n=10 per sex) were obtained from Jackson Laboratory (Bar Harbor, ME) and aged until approximately 24 – 28 weeks prior to study initiation. For experiments that involved anesthesia, mice were initially anesthetized in an induction chamber using isoflurane and then maintained by inhalation of 1-2% isoflurane mixed with oxygen at a flow rate of 200 $mL \cdot min^{-1}$. At the completion of this study, mice were euthanized by exsanguination followed by cervical dislocation in accordance with the Ohio University Animal Care and Use Committee.

Alcohol (Ethanol [EtOH]) Feeding

Ten mice (n=5 per sex) were randomly assigned to receive 20% EtOH in their drinking water (EtOH group) via a no choice design, meaning the EtOH group did not have access to a 100% water bottle (Dekeyser et al., 2013; Song et al., 2002). Mice were initially acclimated to EtOH by increasing the EtOH concentration in 5% increments from 0% to the target 20% (w/v) over the course of approximately two weeks. The 20% concentration was then used until study completion. The target of 20% EtOH in the drinking water was selected because it has been shown to replicate blood alcohol concentrations (BACs) reported in chronic alcoholics (Collins & Neafsey, 2016; Mayo Clinic Laboratories, n.d.; Song et al., 2002) and be an appropriate laboratory model of alcohol-induced organ damage (D'Souza El-Guindy et al., 2010). The remaining ten mice (n=5 per sex) served as control mice (control group) and were given 100% water.

Standard rodent chow was supplied *ad libitum* to both groups. Mouse body mass was measured using a digital scale (Mettler Toledo).

Experimental Design

Experiment 1. To determine whether consuming large amounts of alcohol, prior to developing myopathy, impairs recovery of torque after repetitive bouts of physical stress, EtOH and control mice began performing bouts of eccentric contractions *in vivo* at week 4 (i.e., 4 weeks after start of EtOH feeding). Specifically, mice performed five bouts of 150 maximal eccentric contractions, each bout separated by 2 weeks (Ingalls et al., 2004). *In vivo* tetanic isometric torque of the anterior crural muscles (the dorsiflexors) was measured immediately before and after each injury bout to assess the impact EtOH feeding had on skeletal muscle susceptibility to and recovery from eccentric contraction-induced damage. Baseline tetanic isometric torque was also recorded at week 0, prior to EtOH feeding, to ensure group torques were similar. The last assessment of tetanic isometric torque for experiment 1 was completed 2 weeks (week 14) following the fifth bout of eccentric contractions, after which, experiment 2 began (**Figure 1**).

Experiment 2. To develop chronic alcoholic myopathy, mice continued to consume 20% EtOH (or 100% water) in an unstressed state (i.e., no *in vivo* injuries or torque assessments) for 12 weeks (26 weeks from study initiation) (**Figure 1**). It was recently reported that ~15 weeks of EtOH feeding reduced *ex vivo* muscle force in male mice (Crowell et al., 2019). Therefore, by having mice drink EtOH for 26 weeks, we hypothesized both male and female mice would have consumed enough EtOH to develop myopathy. To determine whether excessive chronic alcohol consumption, while in a

myopathic state, impaired recovery of torque after repetitive bouts of physical stress, mice were re-injured. Briefly, the mice were brought back to the laboratory and five additional bouts of eccentric contractions with tetanic isometric torque measurements were completed, as outlined in *Experiment 1*. At study completion, body composition was obtained using nuclear magnetic resonance (NMR), and fat and lean masses were recorded. Within 5 days of the last assessment of tetanic isometric torque (week 36), mice were euthanized as described in Ethical Approval and Animal Models (see section above). Blood and muscles were also collected for assessment of BACs and wet masses of the anterior crural muscles, respectively.

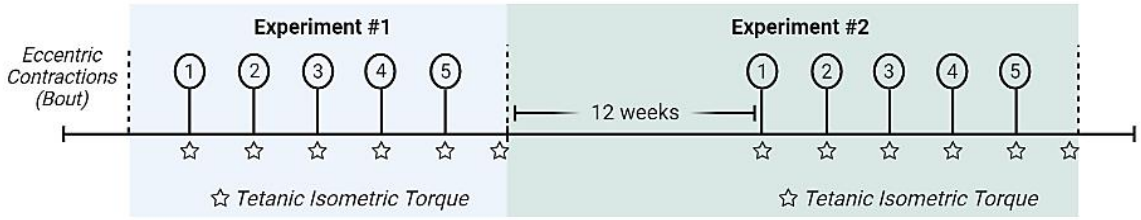

Figure 1. Schematic of the experimental design. For experiments #1 and #2, mice completed 5 bouts of 150 maximal eccentric contractions, totaling 10 bouts. Bouts were separated by 14 days within each experiment. Following experiment #1, mice drank for 12 weeks under basal conditions (i.e., no injuries). Tetanic isometric torque was measured immediately before and after each bout of eccentric contractions. Additionally, tetanic isometric torque was assessed 14 days after the 5th bout for each experiment.

Experimental Methodology

Tetanic Isometric Torque of the Dorsiflexors

In vivo isometric torque of the anterior crural muscles (tibialis anterior, extensor digitorum longus, and extensor hallucis muscles) were assessed as previously described (Baumann et al., 2014; Lowe et al., 1995; Sidky et al., 2021). The anesthetized mouse (see section Ethical Approval and Animal Models) was placed on a temperature-

controlled platform set at approximately 38-40°C to maintain core temperature. The left knee was clamped, and the left foot was secured to a footplate attached to the shaft of the servomotor system (Model 300C-LR; Aurora Scientific, Aurora, Ontario, Canada) with the foot perpendicular to the tibia (defined as 0°). Sterilized needle electrodes were precisely inserted through the skin for stimulation of the left common peroneal nerve and connected to the stimulator and stimulus isolation unit (701C; Aurora Scientific). Stimulation voltage and needle electrode placement were then optimized, and peak isometric torque was recorded (150 ms train of 0.2 ms pulses at 250 Hz).

Eccentric Contractions

Approximately 1–2 min following the tetanic isometric torque measurement (pre-injury), the anterior crural muscles were injured by performing electrically stimulated maximal eccentric contractions (Baumann et al., 2014; Lowe et al., 1995; Sidky et al., 2021). During each eccentric contraction, the foot was passively moved from 0° (positioned perpendicular to tibia) to 19.5° of dorsiflexion, where the anterior crural muscles performed a 100-ms isometric contraction followed by an additional ~20 ms of stimulation while the foot was moved to 19.5° of plantarflexion at ~2,000°·s^{-1} (at 250 Hz). Ten seconds separated each eccentric contraction. A 5-min rest was given after the last eccentric contraction before reassessing tetanic isometric torque (post-injury).

Nuclear Magnetic Resonance (NMR)

Mice were scanned using the Bruker Minispec TD-NMR Analyzer - LF Series. The TD-NMR system acquires and analyzes signals from the sample volume in order to determine fat, free body fluid, and lean tissue values. The system was calibrated before testing

using a standard provided by the manufacturer. The mice were placed into small plastic cylinder tubes with air holes and a tight-fitting plunger was inserted into the cylinder to immobilize the mice. This tube was then placed into the sample chamber of the analyzer until scan was complete, approximately one minute.

Blood Alcohol Concentration (BAC)

Blood was collected at study completion via a cardiac puncture within 1 hour after the dark cycle ended (5:00-7:00 a.m.). Approximately 200 uL- 250 uL of blood was collected into an Eppendorf tube via an 18-gauge sterile needle, allowed to aliquot and then centrifuged at 4°C for 5 min at 10,000 g. Serum was frozen at -80°C until BAC (mg/dl) was determined using an AM1 alcohol analyzer (Analox Instruments Ltd., Lunenburg, MA) (Blednov et al., 2017; Miller et al., 2019; Szumlinski et al., 2019).

Statistical Analyses

A one-way repeated-measures ANOVA was used to assess differences within strains across time, whereas a two-way ANOVA was used to probe for differences between strains across time or injury bout for torque. When significant interactions or main effects were calculated, differences were tested with Holm–Sidak post hoc tests. An α level of 0.05 was used for all analyses. Values are presented as mean ± SEM. All statistical testing was performed using SigmaPlot version 12.5 (Systat Software, San Jose, CA).

RESULTS

Blood Alcohol Concentration (BAC)

Male mice consuming EtOH had BACs ranging from 32.5 – 270.5 mg/dL, which was greater than the control mice with BACs up to 3.7 mg/dL ($p = 0.013$, **Figure 2A**). Similarly, female mice consuming EtOH had BACs ranging from 64.9 – 273.7 mg/dL, while control levels were much lower and did not exceed 8.1 mg/dL ($p = 0.011$, **Figure 2B**).

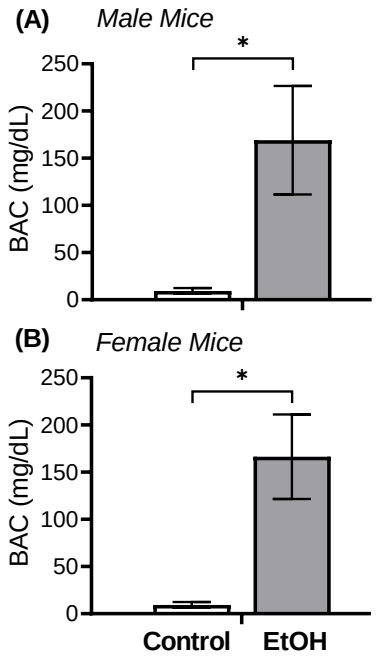

Figure 2. Blood alcohol concentrations (BAC) in male (A) and female (B) mice. Mice on EtOH consumed 20% EtOH for 36 weeks while control only drank water. *Significantly different than the control group ($p<0.05$). Bars are mean ± SEM. n = 4-5 per treatment per sex.

Body Mass

Baseline body mass did not differ between the male control and EtOH groups ($p = 0.904$). The male control group gained weight over the course of the study, weighing more at 4 weeks ($p = 0.025$) and 36 weeks ($p < 0.001$) when compared to baseline. To the

contrary, male mice consuming EtOH did not gain weight over 36 weeks of consumption ($p \geq 0.167$) and weighed less than control mice at weeks 4 ($p = 0.014$) and 36 ($p < 0.001$, **Figure 3A**). Among the female mice, baseline body mass did not differ between the control and EtOH groups ($p = 0.590$). The female control and EtOH groups both gained weight over the course of the study; when compared to baseline, weighing more at week 36 ($p \leq 0.004$) but not week 4 ($p \geq 0.316$). Despite both groups gaining weight, the female EtOH group weighed less than the control group at week 36 ($p = 0.037$, **Figure 3B**).

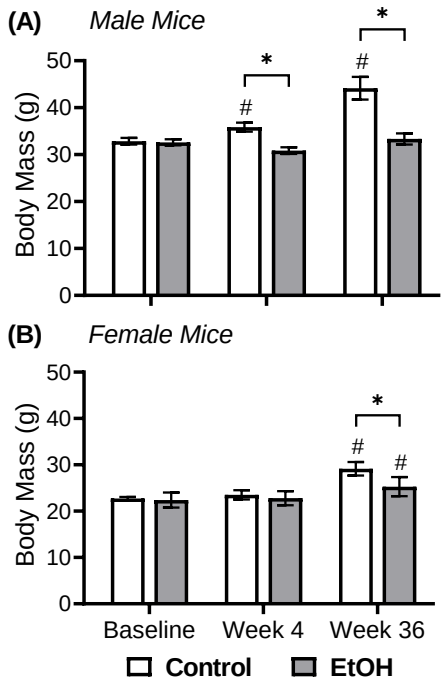

Figure 3. Body mass of male (A) and female (B) mice. Measurements were taken at baseline, 4 weeks of EtOH consumption, and 36 weeks of EtOH consumption. Mice on EtOH consumed 20% in drinking water. *Significantly different than the control group ($p<0.05$). #Significantly different than baseline ($p<0.05$). Bars are mean ± SEM. n = 4-5 per treatment per sex.

Body Composition

Due to the differences in body mass between mice that consumed 20% EtOH vs. 100% water at study completion, body composition was analyzed using NMR at 36 weeks. Male mice on EtOH had less fat mass ($p < 0.001$) and trended to have less lean mass ($p = 0.053$) than that of controls (**Figure 4A**). Conversely, the female EtOH and control groups had similar amounts of fat mass, but as with the males, female mice consuming EtOH trended to have less lean mass than those consuming water ($p = 0.073$, **Figure 4B**).

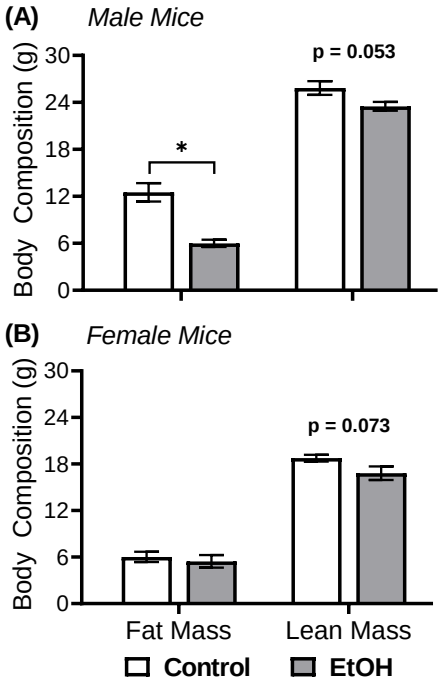

Figure 4. Body composition of male (A) and female (B) mice. Measurements of body composition (fat and lean mas) were analyzed using Nuclear Magnetic Resonance (NMR). Mice on EtOH consumed 20% EtOH for 36 weeks while control only drank water. *Significantly different than the control group ($p<0.05$). Bars are mean ± SEM. n = 4-5 per treatment per sex.

Anterior Crural Muscle Mass

Because chronic amounts of EtOH are known to cause atrophy, wet mass of the anterior crural muscles from the right and left legs were averaged and compared between groups (i.e., EtOH vs. controls). Muscle mass did not differ between the male EtOH and control groups (p = 0.686) (**Figure 5A**). However, muscle mass in the female EtOH group trended to be 15% lower compared to the female control group (p = 0.058, **Figure 5B**).

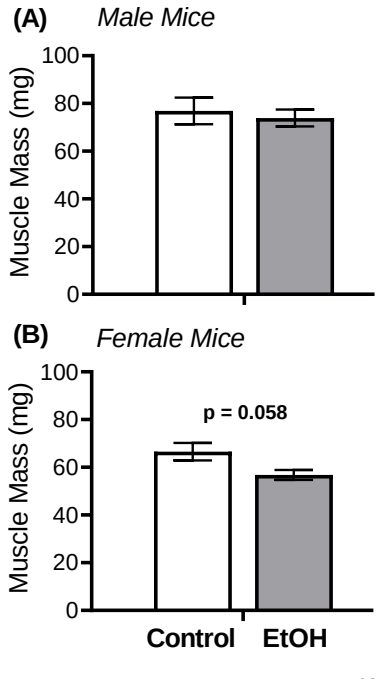

Figure 5. Wet muscle mass of anterior crural muscles in male (A) and female (B) mice. The anterior crural muscles consist of the tibialis anterior, extensor digitorum longus, and extensor hallucis muscles and were averaged from the right and left hindlimb. Mice on EtOH consumed 20% EtOH for 36 weeks while control only drank water. Bars are mean ± SEM. n = 4-5 per treatment per sex.

In Vivo Tetanic Isometric Torque of the Dorsiflexors

Experiment 1

Tetanic isometric torque did not differ between control and EtOH groups, regardless of sex, during the pre-injury assessment for bouts #1 ($p \geq 0.237$, **Figure 6A**) and #5 ($p \geq 0.202$, **Figure 6C**). The eccentric contractions reduced torque immediately after bouts #1 and #5 in male and female mice ($p < 0.001$, **Figure 6A-D**). In both sexes, by day 14, torque did not differ from that of pre-injury values ($p \geq 0.173$, **Figure 6A-D**). Although, during bout #1 in female mice, torque at day 14 trended to be higher than pre-injury torque ($p = 0.078$, **Figure 6B**). No group effects were observed between male control and EtOH consuming mice for bouts #1 ($p = 0.257$) or #5 ($p = 0.953$). Similarly, no group effects were observed between female control and EtOH treated mice for bouts #1 ($p = 0.436$) or #5 ($p = 0.701$).

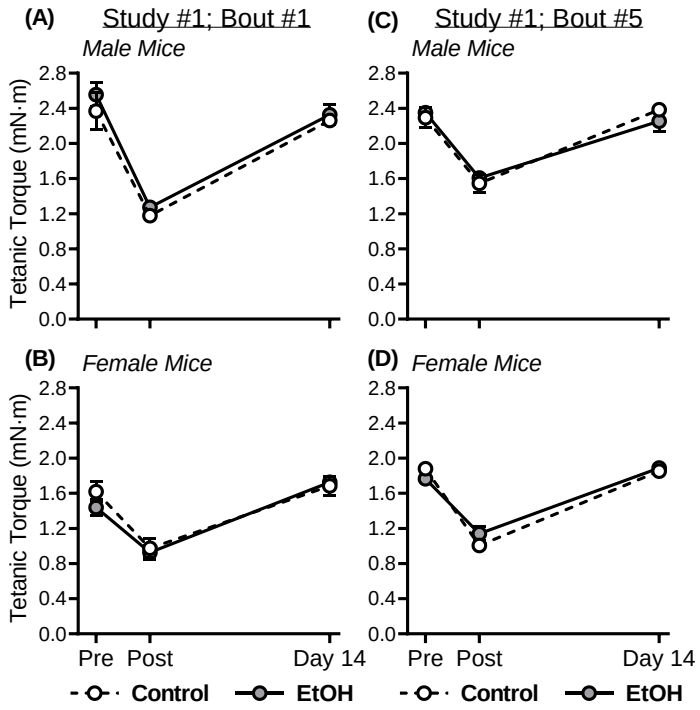

Figure 6. Tetanic isometric torque of the dorsiflexors during experiment #1 bout #1 (A & B) and bout #5 (C & D) in male and female mice. Isometric torque was measured using a tetanic contraction of 250 Hz immediately pre and post the eccentric contractions

and 14 days into recovery. Five total bouts were completed separated by 14 days. During experiment #1, mice consumed 20% EtOH (including EtOH ramping) for 4-14 weeks. Bars are mean ± SEM. n = 4-5 per treatment per sex.

Experiment 2

Following chronic EtOH consumption (i.e., study initiation), pre-injury torque at bouts #1 and #5 did not differ in male control and EtOH groups ($p \geq 0.154$, **Figure 7A & 7C**). In contrast, female mice consuming EtOH produced ~16% less pre-injury torque than controls during bouts #1 ($p = 0.043$, **Figure 7B**) and #5 ($p = 0.032$, **Figure 7D**). Regardless of sex, post-injury torque was lower than pre-injury torque for bouts #1 and #5 in control and EtOH consuming mice (**Figure 7A-D**). For bout #1, torque at day 14 was less than that of pre-injury torque ($p \leq 0.010$, **Figure 7A & 7B**), while day 14 and pre-injury torques did not differ during bout #5 ($p \geq 0.322$, **Figure 7C & 7D**). No group effects were observed between male control and EtOH consuming mice for bouts #1 ($p = 0.660$) or #5 ($p = 0.417$). To the contrary, group effects were observed in female mice during bouts #1 ($p = 0.001$) and #5 ($p = 0.002$); in that, mice consuming EtOH were weaker than controls.

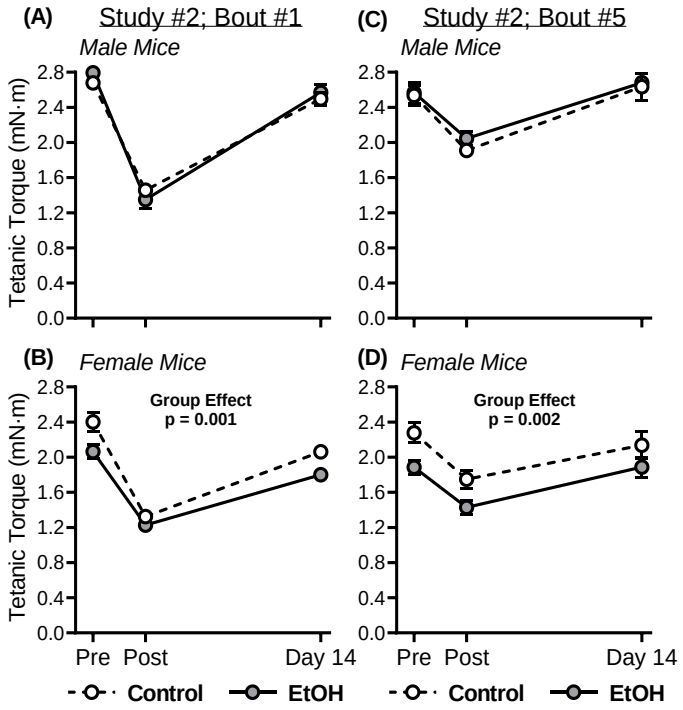

Figure 7. Tetanic isometric torque of the dorsiflexors during experiment #2 bout #1 (A & B) and bout #5 (C & D) in male and female mice. Isometric torque was measured using a tetanic contraction of 250 Hz immediately pre and post the eccentric contractions and 14 days into recovery. Five total bouts were completed separated by 14 days. During experiment #2, mice consumed 20% EtOH (including EtOH ramping) for 26-36 weeks. Bars are mean ± SEM. n = 4-5 per treatment per sex.

DISCUSSION

The overall goal of this study was to determine how chronic EtOH consumption effects recovery of muscle function after repetitive bouts of physical stress (via eccentric contractions) in male and female mice. Three primary findings were observed. First, chronic EtOH intake (prior to developing alcoholic myopathy) does not impair the muscle's ability to recover isometric torque after repeated bouts of eccentric contractions (Experiment #1). Second, and in parallel to the first finding, the muscle's ability to recover isometric torque after repeated bouts of eccentric contractions is not impaired by

chronic EtOH consumption while in a myopathic state (Experiment #2). Third, despite chronic EtOH intake having no measurable impact on recovery of isometric torque following repeated bouts of eccentric contractions, female mice appear to be more sensitive to developing alcohol-induced weakness and atrophy than male mice. These findings suggest that although chronic EtOH causes weakness and atrophy, it does not appear to hinder the muscle's ability to recover isometric torque deficits regardless of contraction bout (up to 10 bouts of 150 maximal eccentric contractions) or duration of EtOH consumption (20% EtOH for ~8 months).

In experiment #1, we hypothesized that recovery of isometric torque following repetitive bouts of eccentric contractions would be impaired in mice consuming chronic amounts of EtOH compared to that of healthy controls (not drinking EtOH). Our results however did not support this hypothesis. Specifically, isometric torque measured either immediately after the eccentric contractions or at day 14 did not differ between control and EtOH groups, which remained true regardless of bout number (**Figure 6**). Although this is the first study to assess recovery of torque after eccentric contractions in EtOH consuming mice, we are aware of ten published research articles that were conducted in humans. Despite two studies by Barnes et al. (Barnes et al., 2010a, 2010b), the other eight are aligned with the present finding and report acute alcohol consumption following resistance exercise does not influence the muscle's ability to functionally recovery (Clarkson & Reichsman, 1990; Haugvad et al., 2014; Levitt et al., 2017, 2020; Martyn & Munsat, 1980; McLeay et al., 2017; Murphy et al., 2013; Poulsen et al., 2007). For instance, recovery of muscular force or power following two identical bouts of heavy

eccentric squats separated by 7 days was similar between men who consumed 1.09 g EtOH·kg^{-1} fat-free body mass or a placebo (Levitt et al., 2020). Others have also shown muscle function is not impaired by EtOH, in addition to other makers of eccentric-contraction induced damage including soreness (Barnes et al., 2010a; Clarkson & Reichsman, 1990; Levitt et al., 2017, 2020; McLeay et al., 2017), creatine kinase (Barnes et al., 2010a, 2010b; Clarkson & Reichsman, 1990; Haugvad et al., 2014; Levitt et al., 2017; McLeay et al., 2017; Murphy et al., 2013; Poulsen et al., 2007), and inflammatory cytokines (Haugvad et al., 2014; Levitt et al., 2017). Together, these studies corroborate our findings that EtOH does not appear to blunt recovery of muscle function after eccentric contractions.

Our second hypothesis (i.e., for experiment #2) was that mice consuming excessive amounts of EtOH, while in a myopathic state, would further exacerbate the muscle's ability to recovery isometric torque deficits following eccentric contractions. As with our first hypothesis, our results also did not support our second hypothesis because recovery of isometric torque did not appear to be compromised in myopathic EtOH consuming mice compared to that of control mice (**Figure 7**). This finding remained true despite excessive EtOH consumption reducing isometric torque and muscle wet mass in female mice (**Figures 5B, 7B & 7D**) and whole-body lean mass in female and male mice (**Figure 4**), independent of the repeated bouts of eccentric contractions. It therefore appears excessive EtOH intake was indeed detrimental to skeletal muscle yet did not blunt the muscle's ability to recover from physical stress (i.e., eccentric contractions or exercise). Only one other study, to our knowledge, has investigated the effects of chronic

EtOH ingestion on skeletal muscle recovery. Dekeyser et al. (2013) found that muscle of male C57BL/6 mice on 20% EtOH for 18-20 weeks were not able to regenerate, as measured by fiber cross-sectional area (CSA), as quickly from a chemical-induced injury (1.2% barium chloride solution) when compared to control mice. Specifically, 14 days post-injury, CSA of the regenerating fibers was reduced ~30% in injured EtOH muscles while injured fibers in control muscles were not different than the CSA of uninjured fibers. It is possible that the contradictory finding between studies [present and Dekeyser et al. (Dekeyser et al., 2013)] may simply be due to type of injury used (contraction- vs. chemical-induced) and the extent of damage the muscle sustained. Nevertheless, our results indicate that continuous EtOH intake while in a myopathic state does not impair the muscle's ability to recover torque deficits after repeated bouts of physical stress.

The present findings that EtOH intake does not hinder the muscle's ability to recover isometric torque deficits following repeated bouts of eccentric contractions was unforeseen based on previous rodent data that reported 1) complete recovery of torque after eccentric contractions was dependent on protein synthesis (Baumann, Rogers, et al., 2016) and 2) EtOH consumption under basal condition reduces protein synthesis rates (Steiner & Lang, 2015b). Using the same *in vivo* protocol as described here, Baumann et al. (Baumann, Rogers, et al., 2016), demonstrated that a single bout of eccentric contractions was a potent activator of mechanistic target-of-rapamycin complex 1 (mTORC1) and ribosomal protein S6 kinase 1 (S6K1) signaling, both vital controllers of protein synthesis rates (Mahoney et al., 2009). By blocking phosphorylation of these proteins using rapamycin treatment, recovery of isometric torque was attenuated by

~20% 14 days into recovery when compared to saline treated group (Baumann et al., 2016). Interestingly, chronic EtOH consumption has also been shown to reduce mTORC1 and S6K1 phosphorylation in rodent skeletal muscle under basal conditions (i.e., EtOH intake with no additional physical stressors) (Lang et al., 2001; Steiner & Lang, 2015b). In fact, after 14 weeks of excessive EtOH intake, protein activity downstream of mTORC1 and S6K1 decreased 37% precent in EtOH fed rats compared to pair-fed control rats (Lang et al., 1999). These results paralleled reductions in translation initiation efficiency and rates of protein synthesis. Therefore, it appears that although EtOH can reduce mTORC1 and S6K1 phosphorylation, both of which contribute to recovery of torque following eccentric contractions, skeletal muscle can overcome these EtOH-induced effects when faced with repeated bouts of physical stress.

While rates of protein synthesis and associated signaling pathways were not assessed in the present study, skeletal muscle of mice consuming chronic EtOH responded to repeated bouts of eccentric contractions similar to that of control mice (no EtOH). Steiner et al. (2015) also reported that excessive EtOH intake does not impair the muscle's ability to respond to stress. Indeed, 14 days of EtOH consumption (up to 36% in Lieber-DeCarli liquid diet) did not prevent overload-induced hypertrophy nor did it disrupt protein synthesis or mTORC1-related signaling. However, it should also be noted that protein synthesis and mTORC1-related signaling were no different between sham-treated control and EtOH mice. Nonetheless, the present results, those from Steiner et al. (2015), and aforementioned human data (Clarkson & Reichsman, 1990; Haugvad et al., 2014; Levitt et al., 2017, 2020; Martyn & Munsat, 1980; McLeay et al., 2017; Murphy et

al., 2013; Poulsen et al., 2007) suggest EtOH intake does not impair the muscle's ability to recover from eccentric contractions or hypertrophy. Some caveats must be considered, as these studies were conducted in healthy muscle of young to middle-aged mice and humans. It is currently unknown how "unhealthy" or aged skeletal muscle will respond to stress while under conditions of excessive EtOH intake.

In closing, we previously theorized that chronic EtOH consumption would hinder recovery of tetanic isometric torque following repetitive bouts of physical stress compared to healthy controls. To the contrary, we discovered that EtOH intake in mice with and without chronic alcoholic myopathy does not affect the capacity of skeletal muscle to recover from repeated eccentric contraction-induced injuries. Despite the reported effects of EtOH intake reducing mTORC1 and S6K1 activity, it appears eccentric contractions provides enough stimulation to allow muscle to overcome these EtOH-induced effects. Altogether, this mouse study of excessive EtOH intake suggests that EtOH may not be as determinantal to skeletal muscle recovery from eccentric contractions or exercise as once predicted.

Chapter 4: Conclusion

The overall goal of this study was to determine how chronic EtOH consumption effects recovery of muscle function after repetitive bouts of physical stress (via eccentric contractions) in male and female mice. Three primary findings were observed. First, chronic EtOH intake (prior to developing alcoholic myopathy) does not impair the muscle's ability to recover isometric torque after repeated bouts of eccentric contractions (Experiment #1). Second, and in parallel to the first finding, the muscle's ability to recover isometric torque after repeated bouts of eccentric contractions is not impaired by chronic EtOH consumption while in a myopathic state (Experiment #2). Third, despite chronic EtOH intake having no measurable impact on recovery of isometric torque following repeated bouts of eccentric contractions, female mice appear to be more sensitive to developing alcohol-induced weakness and atrophy than male mice. These findings suggest that although chronic EtOH causes weakness and atrophy, it does not appear to hinder the muscle's ability to recover isometric torque deficits regardless of contraction bout (up to 10 bouts of 150 maximal eccentric contractions) or duration of EtOH consumption (20% EtOH for ~8 months). Also, despite the reported effects of EtOH intake reducing mTORC1 and S6K1 activity, it appears eccentric contractions provides enough stimulation to allow muscle to overcome these EtOH-induced effects.

Altogether, this mouse study of excessive EtOH intake suggests that EtOH may not be as determinantal to skeletal muscle recovery from eccentric contractions or exercise as once predicted. However, future studies need to be conducted to determine how "unhealthy" or aged skeletal muscle will respond to stress while under conditions of

excessive EtOH intake. Also, more research needs to be done to determine if muscle becomes intolerant or adapts after chronic EtOH consumption, which then allows muscle to recover normally following repetitive injuries.

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