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Pathophysiological mechanisms of alcoholic myopathy - Lessons from rodent models

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Abstract

Skeletal muscle dysfunction is highly prevalent and is one of the earliest pathological tissue changes among people with at-risk alcohol use. Clinical studies to elucidate pathophysiological mechanisms of alcohol-mediated muscle disease are hampered due to ethical considerations, and confounded by nutritional, lifestyle, and comorbid conditions. Rodent models have been developed to study the impact of at-risk alcohol consumption and alcohol-mediated end organ injury, including skeletal muscle dysfunction. This review discusses results from well-established rodent models of alcohol administration and highlights key pathophysiological mechanisms underlying alcoholic myopathy identified in rodent models. Salient pathways include impaired regenerative capacity, altered anabolic/catabolic balance, impaired mitochondrial bioenergetic function, and skeletal muscle morphological and contractile changes.

Key words: *Alcohol, Muscle, Protein synthesis, Stem cell regeneration, Mitochondria*

Alcoholic myopathy, or decreased skeletal muscle mass or function, occurs in 40 to 60 percent of people with at-risk alcohol use (Fernandez-Sola *et al.*, 2007; Preedy *et al.*, 2003) and is one of the earliest pathological tissue changes seen with alcohol use (Lang *et al.*, 2005; Martin *et al.*, 1985). Although alcohol-related muscle disease is nearly five times more common than liver cirrhosis (Estruch *et al.*, 1993), mechanistic data are lacking on its contribution to long-term health, aging, as well as its association with injury, metabolic dysregulation, or disuse atrophy. Ethical issues, complexity of alcohol use patterns, and confounding comorbidities make clinical studies difficult and have led to the development of preclinical models, especially rodents, to understand alcohol-mediated pathophysiological mechanisms underlying human disease. There are always concerns and questions regarding the use of animals in biomedical research and especially in substance use research. However, we obtain reliable data, there is great degree of experimental control and replicability, allowing for mechanistic investigation of human drug use and abuse.

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Why study alcohol and end organ injury?

In the 1930s, E. M. Jellinek, a physiologist, described alcoholism (now referred to as alcohol use disorder; AUD) as a disease. His seminal paper provided clarity and removed stigma associated with AUD, and underscored that it was not due to weakness of will or temperament, moral failing, symptom of mental illness, but that it is a physical illness due to a pharmacologically addictive substance that can affect people from all sections of society (Jellinek and McFarland, 1940). However, at-risk alcohol use has adverse effects on many organ systems, including the liver, lungs, central and peripheral nervous system, immune system, and skeletal muscle. With such wide-ranging effects, it is critical to identify mechanisms leading to alcohol-mediated pathology to better manage and treat alcohol-related disease.

Alcohol administration in rodents

Several reliable models of alcohol administration in rodents have been developed and there are benefits and drawbacks of each. Here, we briefly describe some of the most common models. Some of the models developed allow for excessive drinking and alcohol-seeking behavior demonstrating the rewarding effects of alcohol and their contribution to escalating alcohol use; others are more commonly used to study end-organ effects of alcohol.

1) Two-bottle choice method: Animals are allowed an unrestricted choice between alcohol and water for a predetermined amount of time up to 24 hours per day. Animals consume alcohol voluntarily. Work with these models show heterogeneous populations of animals, like humans, some of which consume larger volumes of alcohol than others, and can help elucidate how certain factors, such as previous patterns of alcohol administration (Younis *et al.*, 2019; Nentwig *et al.*, 2019), influence alcohol preference. These studies have also shown that genetic manipulation by inbreeding or selective breeding can produce animals displaying different alcohol preferences (Grahame *et al.*, 1999).

2) Operant models: Animals must perform a certain task for alcohol intake. For example, animals are trained to press a lever for alcohol to be delivered through the oral route, or in some cases directly into the brain. These models allow for the assessment of motivation to consume alcohol, such as after traumatic brain injury (Stielper *et al.*, 2019) and to elucidate possible factors underlying motivation for alcohol, such as altered glucocorticoid receptor signaling (Pahng *et al*, 2019).

3) Intragastric administration: Animals are administered alcohol via infusion directly into the stomach through surgically implanted intragastric tubes. This method is used to avoid the influence of taste and has been used to study alcoholic liver disease; e.g., Tsukamoto-French model (Tsukamoto *et al*., 1990).

4) Alcohol-containing liquid diet: After transition from solid to liquid diet, alcohol containing liquid diet is the sole source of nutrition in this model. The alcohol content of the liquid diet is increased over the course of several days at the beginning of the experimental period and subsequently maintained at the target level. The most common diet used is the Lieber-DeCarli diet (Lieber and DeCarli,1989) and the control diet is isocalorically matched to the alcohol calories. This strategy is widely used to study end-organ injury, including chronic alcohol-induced skeletal muscle pathology (Levitt *et al*., 2020c; Crowel *et al*., 2016; Lang, 2018).

5) Oral gavage: Animals are orally administered alcohol via gavage. The oral gavage method is commonly used to study acute effects of a single dose of alcohol on muscle pathology or to administer ethanol at binge doses in combination with the Lieber-DeCarli diet; e.g. “binge-on-chronic” or “NIAAA model” (Samuelson *et al.*, 2019; Bertola *et al*., 2013).

6) Systemic injection: This is generally accomplished using an intraperitoneal injection and is commonly used to assess acute effects of alcohol on skeletal muscle (Steiner and Lang, 2015; Steiner *et al*., 2016).

7) Inhalation of alcohol vapor: Animals are placed in chambers and exposed to alcohol vapor for specific periods of time, often referred to as "intermittent ethanol exposure". This is a common method to make rodents dependent on alcohol (Gilpin *et al*., 2008; Mouton *et al.,* 2016).

8) Genetic models: Mice or rats are bred to create lines of animals that are sensitive or insensitive to alcohol and rat lines have been generated to select for alcohol preference. For example, selectively-bred Sardinian alcohol-preferring (sP) and non-preferring (sNP) rats reliably exhibit strong alcohol preference and motivation (sP) or strong avoidance (sNP) of alcohol (Colombo *et al.*, 2006). Other genetic models of alcohol-related behaviors include inbred strains, recombinant inbred strains, and transgenic/ knock-out mice (Mayfield *et al*., 2016).

The metabolic rate of alcohol varies among species, and doses are optimized to generate blood and brain alcohol levels that produce pharmacological effects. For example, that rate of alcohol clearance is much greater in rats compared to humans and further varies among strains (Erickson, 1984; Holford, 1987). Because of the differences in rates of alcohol metabolism, while the method of alcohol administration may not exactly mimic human consumption, it serves to study the effects of alcohol on end organ injury.

Rodent models commonly used to study alcoholic myopathy are chronic Lieber-DeCarli diet feeding, oral gavage, or systemic injections. In addition, *in vitro* studies using established cell lines (e.g. C2C12 mouse-derived myoblasts or primary myoblasts) are used to mechanistically understand how alcohol affects myoblast proliferation, differentiation, metabolism, and function. Here, we will discuss key findings of alcohol-mediated effects on skeletal muscle reported from studies utilizing rodent models.

Muscle stem cell regenerative capacity

Rodent studies have demonstrated that chronic alcohol feeding increases skeletal muscle gene expression of TNFα and interleukin (IL)-6, indicating chronic inflammation (Steiner and Lang, 2015a) . Our published work showed decreased differentiation potential of skeletal muscle stem cells isolated from chronic binge alcohol-administered macaques. This was associated with reduced expression of myogenic genes and impaired myotube formation, indicative of impaired muscle fiber formation (Simon *et al.*, 2014; Simon *et al.*, 2017). The marked dysregulation of myoblast myogenic and inflammatory gene expression and myotube formation with chronic alcohol administration reflects impaired muscle regenerative capacity and is likely to contribute to decreased muscle mass, especially in response to an injury or disuse atrophy. Alcohol and aging are risk factors for traumatic injury and subsequent immobilization-induced muscle atrophy (Lukaszyk *et al*., 2016). In fact, about 10-30% of hospitalized older patients have diagnosed AUD (O'Connell *et al*., 2003). Skeletal muscle recovery may be complicated by alcohol use and altered hormonal status among older individuals (Lukaszyk *et al*., 2016). To examine this question, we utilized Lieber-DeCarli feeding in rats for 10 weeks culminating in 1 week of unilateral hind limb immobilization followed by 3 or 14 days of remobilization (Levitt *et al*., 2020c). Our data indicated that alcohol dysregulates the expression of markers of muscle regeneration following unilateral hind limb immobilization. Although alcohol did not significantly exacerbate the immobilization-mediated decrease in muscle weight, it is possible that underlying differences in regeneration may have occurred. Therefore, when immobilization is indicated, caution is warranted about alcohol use during the immobilization and post-immobilization recovery periods.

Mitochondrial homeostasis and bioenergetics

Alcohol impairs mitochondrial function in tissues including skeletal and cardiac muscle (Guo and Ren, 2010; Kumar *et al*., 2019; Duplanty *et al.*, 2017; Duplanty *et al.*, 2018), and mitochondrial homeostasis is critical in the maintenance of functional metabolic muscle mass (Romanello and Sandri, 2015). In chronic alcohol fed rats, decreased mitochondrial fusion and connectivity, calcium dysregulation,

and impaired mitochondrial bioenergetics and excitation-contraction coupling have been reported (Eisner *et al.*, 2014; Trounce *et al.*, 1990). Studies also show that chronic alcohol decreases muscle cross sectional area with a decrease in total and free glutathione content, decreased glutathione reductase activity and decreased expression of oxidative stress genes (Otis and Guidot, 2010; Otis *et al*., 2007). Together, a number of studies have shown alcohol-mediated oxidative damage in muscle as evidenced by an increase in protein carbonyl, cholesterol hydroperoxide, and malondialdehyde content (Otis *et al*., 2007; Koo-Ng *et al*., 2000).

Alcohol-mediated impaired bioenergetics are not limited to mitochondria. Chronic alcohol administration results in skeletal muscle glycolytic impairments in rodents. For example, activity of the glycolytic enzymes aldolase, pyruvate kinase, and lactate dehydrogenase were significantly decreased in vastus lateralis after chronic ethanol administration (Trounce *et al.*, 1990), mirroring findings in humans (Trounce *et al.*, 1987). In contrast, opposite effects have been observed in white gastrocnemius muscle in male rats, suggesting a potential fiber type specific effect of ethanol on glycolytic enzyme activities (Vila *et al.*, 2001). Using transcriptomics, decreased expression of genes in the glycolytic pathway were also reported after C2C12 myoblasts were treated with 100 mM ethanol, a supraphysiological dose, for 6 or 24 hours (Kumar *et al.*, 2019). Although these previous findings were at the transcriptomic or enzyme activity levels, we recently confirmed that 3 days of treatment with 50 mM ethanol impairs glycolytic function in live myoblasts *in vitro* (Levitt *et al*., 2020).

Skeletal muscle protein synthesis

Clinical studies provide evidence that a major mechanism of alcohol-induced myopathy is altered balance of protein synthesis and breakdown (Steiner and Lang, 2015; Reilly *et al*., 2000; Steiner *et al*., 2015). To identify alcohol-induced changes in specific proteins in the protein synthesis and degradation pathway, liquid alcohol diets have been used. The mammalian target of rapamycin (mTOR) pathway plays a central role in protein synthesis and is important for controlling skeletal muscle mass. mTOR activation activates two signaling pathways; S6 kinase 1 (S6K1) phosphorylation leading to activation of the ribosomal protein S6 and phosphorylation of the eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP1) releasing its inhibition of the translation initiation factor eIF-4E. Chronic alcohol consumption decreases phosphorylation of mTOR itself (Lang *et al*., 2003a), ribosomal protein S6 (rpS6), and phosphorylation of 4E-BP1 in skeletal muscle (Korzick *et al*., 2013). This is associated with a greater proportion of eIF4E in the inactive eIF4E-4EBP1 complex versus the active eIF4E-eIF4G and thus illustrates one possible mechanism by which alcohol decreases protein synthesis. Insulin and insulin-like growth factor (IGF)-1 are anabolic hormones that activate mTOR signaling. Acute ethanol markedly attenuated the insulin and IGF-1 mediated increases in S6K1 and rpS6 phosphorylation but did not simultaneously impair signaling through 4E-BP1 (Kumar *et al*., 2002). It should be noted that phosphorylation was measured at only one time point in that study, so it is possible that an acute effect was present at a different time point. However, ethanol attenuated the leucine-stimulated phosphorylation of 4E-BP1 and eIF4G in addition to mTOR, S6K1, and rpS6 (Lang *et al*., 2003). In addition, rodent models show that alcohol significantly decreases IGF-1 levels in both plasma and muscle, and this decrease is correlated with decreased muscle protein synthesis (Lang *et al*., 1998). Overall, there is strong evidence that alcohol attenuates basal mTOR signaling after chronic administration, acutely attenuates mTOR signaling in response to anabolic stimulation, and may decrease systemic levels of the anabolic hormone, IGF-1.

Skeletal muscle protein degradation

Protein degradation in skeletal muscle is directed primarily by two pathways, the ubiquitin proteasome pathway (UPP) and the autophagic-lysosomal system (Steiner and Lang, 2015; Steiner *et al*., 2015; White *et al.*, 2014). The two ubiquitin ligases or atrogenes,

atrogin-1 and MuRF1 are specific to the muscle and are increased in several catabolic conditions. Both atrogin-1 and MURF1 are increased in acute and chronic alcohol administered rodents (Korzick *et al*., 2013; Vary *et al*., 2008; Vargas and Lang, 2008), possibly reflecting increased protein degradation activity. However, chronic alcohol did not further increase atrogin-1 and MURF1 expression or proteasomal activity in aged rats (Korzick *et al*., 2013). The autophagic-lysosomal system is activated due to cellular stress and mediates degradation of misfolded proteins. Whether alcohol-mediated muscle protein degradation is mediated by autophagy is still not clear. In alcohol-fed mice, there is increased expression of autophagy markers and in vitro treatment of C2C12 myotubes with 100 mM alcohol (a supraphysiological level of alcohol) increased autophagic gene expression within 6h (Thapaliya *et al.*, 2014). However, others have not observed this change in chronic alcohol fed mice (Steiner and Lang, 2015). Similarly, our studies in primary myoblasts derived from chronic alcohol administered macaques do not show changes in autophagic markers compared to myoblasts derived from vehicle administered macaques (Simon *et al.*, 2014). Thus, studies in rodent models suggest that alcohol-mediated decreased protein synthesis and increased protein degradation can potentially contribute to alcoholic myopathy.

Skeletal muscle mass and morphology

Decreased skeletal muscle mass is characteristic of chronic at-risk alcohol use, and structural abnormalities have been observed in muscle biopsy samples from such subjects (Fernandez-Sola *et al.*, 2007). However, studying these phenomena in humans is complicated by differences in nutrition, current and past physical activity, hormonal status, etc. Therefore, rodent models have been employed to examine alcohol-mediated muscle wasting in controlled settings. For example, alcohol-fed rats showed decreased lean body mass compared to pair-fed controls (Korzick *et al*., 2013). A further benefit of studying such effects in rodents is that many individual rodent muscles have a more homogenous muscle fiber type population (e.g. gastrocnemius is composed of ~95% type II fibers) than in humans (e.g., gastrocnemius is composed of ~50% type II fibers). The homogenous fiber type composition allows for easier assessment of the effects of alcohol on different fiber types. Chronic alcohol (15% v/v in drinking water) decreased muscle fiber cross-sectional area across all fiber types in the plantaris, which is composed of ~95% type II fibers in rats (Vila *et al.*, 2001). In the same study, muscle fiber cross-sectional area was unaffected in the type I-dominant soleus and red gastrocnemius muscles. We observed decreased quadriceps mass (white and red portions together) in the non-immobilized hind limb of alcohol-fed rats compared to their pair-fed counterparts (Levitt *et al.*, 2020c). Without immobilization, type II-dominant plantaris mass and total protein were decreased in rats fed ethanol for 12 weeks (Clary *et al*., 2011). Ten weeks of alcohol feeding decreased total protein and RNA in type II-rich gastrocnemius and plantaris but not type I-dominant soleus, with concomitant decreases in expression of the structural proteins titin and nebulin in white gastrocnemius (Hunter *et al.*, 2002), which could further contribute to alcoholic myopathy, particularly in type II muscle. Overall, results from rodent studies confirm that alcohol-induced muscle wasting more severely affects type II-dominant muscle and provide a viable model to elucidate underlying mechanisms.

Skeletal muscle function

Although skeletal muscle mass and function are generally related, a host of factors (bioenergetic, neurological *etc*.) confound this non-linear relationship. Even without sufficient time to decrease muscle mass, acute binge alcohol administration in humans after exercise-induced muscle damage can exacerbate the exercise-induced decrease in force production in men (Barnes *et al*., 2010), although this effect does not appear to translate to women (Levitt *et al.,* 2017), and is not apparent when participants are accustomed to the exercise (Levitt *et al.*, 2020b). For ethical purposes, the doses of alcohol used in human studies of acute binge drinking produce blood alcohol concentrations of only ~0.08-0.12 g/dL. This may be why an additional stimulus (e.g. exercise-induced muscle damage) is needed to uncover effects.

However, human consumption of ethanol can result in blood alcohol concentrations that far exceed those in these studies and may produce acute and cumulative effects on muscle function as muscle weakness is observed in approximately half of patients with AUD (Preedy *et al.*, 2001). Rodent models have been used to examine acute and chronic effects of alcohol on muscle contractile function. In one such study, extensor digitorum longus (EDL) contractile properties post-fatigue were impaired after chronic ethanol feeding but not 2 hours after acute ethanol intoxication @ 3 g/kg (Crowell *et al*., 2019). Previous work examining the acute effects of alcohol on muscle contractile function in rat EDL *in vitro* observed decreased twitch tension at higher (0.4 and 2.5 g% ethanol) but not lower (0.1 and 0.2 g% ethanol) ethanol doses (Taylor *et al.*, 1992). These findings suggest dose dependent effects of alcohol on muscle function. Overall, more work is needed to elucidate effects of alcohol on skeletal muscle contractile function, and rodent models are well-suited for such studies.

Conclusion

Alcoholic myopathy is more prevalent than well-known organ injury like alcoholic hepatitis. Clinical studies in human subjects with AUD are challenging for many reasons including the presence of confounding lifestyle factors, comorbid conditions, and varying patterns and severity of alcohol consumption. Rodent models of alcohol administration have allowed for controlled studies to standardize administration across subjects and minimize confounding factors. The specific experimental question and study design parameters must be considered when selecting an alcohol administration protocol. The most used experimental paradigms to study alcohol-mediated muscle pathology are alcohol-containing liquid diets (chronic model) and intraperitoneal injections or oral gavage (acute models). Using these rodent models, many factors underlying alcoholic myopathy, including impaired regenerative capacity, altered anabolic/catabolic balance, impaired bioenergetic function, and skeletal muscle morphological and contractile changes have been described. Thus, mechanistic studies in rodent models have helped identify key alcohol-mediated pathways that are dysregulated and, in the future, may provide therapeutic targets to ameliorate alcoholic myopathy.

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