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Metabolic Brain Disease

THE BILE DUCT LIGATED RAT: A RELEVANT MODEL TO STUDY MUSCLE MASS LOSS IN CIRRHOSIS

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Abstract:	Muscle mass loss and hepatic encephalopathy (complex neuropsychiatric disorder) are serious complications of chronic liver disease (cirrhosis) which impact negatively on clinical outcome and quality of life and increase mortality. Liver disease leads to hyperammonemia and ammonia toxicity is believed to play a major role in the pathogenesis of hepatic encephalopathy. However, the effects of ammonia are not brain-specific and therefore may also affect other organs and tissues including muscle. The precise pathophysiological mechanisms underlying muscle wasting in chronic liver disease remains to be elucidated. In the present study, we characterized body composition as well as muscle protein synthesis in cirrhotic rats with hepatic encephalopathy using the 6-week bile duct ligation (BDL) model which recapitulates the main features of cirrhosis. Compared to sham-operated control animals, BDL rats display significant decreased gain in body weight, altered body composition, decreased gastrocnemius muscle mass and circumference as well as altered muscle morphology. Muscle protein synthesis was also significantly reduced in BDL rats compared to control animals. These findings demonstrate that the 6-week BDL experimental rat is a relevant model to study liver disease-induced muscle mass loss.

THE BILE DUCT LIGATED RAT: A RELEVANT MODEL TO STUDY MUSCLE MASS LOSS IN CIRRHOSIS

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Running Head : Experimental cirrhosis and muscle mass loss

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Abstract

Muscle mass loss and hepatic encephalopathy (complex neuropsychiatric disorder) are serious complications of chronic liver disease (cirrhosis) which impact negatively on clinical outcome and quality of life and increase mortality. Liver disease leads to hyperammonemia and ammonia toxicity is believed to play a major role in the pathogenesis of hepatic encephalopathy. However, the effects of ammonia are not brain-specific and therefore may also affect other organs and tissues including muscle. The precise pathophysiological mechanisms underlying muscle wasting in chronic liver disease remains to be elucidated. In the present study, we characterized body composition as well as muscle protein synthesis in cirrhotic rats with hepatic encephalopathy using the 6-week bile duct ligation (BDL) model which recapitulates the main features of cirrhosis. Compared to sham-operated control animals, BDL rats display significant decreased gain in body weight, altered body composition, decreased gastrocnemius muscle mass and circumference as well as altered muscle morphology. Muscle protein synthesis was also significantly reduced in BDL rats compared to control animals. These findings demonstrate that the 6-week BDL experimental rat is a relevant model to study liver disease-induced muscle mass loss.

Keywords: experimental cirrhosis, muscle mass loss, protein synthesis, ammonia, hepatic encephalopathy

Introduction

Loss of muscle mass is the most common and clinically significant complication of chronic liver disease (cirrhosis). It is a major contributor to adverse clinical outcomes (both pre and post liver transplantation), including morbidity and mortality (Merli et al. 2002). In addition, muscle wasting leads to poor quality of life and increased susceptibility to infection (Metter et al. 2002; Pichard et al. 2004; Cosquéric et al. 2006; Millwala et al. 2007; O'Brien and Williams 2008; Montano-Loza et al. 2012; Tandon et al. 2012). It has been suggested that liver disease-induced muscle mass loss results from varying contributions including reduced protein synthesis, increased protein catabolism, and an impaired proliferation and differentiation of skeletal muscle progenitor satellite cells (Dasarathy et al. 2002). However, the precise pathophysiological mechanisms underlying the loss of muscle mass in cirrhosis remains to be elucidated.

Hepatic encephalopathy, another complication of liver disease which greatly impacts on patients' quality of life, is characterized by a constellation of symptoms, including cognitive, psychiatric and motor disturbances (Cash et al. 2010). Although the pathogenesis of hepatic encephalopathy is multifactorial including oxidative stress (Görg et al. 2010; Bosoi et al. 2012), inflammation (Shawcross and Jalan 2005; Shawcross et al. 2011; Montoliu et al. 2009), lactate (Bosoi et al. 2014) and gut microbiota (Bajaj et al. 2008), ammonia is a key player as blood-derived ammonia rises to toxic levels in the brain (Butterworth 2002; Bosoi and Rose 2009). The toxicity of ammonia is a result of its direct effect on pH, membrane potential and metabolism which independently or collectively cause cell dysfunction (Lai and Cooper 1991; Bosoi and Rose 2009). As the effects of ammonia are not brain-specific (Lai and Cooper 1991; Norenberg 2003), it has been shown that elevated concentrations of ammonia can also affect other organs and tissues (Kubota et al. 2004; Jia et al. 2014; Rose 2014).

The 6-week bile duct ligation (BDL) rat, a surgical model involving obstruction of the common bile duct, is a well-established experimental model which recapitulates the main features of cirrhosis including, liver failure, hyperammonemia, secondary biliary cirrhosis, ascites, jaundice, brain edema and hepatic encephalopathy (Butterworth et al. 2009; Bosoi et al. 2011; Bosoi et al. 2012). It has been previously suggested that impaired skeletal muscle protein synthesis is the primary reason for loss of muscle mass in rats with portacaval-systemic shunting (Dasarathy et al. 2011). However, muscle mass in the BDL rat has not been extensively evaluated. The present study aims to characterize body composition as well as muscle protein synthesis in the BDL rats with hepatic encephalopathy.

Material and Methods

Animal model

Cirrhosis was induced in male Sprague-Dawley rats (200-225 g) (Charles River, St-Constant, QC) by BDL. The latter is created by obstruction of the common bile duct which reproduces the main features of human cirrhosis. Briefly and as previously described, rats were anaesthetized with isoflurane, and the common bile duct ligated and resected under a dissecting microscope. Sham-operated control rats, matched for weight, were similarly anaesthetized; a laparotomy was performed and the bile duct was isolated (Rose et al. 1999; Bosoi et al. 2011; Bosoi

et al. 2012). Rats were maintained under controlled conditions (22°C, 12 h: 12 h dark-light cycle) with free access to their food and water. Two experimental groups were tested; 1) BDL (n=5) and 2) Sham-operated control rats (SHAM) (n=6). Experiments were conducted following the guidelines of the Canadian Council on Animal Care and were approved by the Animal Protection Committee of the Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM).

Body weight and food intake

Body weight was measured every day of the 6 week experimental protocol using an electronic scale. Food consumption was also monitored every day by the weight of the food.

Body mass composition

Body composition in terms of lean and fat mass was assessed in conscious rats (full body) by *in vivo* scanning and magnetic resonance imaging (EchoMRI 100® Body Composition Analyzer) 6 weeks after the surgeries, according to the manufacturer's protocol. The instrument for composition analysis creates contrast between soft tissues by taking advantage of the differences in relaxation times of the hydrogen proton spins in different environments. Radio pulses cause protons to spin and emit radio signals which are then received and analysed. The amplitude, duration, and spatial distribution of these signals are related to properties of the material scanned. The high contrast between fat, muscle tissue, and free water is further enhanced by application of define composed radio pulses sequences (Nixon et al. 2010).

Gastrocnemius muscle mass, circumference and morphology

At the end of the 6 weeks experimental protocol, rats were sacrificed and the gastrocnemius muscle was dissected and weighed. Muscle (gastrocnemius) mass and circumference were measured using an electronic scale and a scaled thread, respectively. Muscle samples were then fixed in 4% paraformaldehyde buffered with phosphate-buffered saline, decalcified with 10% formic acid, and embedded in paraffin. Longitudinal histology sections were cut with a microtome, and stained with hematoxylin-eosin. Tissue sections were then visualized using microscope.

Protein synthesis

Protein synthesis was quantified as the fractional and absolute protein synthesis rates in the dissected and homogenized muscle and other organs including the brain (frontal cortex), heart, intestine, kidney and liver, using the modified phenylalanine tracer pulse method (Zhang et al. 2002; Dasarathy et al. 2011). In brief, rats were given a small dose (0.5 mg/100 gram body weight) of L-[ring-$^{2}H_{5}$]phenylalanine ip at t=0min, L-[1-^{13}C]Phenylalanine ip at t=30 min and L-[15N]Phenylalanine ip at t=60 min. At t=65min, the rats were killed and blood and tissue collected. The calculation of the fractional protein synthesis was done by using the enrichment in tissue protein samples of L-[ring-$^{2}H_{5}$]phenylalanine, divided by the average enrichment in plasma (from area under the curve calculation of the curve, constructed from the three different phenylalanine isotopes). The enrichment of phenylalanine in plasma and tissue hydrolysates was measured by LC-MS/MS (Engelen et al. 2013; Luiking et al. 2015).

Ammonia

Ammonia levels were measured in arterial plasma using a commercial kit (Sigma, MO, USA). Ammonia levels were assessed based on the reaction with α-ketoglutarate and reduced nicotinamide adenine dinucleotide phosphate in the presence of L-glutamate dehydrogenase. Oxidation rate of reduced nicotinamide adenine dinucleotide phosphate was recorded by the absorbance decrease at 340 nm. Ammonia concentration was calculated according to manufacturer's protocol.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Significance of difference was tested with unpaired t test or ANOVA followed by Bonferroni post-test using GraphPad Prism4 (La Jolla, CA, USA). Probability values of $p<0.05$ were considered statistically significant.

Results

Bile duct ligation-induced cirrhosis leads to decreased gain in body weight

Body weight was significantly lower in BDL animals compared to SHAM from week 2 to the end of the experimental protocol (week 6) (Figure 1). At 6 weeks, BDL rats weighed 422.4 ± 6.15 g compared to 509.7 ± 15.6 g for sham-operated animals ($p<0.001$). Over the 6 weeks, daily food intake was similar (non-significant) between the two groups (area under the curve 1018 g ± 34 g and 1078± 21 g in BDL and SHAM animals, respectively). However, there was a significant difference at week 1 between the two groups (13.4 ± 2.3 g vs 17.6 ± 0.5 g in BDL and SHAM animals, respectively; $p< 0.01$) (Figure 2)

Bile duct ligation-induced cirrhosis provokes altered body composition

Six weeks after surgery, BDL rats displayed a significant decrease in lean (311.2 ± 12.1 g vs 376.5 ± 11.9 g; $t(9)=3.82$, $p<0.001$) and fat (33.9 ± 8.1 g vs 89.3 ± 9.7 g; $t(9)=10.15$, $p<0.001$) mass compared with SHAM animals (Figure 3A and 3B), as measured by magnetic resonance.

Bile duct ligation-induced cirrhosis leads to decreased gastrocnemius muscle mass and circumference as well as altered muscle morphology

Compared with control rats, BDL animals had decreased gastrocnemius mass (1.92 ± 0.05 g vs 2.85 ± 0.10 g; $t(9)=7.60$, $p<0.001$) and smaller gastrocnemius circumference (4.7 ± 0.2 cm vs 5.9 ± 0.1 cm; $t(9)=5.80$, $p<0.001$) (Figure 4A and 4B). A strong correlation was observed between lean and muscle mass in both BDL and SHAM rats ($r=0.8889$; $p=0.0003$) (Figure 4C). Gastrocnemius muscle morphology was analyzed by histological analysis of muscular tissues in SHAM and BDL animals at the end of the 6 week experimental protocol. Hematoxylin-eosin staining revealed disorganized fibres in BDL muscles (Figure 4D).

Bile duct ligation-induced cirrhosis reduces muscle protein synthesis

Muscle protein synthesis, 6 weeks after surgery, was significantly reduced in BDL rats compared to SHAM animals (0.32 ± 0.02 %/h and 0.17 ± 0.07 %/h, respectively; $t(10)=1.81$, $p<0.05$), as evidenced by decreased fractional synthesis rate, whereas protein synthesis in other organs including the brain, heart, intestine, kidney, liver and lung was unaltered (Table 1).

Bile duct ligation-induced cirrhosis increases ammonia levels

Arterial ammonia significantly increased in BDL rats (129.0 ± 14.8 µM vs 42.1 ± 8.2 µM respectively; $t(9)=5.38$, $p<0.001$) (Figure 5).

Discussion

Results of the present study demonstrate that BDL (6 week model) is associated with significant alteration in body composition as evidenced by decreased lean and fat mass, reduced gastrocnemius muscle mass and circumference as well as altered muscle morphology. The strong correlation observed between gastrocnemius mass (weight) and overall lean mass (assessed by MRI) indicates that gastrocnemius is representative of muscle body composition. Interestingly, our results also indicate a significant decrease in fat mass in BDL compared to SHAM rats. This reduction could be a result of fat mass been used as an energy source in order to maintain muscle mass during liver disease. The mechanisms responsible for fat mass decrease in experimental cirrhosis remain to be elucidated.

We found altered muscle morphology in BDL compared to SHAM rats which is most likely due to collapse of a smaller muscle. The mechanisms responsible for this alteration remain elusive. In addition to significant changes in muscle morphology, our results also revealed muscle protein synthesis is significantly decreased in BDL animals compared to SHAM. Impaired muscle protein synthesis may represent a major cause for muscle mass loss in experimental cirrhosis. Interestingly, alteration in protein synthesis was specific to the muscle as the protein synthesis rate was unchanged in the brain, heart, intestine, kidney, liver and lung between the two experimental groups. We speculate that ammonia may exert a deleterious effect on the muscle and contribute to its dysfunction by altering protein synthesis. This is supported with studies demonstrating hyperammonemia induced by portacaval-systemic shunting is associated with reduced muscle mass synthesis (Dasarathy et al. 2011; Davuluri et al. 2016).

Our study also indicate that BDL-induced cirrhosis leads to decreased gain in body weight compared to sham-operated animals, whereas food intake remains similar from week 2 to week 6. The statistical difference in food intake observed at week 1 (a consistent observation found with our extensive experience with this model) (Rose et al. 1999; Bosoi et al. 2011; Bosoi et al. 2012) may be explained by the invasive nature of the BDL surgery along with the recovery phase required for such a surgical procedure. This suggests that nutritional problems occur during the setting of cirrhosis which may include, among other mechanisms, metabolic alterations, malabsorption of nutrients, increased intestinal protein losses, disturbance of substrate utilization and increased energy expenditure (Bémeur et

al. 2010). Specifically, hypermetabolic state and increased energy-protein expenditure and requirements may occur in chronic liver disease. Indeed, the hyperdynamic circulation in cirrhosis leads to systemic vasodilation and an expanded intravascular blood volume which consequently leads to a greater use of macro- and micro-nutrients; hence causing a high energy expenditure and demand. Also, the inflammatory state associated with the inability of the liver to adequately clear activated proinflammatory mediators may result in hypermetabolism (Tilg et al. 1992; von Baehr et al. 2000). Regarding malabsorption, the cirrhotic liver may inadequately synthesize proteins and has diminished storage capacity and an impaired enterohepatic cycle. In addition, portal hypertensive enteropathy may lead to impaired absorption of essential nutrients. Moreover, pancreatic insufficiency, cholestasis, and drug-related diarrhea may all contribute to malabsorption in liver disease. Loss of proteins may result from complications of cirrhosis or from iatrogenic interventions including the use of diuretics for the treatment of ascites and fluid retention as well as the use of lactulose for the treatment of hepatic encephalopathy. Blood loss from oesophageal and gastric varices and from the intestinal lumen due to ulcers or portal enteropathy may also lead to increased protein loss in cirrhosis.

Several factors have been implicated in the pathogenesis of hepatic encephalopathy, including ammonia (Cooper and Plum 1987; Felipo and Butterworth 2002), oxidative stress (Görg et al. 2010; Bosoi et al. 2012), inflammation (Shawcross and Jalan 2005; Montoliu et al. 2009), lactate (Bosoi et al. 2014) and gut microbiota (Bajaj et al. 2008). Ammonia is considered the major factor in the pathogenesis of hepatic encephalopathy as hyperammonemia leads to toxic levels of ammonia in the brain (Cooper and Plum 1987; Felipo and Butterworth 2002). During liver disease, extra-hepatic ammonia metabolism is altered and, with muscle expressing an ammonia-lowering enzyme, glutamine synthetase, muscle plays a critical compensatory role in attenuating hyperammonemia. However muscle wasting can exasperate the degree of hyperammonemia and may play a pivotal role in the risk of developing hepatic encephalopathy (Qiu et al. 2012; Montano-Loza et al. 2014; Rombouts et al., 2016). The underlying causes of muscle wasting in liver disease remain undetermined. However, paradoxically, it has been demonstrated that high ammonia concentrations leads to muscle dysfunction and reduction in protein synthesis (Qiu et al. 2012; Qiu et al. 2013). An understanding of ammonia and its removing vs pathophysiological pathways is totally unclear and remains to be thoroughly investigated.

We conclude that the 6-week BDL experimental model is an excellent model to study liver disease-induced muscle mass loss. Demonstration of similar alterations in human cirrhosis will potentially enhance our understanding of the mechanisms of muscle mass loss in liver disease. Importantly, understanding these mechanisms will allow the identification of therapeutic targets to prevent muscle mass loss and ameliorate the quality of life and the prognostic of patient suffering from liver disease.

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Figures Legends

Table 1 Protein synthesis of organs in rats with bile-duct ligation (BDL) compared to sham-operated controls.

*p<0.05, significantly different from SHAM.

Fig 1 Growth curve of rats with bile-duct ligation (BDL) compared to sham-operated controls.

Mean of daily weights were averaged and expressed as weekly weight. The two way ANOVA indicated an effect of time/week ($F_{5, 54}$ =231.94, p<0.001), an effect of surgery ($F_{1, 54}$=85.93, p<0.001) and interaction ($F_{1, 54}$=2.47, p=0.04). **p<0.01, *** p<0.001, significantly different from SHAM.

Fig 2 Food intake of rats with bile-duct ligation (BDL) compared to sham-operated controls.

Mean of daily food intake were averaged and expressed as weekly intake. The two way ANOVA indicated an effect of time/week ($F_{5, 54}$=98.21, p<0.001) and an effect of surgery ($F_{1, 54}$=11.22, p<0.001), with no interaction ($F_{1, 54}$=1.47, p=0.21).** p<0.01, significantly different from SHAM.

Fig 3 Lean mass (A) and fat mass (B) in rats with bile-duct ligation (BDL) compared to sham-operated controls.

*** p<0.001, significantly different from SHAM.

Fig 4 Gastrocnemius muscle mass (A), muscle circumference (B), correlation between lean mass and gastrocnemius mass (C) and muscle histology of rats with bile-duct ligation (BDL) compared to sham-operated controls.

*** p<0.001, significantly different from SHAM. Bar= 10 µm

Fig 5 Serum ammonia in rats with bile-duct ligation (BDL) compared to sham-operated controls.

* p<0.05, significantly different from SHAM.

Figure

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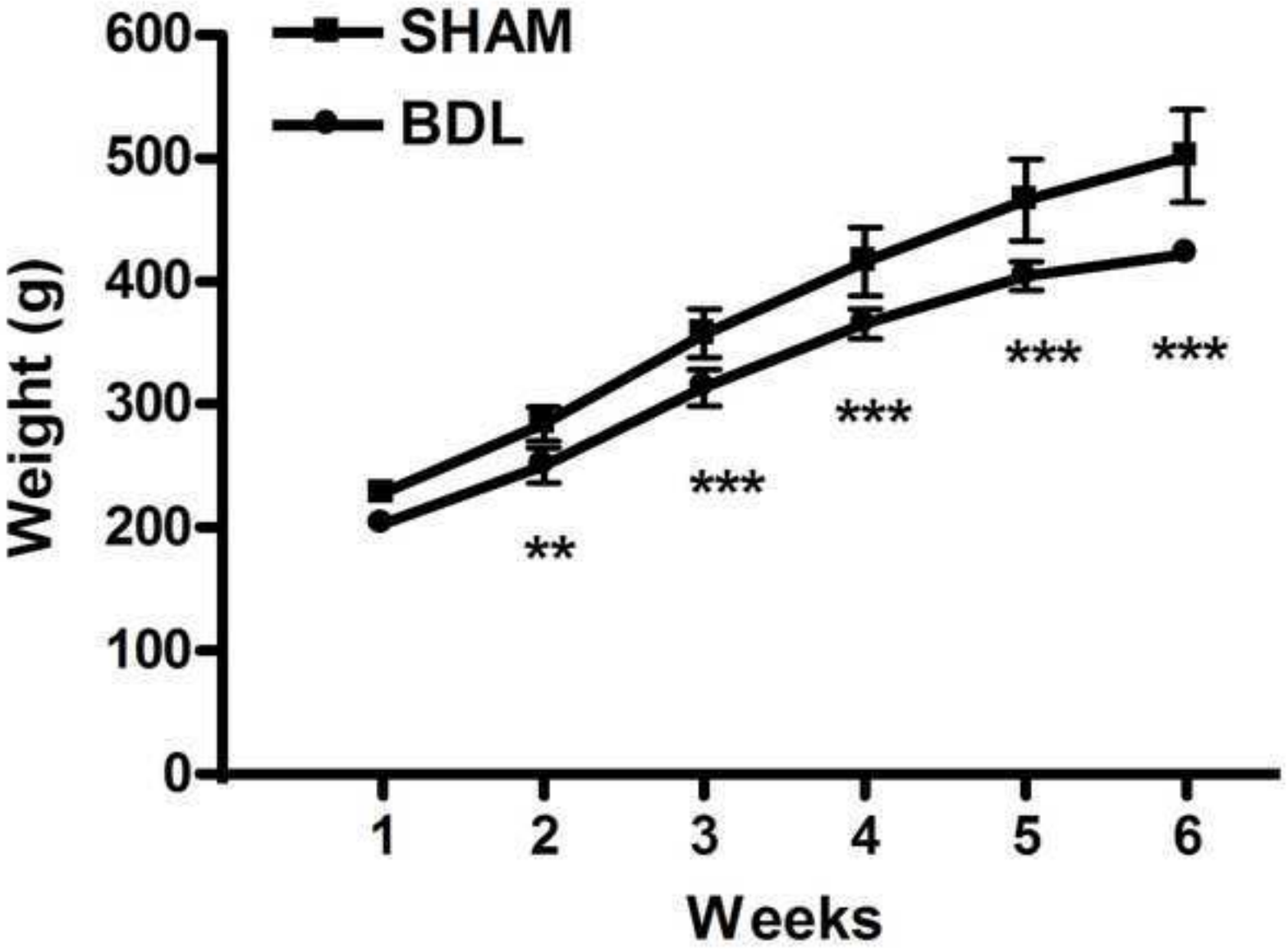

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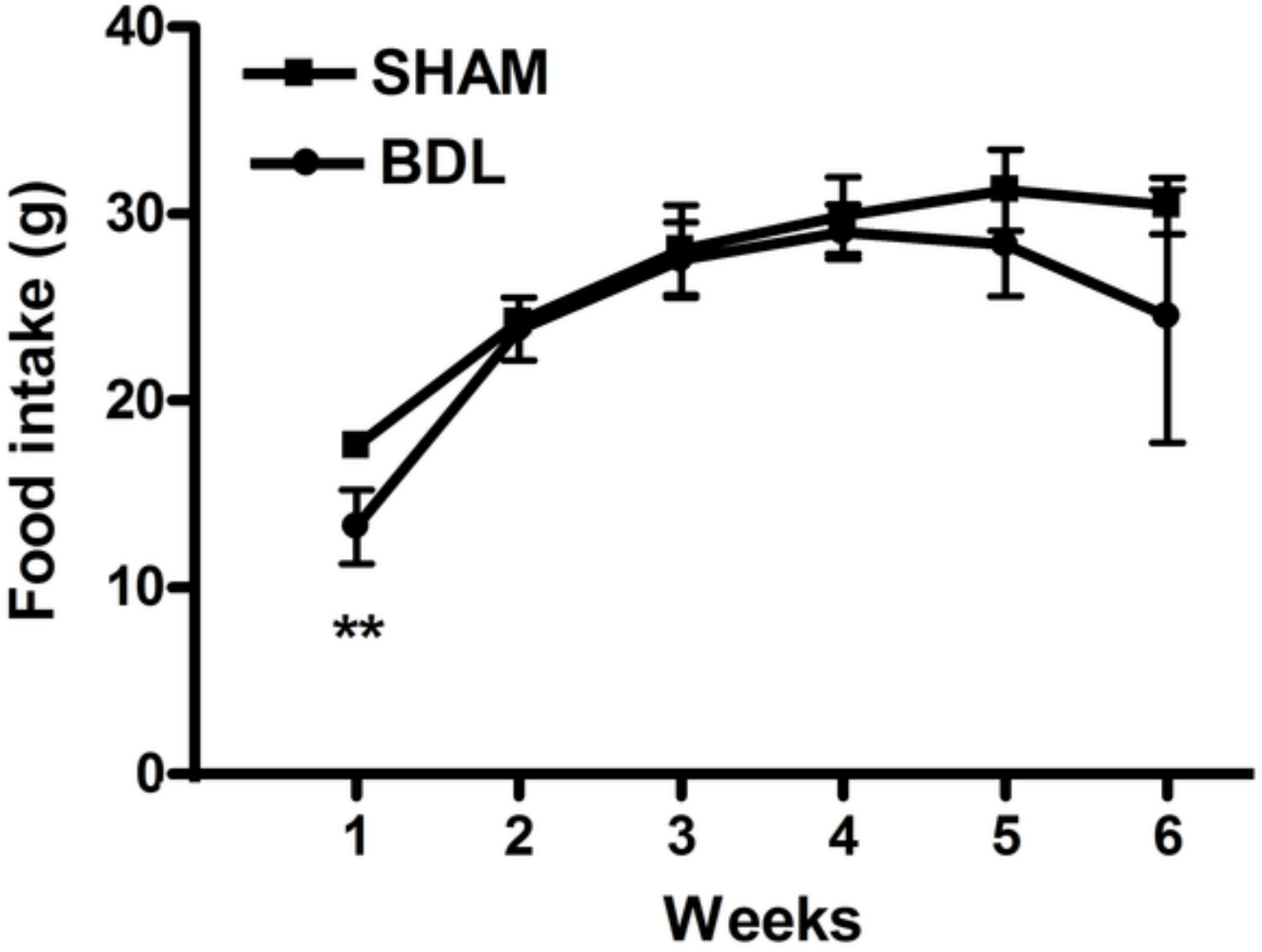

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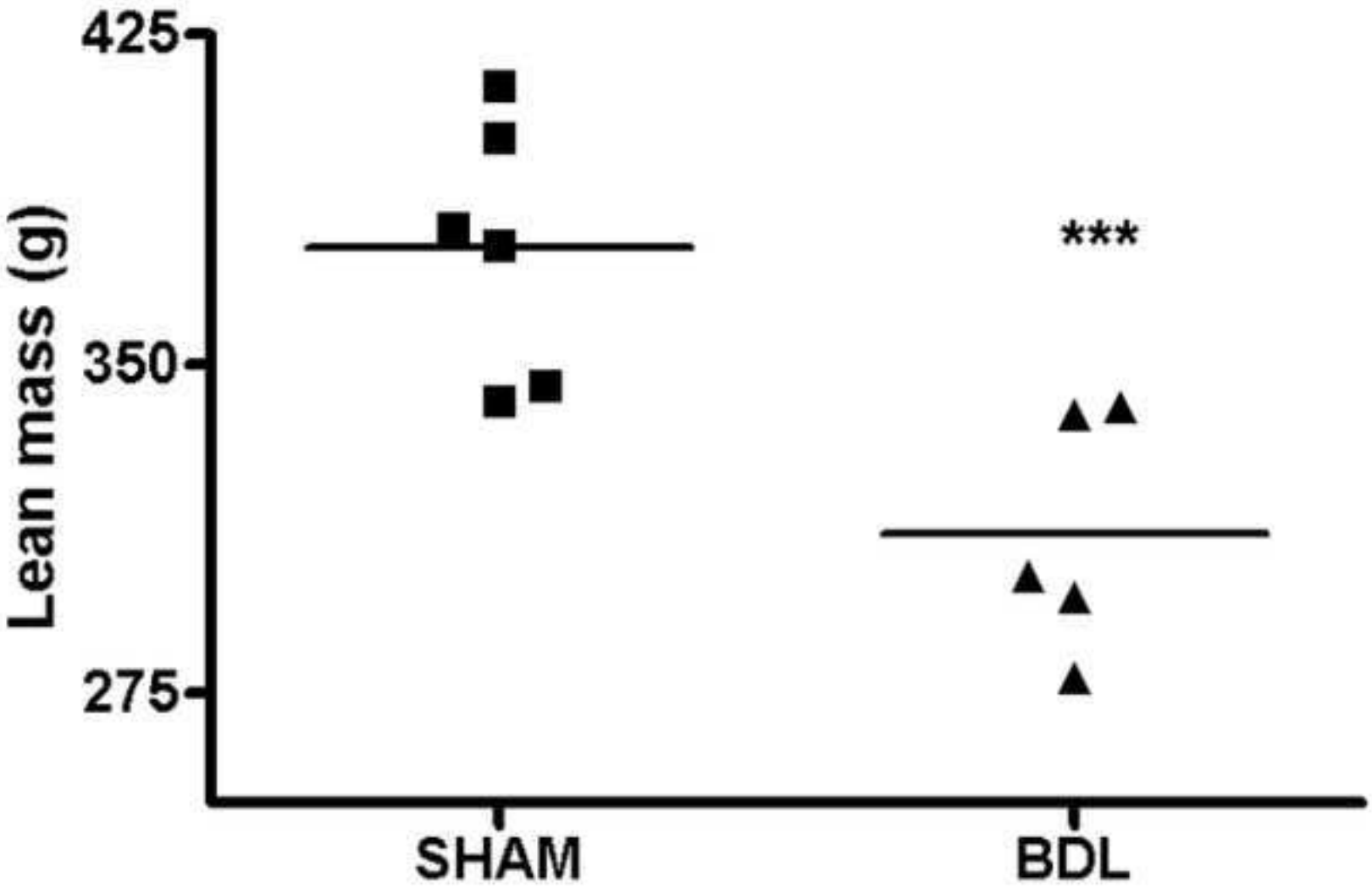

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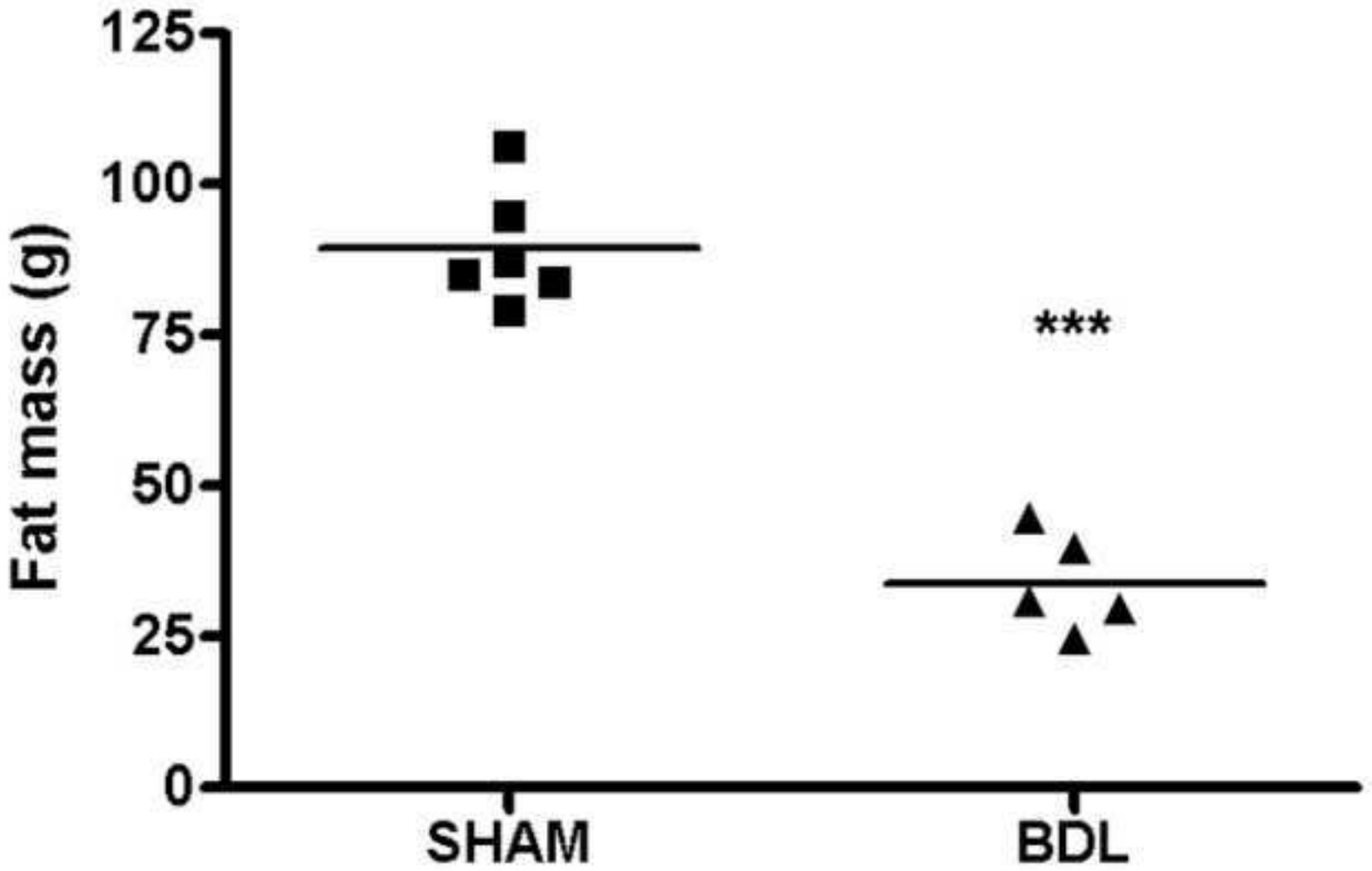

Figure

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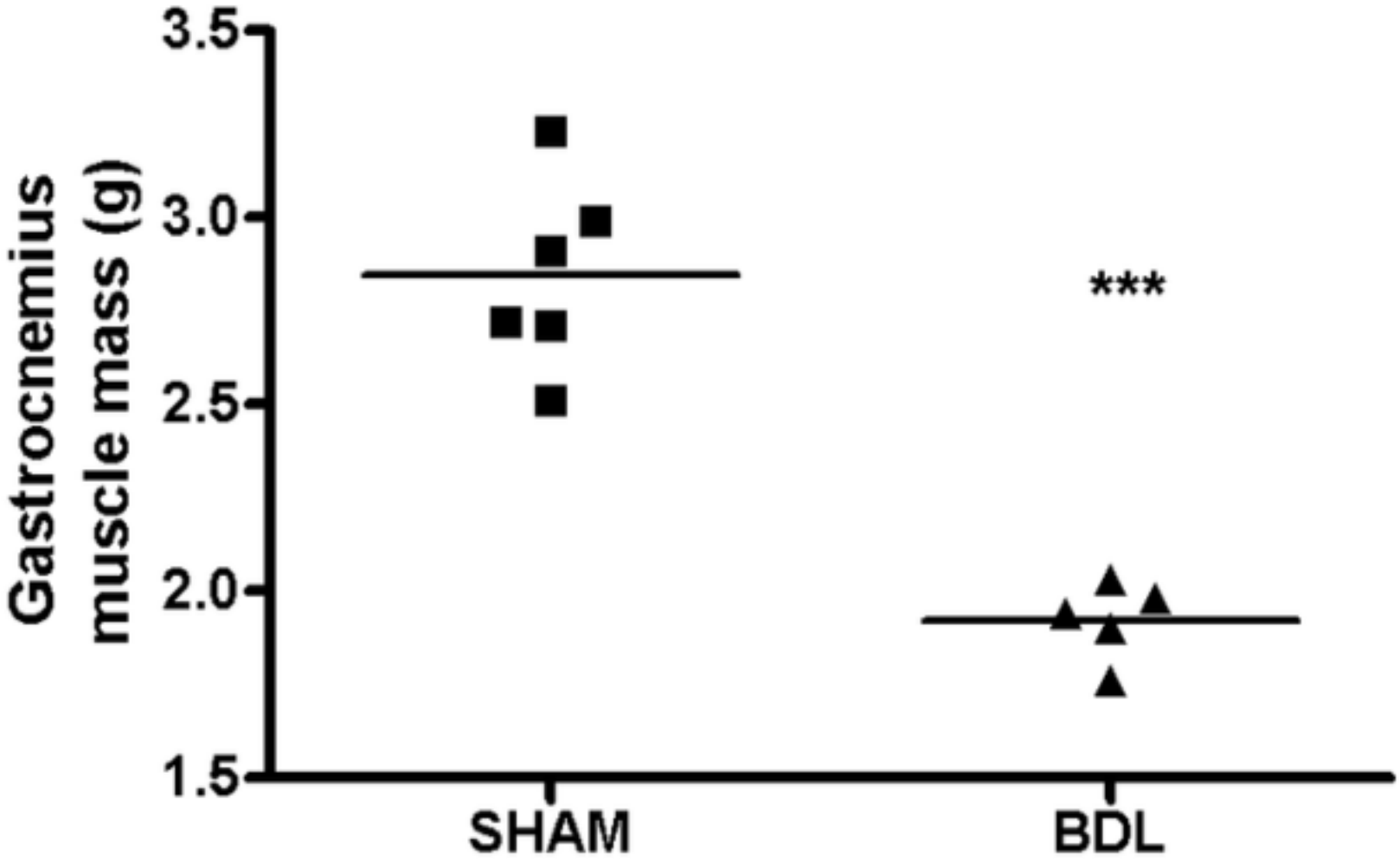

Figure

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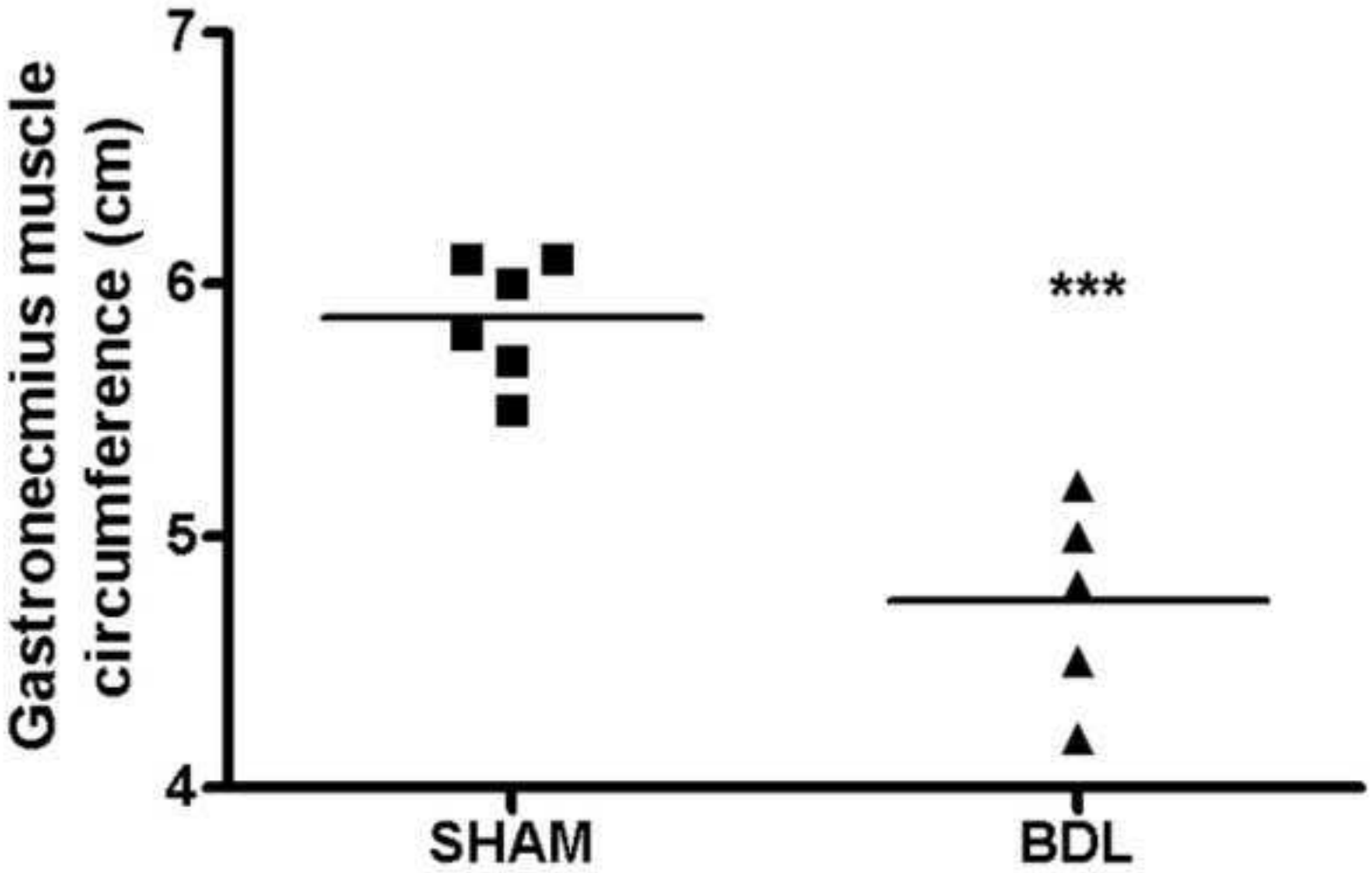

Figure

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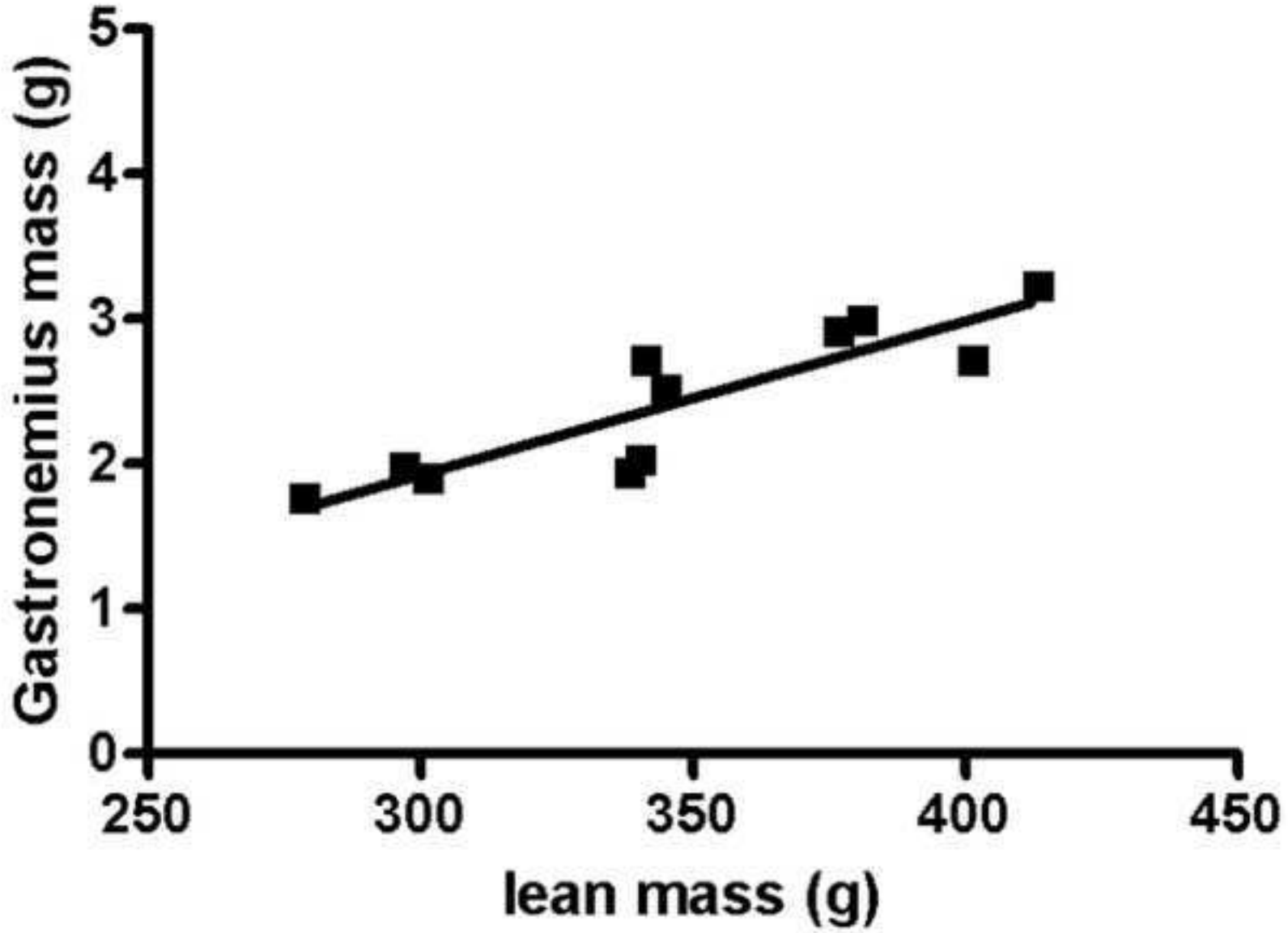

Figure

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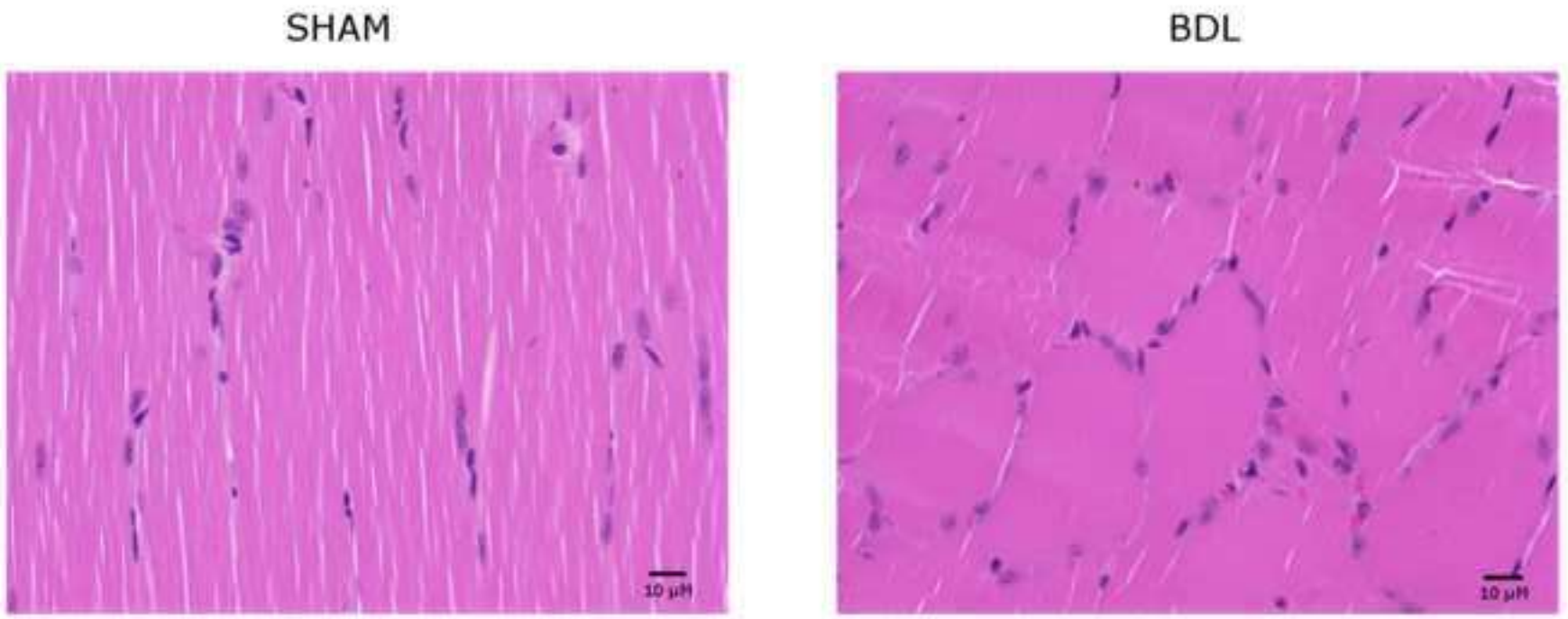

Table

	SHAM	BDL
Frontal Cortex (%/hour)	0,63 ± 0,25	0,56 ± 0,07
Heart (%/hour)	0,68 ± 0,31	0,64 ± 0,14
Intestine (%/hour)	4,46 ± 1,21	3,42 ± 1,34
Kidney (%/hour)	2,34 ± 0,92	1,73 ± 0,29
Liver (%/hour)	3,45 ± 1,29	2,95 ± 0,67
Lung (%/hour)	1,08 ± 0,39	1,25 ± 0,31
Muscle (%/hour)	0,32 ± 0,19	0,17 ± 0,07 *