



Cholecalciferol in ethanol-preferring rats muscle fibers increases the number and area of type II fibers

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ARTICLE INFO

Keywords:

Ethanol-preferring rats
Cholecalciferol
I and II type muscle fibers
UChB rats

ABSTRACT

The chronic use of ethanol causes neuropathy and atrophy of type II fibers and promotes vitamin D decrease. This study evaluated cholecalciferol effects on the deep fibular nerve and extensor digitorum longus (EDL) muscle using an UChB ethanol-preferring rats model. Blood analyses were carried out to measure levels of 25-hydroxycholecalciferol (25(OH)D), calcium (Ca^{2+}), Phosphorus (P), and parathyroid hormone (PTH). It was used EDL muscle to evaluate oxidative stress. The deep fibular nerve and EDL muscle were used for morphologic and morphometric assessment. 25(OH)D plasma levels were higher in the supplemented group and no alterations were observed in other parameters including the oxidative stress evaluation. The G ratio remained constant which indicates nervous conduction normality. Cholecalciferol supplementation promoted an increase in the number and area of type II fibers and a decrease in the area of type I fibers. In the studied model, there was neither alcoholic myopathy nor neuropathy. The EDL muscle glycolytic patterns in the high-drinker UChB rats may be associated with the differential effects of cholecalciferol on metabolism and protein synthesis in skeletal muscle.

1. Introduction

Ethanol consumption is a global health problem and can be associated with a large number of chronic diseases (5%) and deaths worldwide (3.8%) (Ramadori et al., 2017; Rocco et al., 2014; Stickel et al., 2017). The production of reactive oxygen species (ROS) is associated with ethanol metabolism (Hernandez et al., 2016). ROS are reactive chemical entities produced as intermediaries in oxidation-reduction reactions (redox). An imbalance in the production and elimination of ROS by antioxidant systems causes oxidative stress and pathophysiologic alterations (Marchi et al., 2014).

One typical alteration related to ethanol intake is chronic alcoholic myopathy that occurs in 40–60 percent of chronic alcoholics (Fernandez-Sola et al., 2007; Simon et al., 2017; Urbano-Marquez and Fernandez-Sola, 2004) which leads to muscular atrophy of type II fibers. Type II muscle fibers are more affected than type I fibers (Adachi et al., 2003; Duran Castellon et al., 2005; Gonzalez-Reimers et al., 2010; Nemirovskaya et al., 2015; Otis et al., 2007; Otis and Guidot, 2009;

Preedy and Peters, 1990; Reilly et al., 2000; Zinovyeva et al., 2016), and this atrophy develops independently of alcohol-induced disorders (Zinovyeva et al., 2016).

Neuropathy is another frequent alteration in chronic alcoholics that is present in 25–66 percent of these individuals (Ammendola et al., 2001; Chopra and Tiwari, 2012). This disease comprises axonal abnormalities with Wallerian degeneration and fiber myelination reduction (Yerdelen et al., 2008), as well as axonal loss (Mellion et al., 2013; Nguyen et al., 2012).

Many researchers have associated a decrease in vitamin D concentration to chronic alcohol intoxication reported in alcoholics or animals treated with ethanol (Fisher and Fisher, 2007; Gonzalez-Reimers et al., 2010, 2015; Miroliaee et al., 2010; Neupane et al., 2013; Nicolas et al., 2003; Quintero-Platt et al., 2015; Santori et al., 2008; Wijnia et al., 2013). Vitamin D receptors have been identified in many tissue types, including skeletal muscle (Bischoff et al., 2001; Ceglia and Harris, 2013; Simpson et al., 1985; Stockton et al., 2011). Studies have shown that vitamin D modulates the proliferation and differentiation of

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<https://doi.org/10.1016/j.acthis.2018.09.004>

Received 3 April 2018; Received in revised form 6 September 2018; Accepted 7 September 2018

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muscle cells and regulates muscle contractile function (Boland et al., 2002; Buitrago et al., 2012; Garcia et al., 2011; Stratos et al., 2013). Vitamin D is associated with a decrease in type II muscle fiber atrophy (Sato et al., 2005) and an increase in the diameter and percentage of type II fibers (Ceglia, 2009; Chatterjee, 2001). It reintegrates muscle tissue (Annweiler et al., 2010), increasing its strength and it has also positive effects on the myelination of nerve fibers (Chabas et al., 2013; Montava et al., 2015).

Vitamin D in physiologic concentrations acts to protect the cells against oxidative damage (Bhat and Ismail, 2015; Chandrashekar et al., 2015; Codoner-Franch et al., 2012). Antioxidant imbalance as well as an increase in the lipid peroxidation and production of free radicals and ROS are also present in chronic alcoholism (Adachi et al., 2000; Fernandez-Sola et al., 2002; Koo-Ng et al., 2000; Mansouri et al., 2001; Preedy et al., 2001), with increased oxidative stress in muscle tissue (Fujita et al., 2002).

Many animal models have been used in studies on ethanol intake (Fagundes et al., 2016; Fernandez et al., 2016; Fritz and Boehm, 2016; Moore and Lynch, 2015; Peris et al., 2015; Portari et al., 2016; Shukla et al., 2015). The ethanol-preferring rat model, UCh, has been selectively bred at the University of Chile for decades (Mardones and Segovia-Riquelme, 1983). These rats, which comprise two varieties (low – UChA and high - UChB ethanol consumers of 10% ethanol solution) of inbred animals, are derived from the original Wistar rat strain and have been considered a good model for the study of alcoholism.

The aim of this study was to analyze the effects of cholecalciferol supplementation both on extensor digitorum longus (EDL) muscle with focus on type I and II fibers and on deep fibular nerve using UChB ethanol-preferring rats.

2. Materials and methods

2.1. Animals and experimental design

The animals of the UChB lineage were selected and standardized (Mardones and Segovia-Riquelme, 1983) based on ethanol intake. 60-day-old rats were given free choice between two bottles containing either water *ad libitum* or 10% ethanol solution *ad libitum* (measured weekly) for 15 days. Animals whose average intake was higher than 2.0 ml of 10% ethanol/100 g (g) of body weight/day were selected for the UChB lineage (Quintanilla et al., 2006) (Fig. 1A).

After selection, the animals received no more water and ethanol bottles were maintained until 90 days. When the animals completed 90-day-old twenty male adult UChB rats were divided into two groups: Vitamin D (UV) group that received 12.5 µg/kg (500 UI) cholecalciferol

(C9756-Sigma), which is the main dietary source of vitamin D (Desmarchelier et al., 2016) dissolved in 0.3 ml olive oil administered orally and Control (UC) group that received the vehicle (Salum et al., 2012). Both groups received the treatment by gavage and had continuous access to 10% ethanol for 75 consecutive days (Fig. 1B).

Animals were maintained at the Anatomy Department, Biosciences Institute, UNESP, Botucatu-SP and kept in polyethylene boxes (40 × 30 × 15 cm) covered with wood shavings under controlled conditions of luminosity (12 h light, 12 h dark) and temperature (20–25 °C), receiving 10% ethanol and industrialized food (Provence®) *ad libitum*. All experimental procedures were approved by the Committee on Animal Research and Ethics of the Biosciences Institute, UNESP, Botucatu (Protocol number 531).

In the experimental period, the nutritional parameters of ethanol (ml) and food (grams) intakes were evaluated weekly. After 75 days of experimental period, the animals were fasted for 12 h, weighed and anesthetized with Ketamine/Xylazine (90 mg/kg and 10 mg/kg, respectively) intraperitoneally. They were euthanized by decapitation for further analysis.

2.2. Nutritional parameters

Consumption of the 10% ethanol solution (ml) and food (grams) as well as weight changes were evaluated weekly. At the end of the experiment, the extensor digitorum longus (EDL) muscle was removed and weighed.

2.3. Blood analysis

The plasma/serum was obtained, and the levels of 25-hydroxyvitamin D levels (25(OH)D) that is the first hydroxylation of cholecalciferol to become hormonally active and to analyze vitamin D status is usually estimated by measuring the plasma 25(OH)D concentration. The levels of calcium (Ca²⁺), phosphorus (P) and parathyroid hormone (PTH) also were measured at VitaeLab in São Paulo- SP/Brazil.

2.4. Oxidative stress

The EDL muscles were dissected, frozen in liquid nitrogen, and stored at –80 °C. After the samples were homogenized, a lucigenin-derived chemiluminescence assay was performed to detect O²⁻ levels as described previously (Yogi et al., 2010). The luminescence was measured in a luminometer Orion II (Berthold Detection Systems, Pforzheim, Germany). The results were expressed as relative light units (RLU)/mg protein. The measurement of Thiobarbituric Acid Reactive

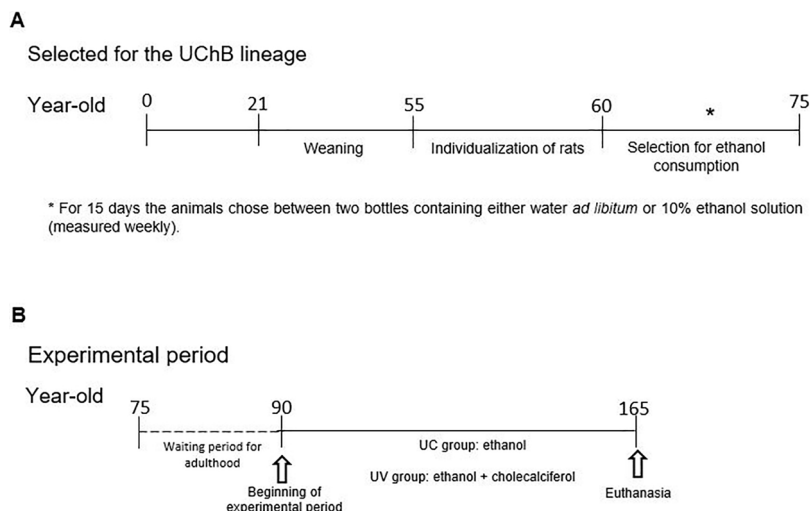


Fig. 1. Experimental diagram (A) Schematic representation of birth until selection period of UChB rats. (B) Schematic representation of experimental period.

substances (TBARS) in the EDL muscle was performed using a commercially available kit (#10009055, Cayman Chemical, Ann Arbor, MI, USA) as described previously (Gonzaga et al., 2015) and expressed as nmol/mg protein. SOD (superoxide dismutase) and CAT (catalase) activity and GSH (reduced glutathione) levels were determined as described previously (Gonzaga et al., 2014).

2.5. Morphological and morphometric analysis

2.5.1. The deep fibular nerve and EDL muscle fibers

The EDL muscle fragments were covered with neutral talcum powder and frozen in liquid nitrogen (stored in freezer at -80°C). Sequential histological slices were obtained using a cryostat Leica CM 1800 at -25°C at $8\ \mu\text{m}$ thick and stained with hematoxylin and eosin and immunoperoxidase to analyze I (slow) fibers (1:180, product code: NCL-MHCs, Novacastra) and II (fast) fibers (1:130, product code: NCL-MHCf, Novacastra). A H-Histofine Rat display system (Multi - Nichirei) was used, and a solution containing DAB (3,3-diamino-benzidine tetrahydrochloride), chromogen (1:50) and hematoxylin was used as the counter-staining. To obtain approximately 200 fibers, 5–6 fields at $200\times$ magnification were photographed per animal in each experimental group. The fiber types were calculated, and the fiber area was measured.

After removal of the deep fibular nerves, they were fixed and stained with 1% osmium tetroxide solution following the histological routine. To facilitate the visualization of the entire sectioned nerve, $100\times$ magnification was used. The number of axons, the diameter of the axons and the nerve fibers were obtained and myelin thickness (diameter fiber - diameter axons/2) and the G ratio were calculated (diameter axons/diameter fiber). The free software “Image J” (Schneider et al., 2012) (<http://rsbweb.nih.gov/ij/>) was used for the muscle and nerve quantitative analyses of the outlined parameters.

3. Statistical analyses

Software Statistical Analysis System (SAS® v.9.3, Cary, North Carolina) was used to obtain the statistical analyses and a level of $p \leq 0.05$ was considered statistically significant. Data were expressed as the means \pm standard error of the means. Two way ANOVA repeated measures with two groups followed by Tukey multiple comparison test were applied to evaluate the interaction among weeks concerning the means of body weight, food consumption and ethanol intake. Student's *t*-test was used to determine differences in weight gain, weight EDL muscle, blood analyses, antioxidant enzyme activity in the EDL muscle and morphometric analyses of the nerve and muscle.

4. Results

4.1. Nutritional parameters

Body weight was evaluated weekly, did not differ significantly across the two groups ($p > 0.005$). UC and UV groups gain similar weight body between 1 and 10 week (*UC week 1 vs week 10 and **UV week 1 vs week 10, $p < 0.0001$) (Fig. 2 and Table 1). The EDL muscle weight did not vary between the groups ($p > 0.05$, Table 2).

In relation to the food consumption (g/week, Fig. 3A), there was no significant difference between the groups ($p = 0.6165$) nor across the experiment ($p = 0.8179$). There was a clear increase in ethanol intake (ml/week) in both groups (*UC and UV week 1 vs week 10; $p < 0.0001$). Ethanol intake peaked in week 4 and remained constant until week 10 in both groups (**UC and UV week 1 vs week 4, $p < 0.0001$). No difference in ethanol intake was observed between the two groups ($p = 0.5237$, Fig. 3B) in week 10.

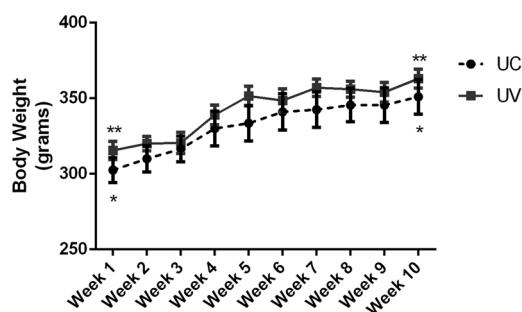


Fig. 2. The body mass over the experimental period (**indicate significant differences between week 1 vs week 10 of UC group and *indicate significant differences between week 1 vs week 10 of UV group, both $p < 0.0001$) Data were expressed as the means \pm standard error of the means. Two way ANOVA repeated measures with two groups followed by Tukey multiple comparison test.

Table 1

Weight gain (grams) at the beginning and the end of experimental period. Data were expressed as the means \pm standard error of the means. Student's *t*-test.

| Weight Gain (grams) | |
|---------------------|----------------|
| | UC |
| Grams | 45.0 \pm 3.7 |
| | UV |
| Grams | 44.4 \pm 2.9 |

Table 2

EDL muscle weight (grams). Data were expressed as the means \pm standard error of the means. Student's *t*-test.

| EDL muscle (grams) | |
|--------------------|-------------------|
| | UC |
| Grams | 0.187 \pm 0.009 |
| | UV |
| Grams | 0.182 \pm 0.004 |

4.2. Blood analyses

In relation to blood analyses the results showed that the 25(OH)D values were higher in the UV group ($p = 0.0003$). There was no differences in the concentrations of calcium, phosphorus and parathyroid hormone ($p > 0.05$). (Table 3).

4.3. Antioxidant enzyme activity in the EDL muscle

The antioxidant enzyme activity showed no significant difference in the levels of GSH, SOD and CAT activity, lucigenin or TBARS between the groups ($p > 0.05$) (Table 4).

4.4. Morphological and morphometric analyses

4.4.1. The deep fibular nerve

The morphological analysis of the deep fibular nerve revealed a prevalence of myelinic fibers with bundles of organized myelinated axons containing neurofilaments distributed homogeneously throughout the entire analyzed area of the nerve. Integral and concentric myelin sheaths without signs of demyelination were present. The axoplasm and endoneurium were well preserved. The general morphology was within the normal pattern and showed no differences between the groups (Fig. 4A and B).

The morphometric analyses of the number of axons, thickness of myelin sheath and G ratio, showed no statistically significant differences between the groups. In the group supplemented with cholecalciferol, there was decrease in the average diameter of the nervous fibers ($p = 0.0050$) and axons ($p = 0.0012$) (Fig. 4C–G).

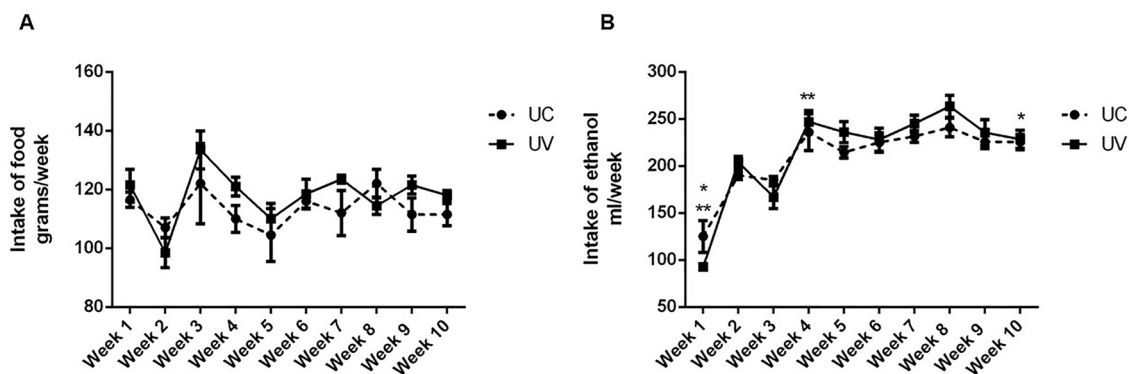


Fig. 3. A: Intake of food (g/week) the experimental period. B: Intake of ethanol (ml/week) the experimental period (* indicate significant differences between week 1 vs week 10 of UC and UV groups and ** indicate significant differences between week 1 vs week 4 of UC and UV groups, both $p < 0.0001$). Data were expressed as the means \pm standard error of the means. Two way ANOVA repeated measures with two groups followed by Tukey multiple comparison test.

Table 3

Blood analyses. Data were expressed as the means \pm standard error of the means. Student's *t*-test.

| Blood analyses | UC | UV |
|-----------------------------|----------------|------------------|
| Vitamin 25(OH)D (ng/ml) | 10.7 \pm 4.5 | 51.6 \pm 8.7 * |
| Calcium (mg/dl) | 10.2 \pm 0.1 | 10.8 \pm 0.08 |
| Phosphorus (mg/dl) | 8.8 \pm 0.4 | 9.8 \pm 0.1 |
| Parathyroid hormone (pg/ml) | 1.0 | 1.0 |

* $p = 0.0003$.

Table 4

Antioxidant enzyme activity in the EDL muscle. Data were expressed as the means \pm standard error of the means. Student's *t*-test.

| Antioxidant enzyme activity in the EDL muscle | | | | | |
|---|-------------------------------------|---|---------------------------------|----------------------------------|-----------------------------------|
| | GSH (μ g/ mL/mg protein) | SOD (inhibition rate %/ml of plasma) | CAT (unit/ mg of protein) | Lucigenin (RLU/mg protein) | TBARS (nmol/ mg of protein) |
| UC | 17.5 \pm 1.5 | 14.1 \pm 0.6 | 13.8 \pm 0.8 | 72.4 \pm 3.5 | 1.8 \pm 0.1 |
| UV | 18.8 \pm 1.0 | 15.1 \pm 0.9 | 12.2 \pm 1.2 | 78.3 \pm 13.0 | 2.0 \pm 0.1 |

4.4.2. EDL muscle fibers

The HE morphological analysis showed normal muscle fibers that presented a polygonal shape with peripheral nuclei, and preserved endomysium and perimysium. There were no alterations after the experimental period in relation to the all groups studied (Fig. 5A).

The immunohistochemistry of the muscle fibers for fast and slow fibers in all experimental groups demonstrated a mosaic pattern with a predominance of type II fibers, compatible with the EDL muscular characteristic (Fig. 5B and C). After cholecalciferol supplementation, there was an increase in the number of type II fibers ($p = 0.0199$) and no difference in the number of type I fibers. There was a reduction in the type I fiber area ($p = 0.0002$) and an enhancement in the type II fiber area ($p < 0.001$) (Fig. 5D–G)

5. Discussion

This is the first study to investigate the effects of cholecalciferol supplementation on UChB ethanol-preferring rat model. The main finding after supplementation with cholecalciferol was an increase both in the number and area of type II fibers and a decrease in the area of type I fibers, in the EDL muscle. These results show that the cholecalciferol influenced the answer of the UChB ethanol-preferring rat model muscle fibers, as evidenced with its increase in blood

concentrations (Ceglia and Harris, 2013; Gonzalez-Reimers et al., 2010; Salum et al., 2013; Seida et al., 2014).

These findings are in agreement with other studies that used vitamin D supplementation. In human studies, it was observed, specifically in older women, an increase in the area and composition of type II muscle fibers as well as an increase in subtype of type II (IIa) muscle fibers related to number and to cross-sectional area after treatment with the active vitamin D analogue, 1 alpha-hydroxycholecalciferol (Sørensen et al., 1979). An increase in the relative number and size of type II fibers was also observed in women after stroke (Sato et al., 2005). Agergaard et al. (2015) studying the association of cholecalciferol intake with resistance training in young and elderly men, observed improvement in muscle quality and that its intake in young men influenced a skeletal muscle remodeling with an increase of type I and II area fibers. So it is possible to consider a positive effect of cholecalciferol intake on type II muscle fibers.

In this study the muscle used was a typical glycolytic muscle with a predominance of type II fibers. There are differences in the mitochondria quantity related to oxidative and glycolytic muscular fibers and their metabolic responses should be taken into consideration (Schiaffino and Reggiani, 2011). In addition the supplementation of vitamin D is able to promote an improvement in parameters of mitochondrial function (Sinha et al., 2013) and according to Ray et al. (2016) its effects are muscle-specific.

In human muscle was verified that the mitochondrial form, distribution, and function were influenced by vitamin D (Ryan et al., 2015). The vitamin D effects on the skeletal muscle cell have been related even with metabolism and protein synthesis, and it is important for maintenance of the mass, strength and speed of contraction of skeletal muscle (Pedrosa and Castro, 2005). On the other hand, ethanol is considered a potent inhibitor of muscle protein synthesis (Preedy et al., 2001) and its effect is more intense on type II than on type I muscle fibers (Gonzalez-Reimers et al., 2010).

Many authors have described that chronic ethanol users have low levels of vitamin D (Gonzalez-Reimers et al., 2010, 2015; Mercer et al., 2012; Turner, 2000). Vitamin D (cholecalciferol) is enzymatically converted into circulating form (25-hydroxyvitamin D/25(OH)D) in the liver and after into its active form in the kidney (1 α ,25-dihydroxyvitamin D3/1,25-(OH)2D3). Its conversion is regulated by the blood calcium and phosphorus levels, under the influence of the parathyroid hormone (PTH) (Plum and DeLuca, 2010). In this study, only the circulating form of vitamin D was quantified, disabling the correlation between these parameters. The calcium, phosphorus, and PTH levels of the blood analyses showed no difference between the groups. Domingues-Faria et al. (2014) found that vitamin D depletion had no effect on serum phosphorus or calcium levels. Concerning the PTH value, it can be concluded that the similarity between groups may be considered a limitation of the current study due to the insensitivity of

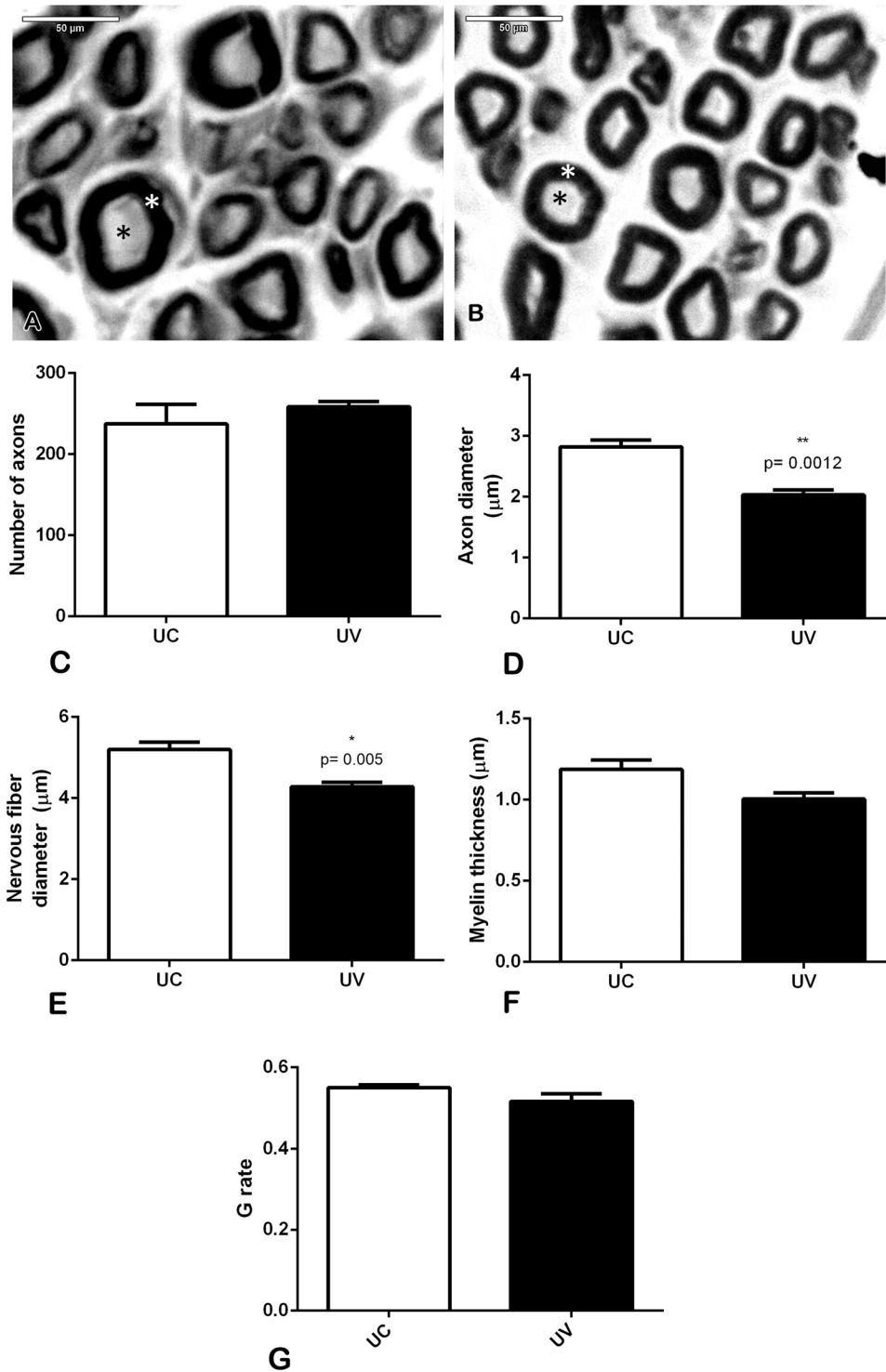


Fig. 4. A–B: Photomicrographs of cross-sections of the deep fibular nerve of the experimental groups stained by osmium tetroxide (Scale bar = 50 μm). Myelinic fibers (Black asterisk), Myelin sheaths (White asterisk). A: UC group; In B: UV group. C–D: Morphometric analysis of deep fibular nerve where C: Axon number; D: Axon diameter (μm); E: Nervous fiber diameter (μm); F: Thickness of myelin sheath (μm) and G: G rate. Data were expressed as the means ± standard error of the means. Student's *t*-test, * *p* = 0.0050 and ** *p* = 0.0012.

the detection method used.

Alteration in the nerve as axonal degeneration (Ertem et al., 2009; Mellion et al., 2013; Nguyen et al., 2012) and a reduction in axon number and myelination (Yerdelen et al., 2008) have been common findings in chronic alcoholism. In the present study, no histological abnormality or change in the number of axons was observed. Although a decrease in the diameters of the fibers and axons was found in the UV

group, there was no significant change in the G ratio. The G ratio is a parameter related to the conduction velocity of the nervous impulse. Low values (approximately 0.4) usually indicate axonal degeneration, while high values (approximately 0.7) indicate a normal conduction velocity (Mendonca et al., 2003).

Our results showed an increase in body weight during experimental period. This may be due to the animal growth and related to an increase

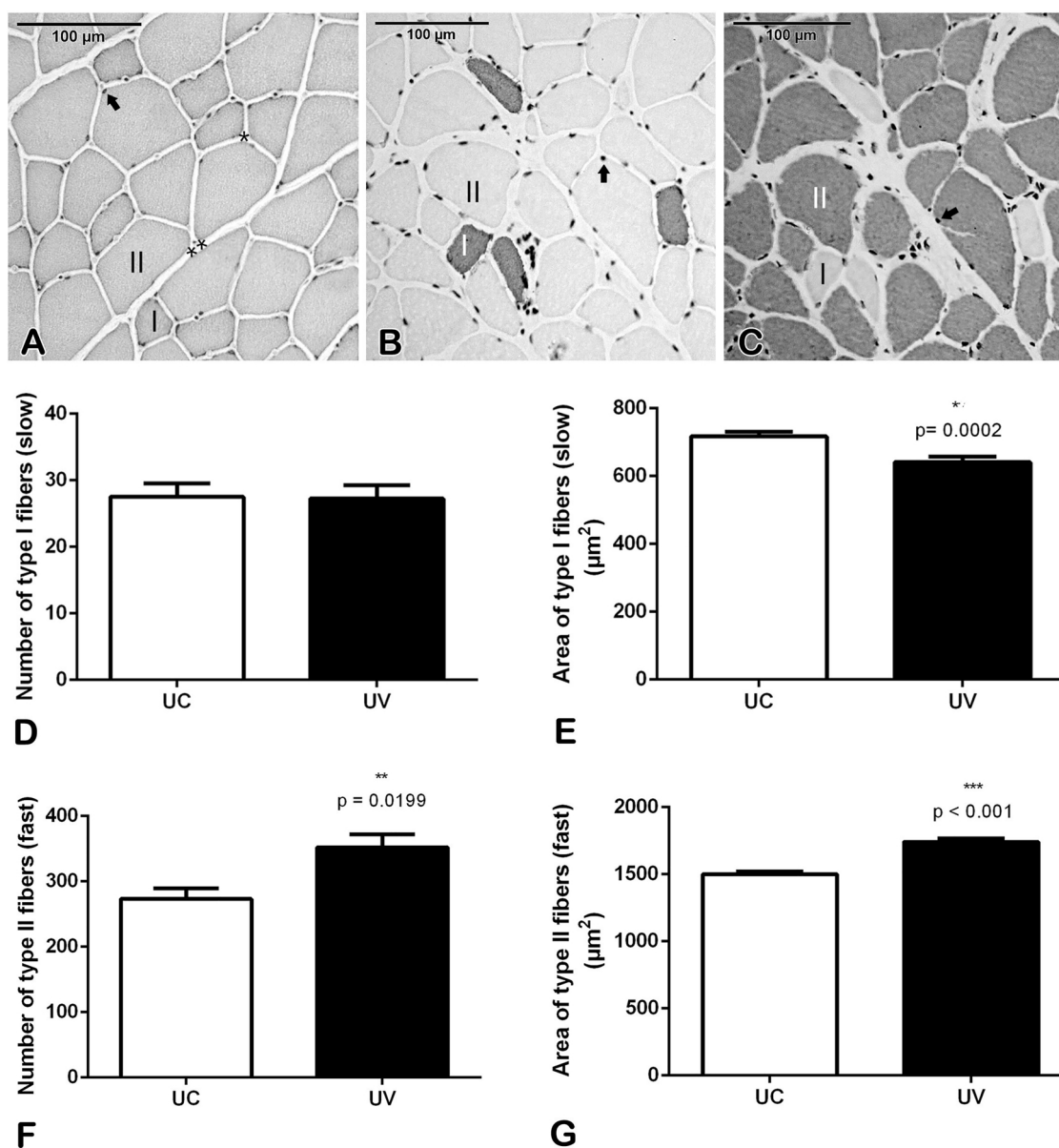


Fig. 5. A–C: Photomicrographs of cross-sections of the muscle fibers of the UChB Control group in the EDL muscle (Scale bar = 100 μm). Endomysium (*), Perimysium (**), Peripheral nuclei (arrow), Type I fibers (I), Type II fibers (II). A: Fibers stained with hematoxylin and eosin; B: Immunohistochemistry of the type I fibers; C: Immunohistochemistry of the type II fibers. D–G: Morphometric analysis of EDL muscle, where D: Number of type I fibers (slow); E: Area of type I fibers (slow) (μm²); F: Number of type II fibers (fast) and G: Area of type II fibers (fast) (μm²). Data were expressed as the means ± standard error of the means. Student's *t*-test, * *p* = 0.0002; ** *p* = 0.0199 and *** *p* < 0.001.

in adipose tissue as a consequence of ethanol intake (Lukasiewicz et al., 2005; Traversy and Chaput, 2015) for seven times a week (Sayon-Orea et al., 2011).

The results found in this experimental protocol indicated that neither cholecalciferol nor ethanol influenced food intake. There was an increase in ethanol intake during the experiment, with a peak in both groups at week 4. The results showed that UChB animals showed the similar ethanol intake as in previous studies (Chuffa et al., 2013; Quintanilla et al., 2016; Sotomayor-Zarate et al., 2013). The same evolution observed in the UC group was seen in the UV group after cholecalciferol supplementation. This result can be justified as their intake tended to stabilize at day 15 but it was still 25–30% higher at the end of day 30 (Karahanian et al., 2011).

No alteration was found in GSH, SOD, CAT, lucigenin or TBARS activities in the animals in this study. Whereas the chronic alcoholism plays a role in oxidative damage, the vitamin D can act as an

antioxidant (Adachi et al., 2000; Bhat and Ismail, 2015; Chandrashekar et al., 2015; Chatterjee, 2001; Codoner-Franch et al., 2012; Fernandez-Sola et al., 2002; Koo-Ng et al., 2000; Mansouri et al., 2001; Preedy et al., 2002, 2001). Considering that alcoholic myopathy is associated with oxidative damage (Duran Castellon et al., 2005; Fernandez-Sola et al., 2002; Fujita et al., 2002; Gonzalez-Reimers et al., 2010) and in this study no myopathy signals were observed, it could explain the similar results related to the antioxidant enzyme activities.

In summary, even though it has not been observed compatible changes related to myopathy or neuropathy alcoholic, the main results of this study concerning the increase in number and area of type II muscle fibers in UV group may be associated with the differential effects of vitamin D on metabolism and protein synthesis in skeletal muscle. So cholecalciferol supplementation in the UChB ethanol-prefering rat model may play an important role to promote glycolytic pattern in EDL muscle.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

Pró-Reitoria de Pesquisa - RENOVE UNESP (0252/010/14).

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