Effect of ethanol in the *digastricus* muscle in rats (*Rattus norvegicus*): an histoenzimologic evaluation

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ABSTRACT

It is estimated that from 1 million of adult people in the western hemisphere, 20,000 present skeletic muscle abnormalities from abusive alcohol consumption. In this case a progressive muscle weakness sets in specially in the type II fibers of the thigh, shoulder and hip muscles. In the masticatory muscles the alcohol effects are unknown. This research aimed to evaluate possible type II myofibers alterations in the digastricus muscle in order to compare with the trunk muscle. Muscle fragments from digastricus and abdominal rectus muscles of 5 animals were harvested per grouping: Control (C), Alcoholic (A) and Isocaloric (I) groups. The fragments were submitted to the myosin ATPase (acid and alkaline pre incubation) and NADH-TR reactions. The fibers were classified into FG (fast-twitch-glycolitic) FOG (fast-oxidative-glycolitic) and SO (slow-oxidative). The results showed significative difference in the FG and FOG areas of the digastricus muscle among the studied groups and only in the FG fibers in the rectus abdominalis muscle. It was also

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observed a significative difference in the FG and FOG fibers frequency in the abdominal rectus muscle. Based in these results we conclude that the alcohol produces an atrophy in the masticatory fast fibers (FG and FOG fibers) similar to the trunk muscle.

KEY WORDS: type of fibers; masticatory muscle; *digastricus* muscle; alcoholism; rats

INTRODUCTION

The severe effects on striated muscles produced by abusive ingestion of alcohol were discovered a century and a half ago. However, thirty years ago there were only a few specific studies in the field. Studies conducted in Great Britain and Spain, however, revealed that such miopaty is common (PREEDY et al., 2001).

It is estimated that out of the 1 million adults in the western hemisphere, 20,000 present muscle skeletic anomalies due to alcohol consumption (REILLY et al, 1995). The incidence of the disease is similar in men and women and the estimations depend on the parameters used for the diagnosis. Indeed, there are many sub-clinical cases that are not identified by common criteria. In these cases patients show only a reduction in the muscular strength including, in some cases, some degree of muscle atrophy but without clinical symptoms (URBANO-MÁRQUEZ et al., 1989; SACANELLA et al., 1995).

Studies have been conducted to show the effect of alcohol in the striated muscle in muscle from the trunk and limbs and in these the alcoholic miopathy is characterized by a selective atrophy of fibers type II. Studies reveal that fiber I is minimally affected and can, in some cases, show a compensatory hipertrophy although type I fiber atrophy always occur in severe cases of alcohol ingestion (PREEDY et al., 1999).

Salisbury et al., (1992) observed that ethanol cause an specific miopathy in rats affecting selectively the type II fibers. According to these authors such alterations have a straight correlation with the anomalies observed in the chronic alcoholic miopaties in humans.

For some time the histoenzimologic data of trunk and limb members were extrapolated to chewing muscles, but soon authors concluded that some differences were clear between them (MAO et al., 1992; TUXEN et al., 1992) such as the presence, in muscles of the mandible, of a great amount of a same histochemical type of

fiber in a same fascicle or even occupying the entire fascicle. Finding similar to that in limb muscles suggest a process of denervation followed by reinervation (DUBOWITZ; BROOKE, 1973).

Taking into consideration the point discussed above, the present study aims to observe the frequency and size of the histoenzimologic fiber types found in the *digastricus* muscle of rats and whether ethanol produces alterations in the area or in the frequency of these types of fibers.

MATERIAL AND METHODS

Fifteen adult male Wistar rats (*Rattus norvegicus*), 3 months in average old and weighting approximatelly 200 g provided by the Biotery fo the University of São Paulo – campus Bauru – SP, were used.

After 90 days of age rats were growth separately in metabolic cages in order to control the daily consumption of ration, liquid and the animal weight. The room had artificial light controlled by a timer in cycles bright/dark of 12 hours, exhauster and air conditioner with average temperature of 21°C controlled by thermometer Incoterm® for room temperature.

Animals were isolated in three groups of five animals each as follows:

– Normal control group (N) – animals received water as liquid diet.
– Nutritional paired isocaloric group (I) – animals received water with saccharose as liquid diet.
– Alcoholic – chronic alcoholic group (A) – animals received a mixture of 25% ethylic alcohol diluted in water.

The alcoholic model adopted was “semi-voluntary” in which the administration of diluted alcohol was done as the sole liquid food available to the animal.

The alcoholic group (A) was submitted weekly to periods of progressive adaptation to 6%, 15% and 25% alcohol to prevent their death (CAGNON, 1993).

The alcohol used was absolute alcohol from Merck Laboratories.

The Nutritional paired isocaloric group (I) received the average amount of food (ration and liquid) amount by the alcoholic group in the previous day. In this group the treatment was started one day after the alcoholic group to find out the amount of alcohol and ration in order to calculate the saccharose diet (da SILVA, 1987).

The normal control group (N) received water and ration *ad libitum*.

Along the experiment animals of the three groups received consistently the same solid diet (Nuvilab CR 1 ration from NUTIVAL).

After 120 days of treatment animals were sacrificed for the removal of samples in the middle region of the *digastricus* and *rectus abdominalis* muscles to avoid intramuscular variability, according to the directions in a study by Hiiemae (1971). The latter muscle was used as control once the effect of ethanol on trunk muscles is already known.

Animals were sacrificed by anesthesia with an intraperitoneal initial dose of 30 mg/Kg of pentobarbital (Hypnol®). An extra doses of 70 mg/Kg was given for sacrifice.

From the samples several transversal to the longitudinal fiber axis10 mm slides were obtained, which were submitted to the m-ATPase reaction (with acid and alkaline pre-incubation) following the methodology of Werneck (1981) and the NADH-diaphorase according to Pearse (1968) modified by Dubowitz and Brooke (1973).

Fibers were classified according to Peter et al. (1972) in FG, FOG and SO. Several slides fields from the m-ATPase (in alkaline and acid pH) and NADH-TR reaction and a Zeiss micrometric scale were photographed with 10x magnification in an Olympus P-330N microscope. The identification and classification of muscle fibers were made by means of the copies of the slides submitted to the NADH-diaphorase and APTase in alkaline and acid pH reactions.

The microscopic files were selected at random and were fibers 300 identified and classified. Forty fibers of each type for each slide, for each muscle and each animal were used to calculate the area. The area calculation was done with an image analysis system model Image-Pro Plus 4.1 connected to a Pentium III microcomputer.

Data obtained on the frequency and area of the different types of fibers were submitted to variance analysis to evaluate the presence or not of significant differences among the studied groups and muscles. Whenever a significant difference was detected it was used the Test of Turkey was used to identify them (SNEDECOR; COCHRAN, 1980).

The present study was cleared by the Committee on Ethics in Research of the University of the Sacred Heart (USC).

RESULTS

Weight of animals at the beginning of the experiment and at the moment of sacrifice and the weight gain can be seen in TABLE 1.

TABLE 1 – Average weight of animals (g) in the beginning of the experiment and at the moment of sacrifice and the gain in weight (%) in groups N, A and I.

	Normal		Alcoholic		Isocaloric	
	mean	sd	mean	sd	mean	sd
Initial weight	271.2	28	258.3	18.6	276.68	27.2
Final weight	413.9	39	282.5	48.4	345.88	28.3
Weight gain in %	53.3	14,8	9.36	18	25.4	9.8

Dp – desvio padrão

The amount of calories ingested daily by groups is presented in TABLE 2.

TABLE 2 – Mean value for calories (Kcal) ingested daily by animals for groups N, A and I.

	Normal		Alcoholic		Isocaloric I	
	mean	Sd	mean	Sd	mean	Sd
Ingested ration	23.36	1.43	10.49	1.53	9.34	1.57
Produced calories	63.97		28.80		25.69	
Ingested liquid	49.65	4.82	22.84	3.70	21.41	4.03
Produced calories			32.44		30.71	
Total of calories	63.97		61.24		56.4	

Dp – desvio padrão

The nitrogen balance was calculated from the stools and urine from the animals and revealed the following values: Group N = 1.24, Group A= -3 and group I = -3.5

The blood test revealed presence of alcohol only in members in group A in average 0.22 g/l of blood (human index).

The fibers of the *digastricus* muscle and the *rectus abdominalis* responded in a similar way in all the studied groups and the results can be seen in TABLE 3.

TABLE 3 – Result of the m-ATPase and NADH-Tr reactions in samples of *digastricus* and rectus abdominalis muscle in groups N, A and I.

Fibers type	m-ATPase pH 10.6 Figure 1	m-ATPase pH 4,6 Figure 2	m-ATPase pH 4,45 Figure 3	NADH-Tr Figure 4
FG	+++	++	+	+
FOG	+++	+	+	++
SO	+	+++	+++	+++

+ weak reactivity ++ mild reactivity +++ strong reactivity

Based in the results of the reactions it is possible to say that the fibers of the *digastricus* muscle have the following characteristics:

– FG fibers – intense reactivity to m-ATPase with pre-incubation at pH 10.6 (FIGURE 1), moderate reactivity to m-ATPase with pre-incubation at pH 4.6 (FIGURE 2) and poor reactivity to m-ATPase with pre-incubation at pH 4.45 (FIGURE 3) as well as to BADH-Tr (FIGURE 4).

– FOG fibers – intense reactivity to m-ATPase with pre-incubation at pH 10.6 (FIGURE 1), poor reactivity to m-ATPase with pre-incubation at pH 4.6 (FIGURE 2) and pH 4.45 (FIGURE 3) and moderate reactivity to NADH-Tr (FIGURE 4).

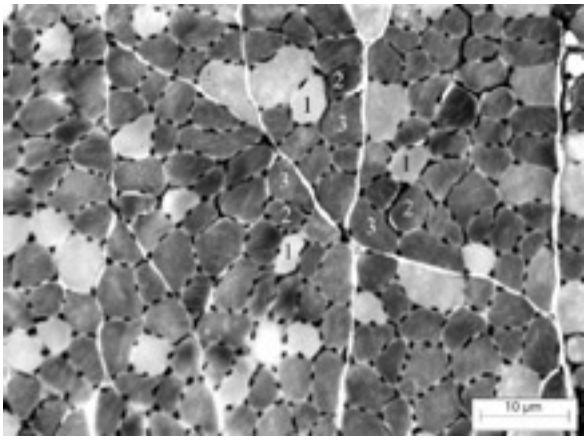

FIGURE 1 – Alcalin m-ATPase reaction in *digastricus* muscle (ph 10.6).
1 = SO fiber; 2 = FOG fiber and 3 = FG fiber.

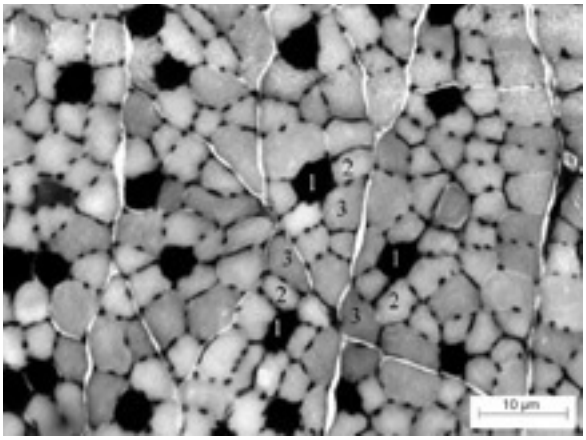

FIGURE 2 – Acid m-ATPase reaction in *digastricus* muscle (ph 4.6).
1 = SO fiber; 2 = FOG fiber and 3 = FG fiber.

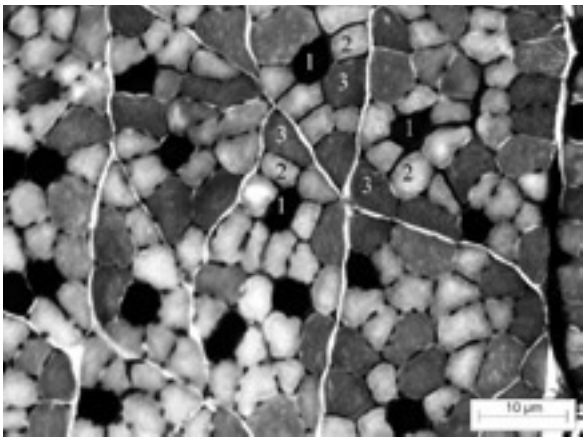

FIGURE 3 – Acid m-ATPase reaction in *digastricus* muscle (ph 4.45).
1 = SO fiber; 2 = FOG fiber and 3 = FG fiber.

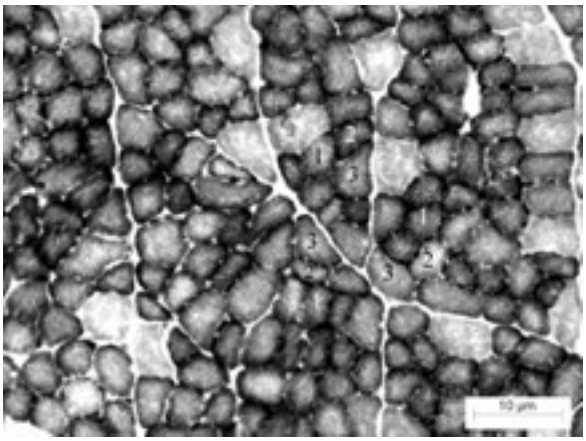

FIGURE 4 – NADH-Tr reaction in *digastricus* muscle.
1 = SO fiber; 2 = FOG fiber and 3 = FG fiber.

– SO fibers – weak reactivity to m-ATPase with pre-incubation at pH 10.6 (FIGURE 1) and intense reactivity to the others reactions (FIGURES 2, 3 and 4).

The mean values for area and frequency of the different fibers in the *digastricus* muscle, in groups N, A and I can be seen in TABLE 4 and 5, respectively.

TABLE 4 – Area (μm^2) of the different fibers types in the *digasticus* muscle of animals of groups N, A and I.

Fibers	Normal		Alcoholic		Isocaloric		F	P
	mean	sd	mean	sd	mean	sd		
FG	1.33b	0.17	0.99a	0.22	0.96a	0.15	6.50	0.012*
FOG	0.82b	0.10	0.65a	0.13	0.66	0.06	4.82	0.029*
SO	0.72	0.14	0.67	0.18	0.62	0.06	0.72	0.506

sd = standard deviation - Groups with same letter do not have statistical difference between them.

Regarding data for *digastricus* muscle the ANOCA revealed significant statistical difference between the groups in the area of fibers of types FG and FOG. However, there was no significant statistical difference between the groups in terms of area in the fibers of type SO.

TABLE 5 – Frequency (%) of the different types of fibers of the digastricus muscle in animals of groups N, A and I.

Fibers	Normal		Alcoholic		Isocaloric		F	P
	mean	sd	mean	sd	mean	sd		
FG	30.9	4.54	34.8	4.44	32.2	5.60	1.24	0.321
FOG	63.1	5.41	56.3	5.47	59.9	4.57	1.39	0.285
SO	6.0	2.42	8.9	1.97	7.9	2.73	2.01	0.176

sd = standard deviation

In the area of fibers of the FG type the Test of Turkey revealed that the significant statistical difference was between groups N and A and N and I. In the fibers of type FOG this test identified statistical significant difference between groups N and A.

In what regards the frequency of the different fibers found in the *digastricus* muscle the ANOVA did not detect statistical significant difference among the groups.

Data on area average and frequency average of the different types of fibers in the *rectus abdominalis* muscle in groups N, A and I can be seen in TABLES 6 and 7.

TABLE 6 – Área (μm²) of the different types of fibers of the rectus abdominalis muscle in animals of groups N, A e I.

Fibers	Normal		Alcoholic		Isocaloric		F	P
	mean	sd	mean	sd	mean	sd		
FG	6.43a	0.91	3.31b	0.91	4.77c	0.38	20.40	0.000*
FOG	2.72	0.82	1.87	0.46	1.90	0.19	3.78	0.053
SO	0.93	0.14	0.69	0.15	0.83	0.32	1.60	0.241

sd = standard deviation - Groups with same letter do not have statistical difference between them.

TABLE 7 – Frequency of the different types of fibers in the rectus abdominalis muscle of animals of groups N, A e I.

Fibras	Normal		Alcoholic		Isocaloric		F	P
	mean	sd	mean	sd	mean	sd		
FG	63.6a	1.84	60.9ab	4.09	57.7b	5.59	4.04	0.045*
FOG	22.3a	3.84	23.7ab	2.18	28.3b	5.16	4.08	0.044*
SO	14.1	2.98	15.4	3.80	14.1	5.93	0.13	0.873

sd = standard deviation - Groups with same letter do not have statistical difference between them.

Regarding the area of the different types of fibers in the *rectus abdominalis* muscle ANOVA detected statistical significant difference among the groups only for fibers type FG. However, values for FOG fibers were close to a statistical significant difference. The test of Tukey revealed that the difference in area for the FG type was between groups N and A and N and I and also between A and I.

In what refers to data about the frequency of the different types of fibers in the *rectus abdominalis* muscle the ANOVA test revealed statistical significant difference among the groups both to

fibers FG and FOG. The test of Tukey showed that this difference was between the groups N and I, both in fibers FG and FOG.

The Pearson correlation of data revealed a correlation between the area of FG fibers and the weight of the animals in the *rectus abdominalis* muscle (r = 0.7605; p = 0.001). For the *digastricus* muscle, in which the correlation was not detected, values were close to the correlation (r = 0.4780; p = 0.072).

DISCUSSION

Many studies on the effects of alcohol in the striated muscle are available in the literature. Some of them were done in humans and other in animals, and rat is the most indicated animal as an ideal experimental model since it presents a selective atrophy for type II fibers similar to what occur in human (TROUNCE et al., 1990; PREEDY et al., 1990; SALISBURY et al., 1992; PREEDY et al., 1994).

It is recognized that the striated muscles respond to the functional demand (PHELPS, 1984). Thus, the percentage and the area of the different types of fibers found in chewing muscles varies from animal to animal according to their feeding habits.

In histoenzimologic research on the chewing muscles, the *digastricus* (anterior face) is one of the least studied.

Among non-human primates the most frequent type of fiber is variable. Some authors consider that those of slow contraction (types I or SO) (CLARCK; LUSCHEI, 1981; MAXWELL et al. 1981) are more frequent. For others, the FG types are more frequent (ANDRO et al., 1994). In what regards the fiber area, opinions are less variable, pointing type IIB (MAXWELL et al., 1981) or FG (ANDREO et al., 1994) as the ones with greater area.

In humans type IIB fibers were more frequent (ERIKSSON et al., 1982).

In rats the *digastricus* muscle is considered as a mandible depressor (SFONDRINI et al., 1996) and there are few studies on histoenzimology of its fibers: Bennet et al. (1977), in a study with adult and growing animals, observed only two types of clearly distinct fibers; and Rokx et al. (1984), who classified fibers of this muscle in four types being three of quick contraction and one of slow contraction. These authors observed that the *digatricus* muscle (anterior belly) had a high percentage of rapid contraction fibers; Kiliaridis et al. (1988), studying the influence of the consistency of food in the area of different fiber types in this muscle observed that the fibers of quick contraction (FG and FOG) were not influenced by this factor.

In the present study, in the *digastricus* muscle of normal, alcoholic and isocaloric rats it was observed that quick contraction fibers (FG+FOG) are more frequent that those of slow contraction what agrees with results by Roky et al. (1984) and that they are also greater in area.

In relation to the effect of alcohol on the *digastricus* muscle it can be said that it was similar to the one on *rectus abdominalis* muscle, which is a somitic muscle, showing a statistical significant reduction in the area of fibers of rapid contraction (FG and FOG) what was not observed in the slow contraction fibers (SO). These data are similar to those reported in previous studies (TROUNCE et al., 1990; CHEN et al., 1991; PREEDY et al., 1994; SESTOFT et al., 1994; REILLY et al. 1998) on muscles of trunk and limbs.

In the *rectus abdominalius* muscle the reduction in the number of fibers of the FOG type was not statistically significant although values were close to that. Perhaps with a greater sample this could have occurred.

The results in the alcoholic and isocaloric groups were similar both for the *digastricus* muscle and the *rectus abdominalis*. Although greater in the isocaloric group, the results were never with statistical difference between them but the area of FG fibers in the *rectus abdominalis* muscle.

In trunk and limb muscles the presence of grouping in the fibers, indicating denervation followed by reinervation, has been confirmed in muscle biopsies of alcoholic patients or animals (FERRAZ et al., 1989; SHARMA et al., 1990; CHEN et al., 1991) and refuted by others (SALISBURY et al., 1992; PREEDY et al., 1994). In the present study this aspect could not be considered since the presence of grouping are said to be normal in chewing muscles (DUBOWITZ; BROOKE, 1973).

In what regards the effect of alcohol on slow contraction fibers, or type I fibers, the literature shows conflicting data. Some authors consider that this type of fiber is not affected by alcohol (MARTIN et al., 1985; FERNÁDEZ-SOLÁ et al., 1995; REILLY et al., 1998) and others consider that type I fibers may suffer slight hypertrophy (PREEDY et al., 1989; SALISBURY et al., 1992; PREEDY; PETER, 1994). According to Preedy et al. (1994), in special circumstances, a chronic alcoholic may present atrophy of type I fibers but always in a lesser extent than for type II fibers. In type SO fibers in the present study there was a slight reduction in the area of these fibers but the difference was not significant form the statistical point of view between the groups.

The selective effect of atrophy of rapid contraction fibers in alcoholic patients have been considered as independent of their nutritional status by some authors (MARTIN et al., 1985; SALISBURY et al., 1992). However, Carpenter and Karpati (1984) considered that in chronic alcoholics the development of atrophy of type II fibers could be due to other causes such as of malnutrition. These authors reported also that, in previous studies, the atrophy of type II fibers was detected in about 30% of chronic alcoholics being more frequent in cases with associated malnutrition, peripheral neurophaty and other diseased s related to ethanol.

Despite the objective of the present study not being related to the nutritional status of the rats an important statement should be made regarding the isocaloric rats, the ones considered as control group. Animals of this group experience less development when compared to rats from group N, similar to the results of Preedey et al. (1988). According to these authors this aspect can affect the skeletal fibers. Therefore, in this study the obtained data in animals of the alcoholic group are similar to those of the isocaloric group, which, to some extent, can be considered as undernourished. Goldspink and Ward (1979) stated that food below an optimal amount induce morphological differences in skeletal muscle.

It should be remembered that, according to Bunout et al. (1987) the alcoholic miopaty can be partially attributed to a negative nitrogen balance in alcoholic patients. In the present study, the nitrogen balance of both alcoholic and isocaloric groups was negative. It was also observed that the isocaloric group, although receiving a smaller average daily caloric intake than the alcoholic group (56.4 Kcal e 61.24 Kcal respectively), had a greater weight gain despite the fact that the difference was not statistically significant.

Researchers consider that the type IIB (anaerobic glycolytic rapid contraction fibers) are the rapid contraction fibers that are most affected by alcohol, leading to a greater reduction in their size (TROUNCE et al., 1990; CHEN et al., 1991; PREEDY et al., 1994; SESTOFT, et al., 1994; REILLY et al., 1998;), which is similar to the finding of the present study in which the FG fibers were those with greater reduction in size in the studied muscles.

Some studies stated that the consumption of alcohol can shape the expression of the myosin isoform (MHC) of striated muscles, increasing the expression of type I MHC and decreasing the expression of the types IIA and IIB MCH (SESTOFT et al., 1994). In the present study there was an increase n the frequency of SO (I) fibers in the group A of both studied muscles, although this difference was not statistically significant.

A fact that calls attention and deserves further studies is that in the *digastricus* muscle the decrease in the number of FG fibers in the alcoholic group was 25.6% whereas in the muscle *rectus abdominalis* was 48.5%. What is the explanation to this finding: the embryologic origin; the different inervation; or the different functional demand?

CONCLUSION

Based in the results from the present study it is possible to conclude that the digastricus muscle of rat is formed by three types of fibers, two of rapid contraction (FOG and FG) and one of slow contraction and that the fibers of rapid contraction are more frequent (FOG>FG) and have greater area (FG>FOG) than that of slow contraction (SO); ethanol causes atrophy of fibers of rapid contraction similar to the one observed in the trunk muscles; however, the modification in the chewing muscles seems to be less than in the trunk muscles both in the size and frequency of fibers.

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