Profile of the cell body of nadh-diaphorase positive myenteric neurons from the stomach of rats (*Rattus norvegicus*) subjected to chronic alcoholism

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ABSTRACT. The purpose of this work was to compare the profile of the cell body of the NADH-diaphorase positive myenteric neurons from the stomach of rats. It was used 10 animals (*Rattus norvegicus*), from groups a) control (n=5), that during 210 days had *ad libitum* supply of chow with normal protein level (22%) and water; and b) experimental (n=5), that during 210 days had *ad libitum* supply of chow with normal protein level (22%) and sugar cane brandy diluted to 30 Gay Lussac (30º v/v). The stomachs collected and subjected to the technique for neuronal staining. The cell body of the neurons (n=1.000) was measured through a computerized system of image analysis. The profile of the neurons from the control rats ranged from 60.16 and 638.64 μm^2. In the experimental group the values ranged from 40.84 to 599.15 μm^2. We observed a significant decrease on the cell body size, increase of the small neurons and decrease of the large neurons.

Key words: stomach, myenteric neurons, rat, alcoholism.

RESUMO. **Perfil do corpo celular de neurônios mioentéricos nadh-diaforase positivos do estômago de ratos (*Rattus norvegicus*) submetidos ao alcoolismo crônico**. O objetivo do trabalho foi comparar o perfil dos corpos celulares dos neurônios mioentéricos NADH-diaforase positivos do estômago de ratos. Utilizou-se 10 animais (*Rattus norvegicus)*, provenientes dos grupos: a) controle (n=5), que durante 210 dias receberam, *ad libitum,* dieta com teor protéico normal (22%) e água; e b) experimental (n=5), que durante 210 dias receberam, *ad libitum*, ração com teor protéico normal (22%) e aguardente-de-cana diluída a 30 Gay Lussac (30º v/v). Os estômagos coletados foram submetidos à técnica de evidenciação neuronal. A mensuração do perfil dos corpos celulares dos neurônios (n=1.000) foi através de um Sistema Computadorizado de Análise de Imagem. O perfil dos corpos celulares dos neurônios do grupo controle ficou entre 60,16 a 638,64 μm^2. No grupo experimental variou de 40,84 a 599,15 μm^2. Constatamos redução significante no tamanho do corpo celular, aumento de neurônios pequenos e diminuição de neurônios grandes.

Palavras-chave: estômago, neurônios mioentéricos, rato, alcoolismo.

Introduction

The myenteric plexus, an integral part of the enteric nervous system, has a fundamental role on the functions carried out by the gastrointestinal tract. This plexus has varied organization, location and constitution, when its phylogenesis (Irwin, 1931; Matsuo, 1934; Burnstock, 1959; Rash and Thomas, 1962; Bennett and Cobb, 1969; Gabella, 1979; Souza *et al*., 1982, 1988; Christensen *et al*., 1984; Timmermans *et al*., 1991; Molinari *et al*., 1994; Stabille *et al*., 1998) or its appearance in the organs of the gastrointestinal tract (Gabella, 1972; Barbosa, 1973; Faussone-Pellegrini, 1992; Sant'Ana *et al*., 1997a; Miranda-Neto *et al*., 2001) are concerned.

As for the rat stomach, the ganglia of the myenteric plexus of the aglandular region are found between the longitudinal and circular smooth muscle layers of the outer muscle tunica; on the glandular region they are also found embedded in the longitudinal layer of the muscle tunica (Oliveira *et al*., 2000). The organization, the arrangement of the ganglia, and the number of myenteric neurons in

the plexus are different when the aglandular (Oliveira *et al*., 2000) and glandular (Fregonesi *et al*., 1998; Oliveira *et al*., 2000) regions of the stomach are compared. In the aglandular region, it is observed a greater density of neurons next to the central region of the limiting fold than on the region next to the greater gastric curvature (Molinari *et al*., 2002); on the glandular region, the neuronal density is greater next to the lesser gastric curvature (Fregonesi *et al*., 1998; Oliveira *et al*., 2000).

The morphology and the number of neurons of the myenteric plexus have been also studied in animals subjected to different conditions, such as protein malnutrition (Santer and Conboy, 1990; Natali and Miranda-Neto, 1996; Sant'Ana *et al*., 1997b; Miranda-Neto *et al*., 1999), protein and vitamin malnutrition (Sant'Ana *et al*., 2001), diabetes (Zanoni *et al*., 1997; Furlan *et al*., 1999; Fregonesi *et al*., 2001), Chagas disease (Maifrino, 1996; Moraes, 1997), and aging (Gabella, 1971; Santer and Baker, 1988; Santer, 1994; Gomes *et al*., 1997; Johnson *et al*., 1998). However, we did not find in the literature any work mentioning the effects of alcohol on the morphology of the myenteric neurons from the stomach.

It is known that alcohol is a toxic substance capable of producing changes in various organs, including the digestory system. Acute effects of alcohol on the gastric mucosa were first studied by Beaumont in 1933 and Wolf and Wolff in 1944 (Mincis *et al*., 1995).Gastric absorption of alcohol is related to the alcohol dehydrogenase (ADH) activity of the stomach, that is, the greater the activity of this enzyme, the smaller the ethanol absorption (Mincis, 1994).

Among other effects, ethanol impairs vitamin absorption, which can lead to peripheral neuropathies, initially due to axonal changes and often followed by myelin sheath and even pericaryon destruction (Levy, 1991). In mice subjected to chronic alcoholism, it was observed degeneration of the intrapancreatic nerve fibers (Berger and Fehér, 1997).

Once the myenteric plexus is formed by neurons and nerve fibers located in the muscle tunica, and alcohol has a damaging action on the mucosa and also causes neuropathies, we believe that it is of great interest the study of the action of alcohol on the morphology of the neurons from this plexus, because it is fundamental for the perfect functioning of the stomach. In this way, the purpose of this work was to evaluate the profile of the cell bodies of the NADH-diaphorase (NADH-d) positive myenteric neurons from the stomach of rats subjected to experimental chronic alcoholism during 210 days, and thus establish possible effects of alcohol on the morphometry of the neuronal cell body.

Material and method

It was used the stomach of 10 laboratory rodents, adult male *Rattus norvegicus*, albine, from the Wistar strain, supplied by the Central Biotery of the State University of Maringá, state of Paraná. The animals, with 60 days, were placed in the Sector Biotery of the Department of Morphophysiological Sciences/UEM and kept in individual cages under constant temperature and light-dark cycles of 12 hours, during 210 days. They were divided in two groups: a) control animals (n=5), receiving NUVILAB rat chow (recommended by the National Research Council and National Institute of Health – USA) with normal protein level (22%) and water, both *ad libitum*; and b) experimental animals (n=5), having NUVILAB chow with normal protein level (22%) and sugar cane brandy (Trademark "51", 39 GL, Muller Industries, Pirassununga, state of São Paulo, Brazil) diluted to 30 Gay Lussac (30º v/v) (Cagnon *et al*., 1996; Martinez *et al*., 1997; Pereira, 1997), both *ad libitum*. The rats were handled according to the rules established by the Brazilian College of Animal Experimentation (COBEA) and the Law 6638 of 08/05/1979 (Brasil, 1979).

After the 210-day period the animals were killed through inhalation of ethylic ether. The stomach of the 10 animals were washed, filled with Krebs solution (pH 7.3), washed twice (10min each) in Krebs and immersed for five min in Triton X-100® at 0.3% in Krebs. They were washed twice again (10 min each) in Krebs and incubated for 45min for evidenciation of the NADH-d. This medium contained in each 100mL; 25mL stock solution of Nitro Blue Tetrazolium (NBT: Sigma, St. Louis, USA) at 0.5%; 25mL phosphate buffer 0.1M pH 7.3; 50ml distilled water and 50mg β-NADH (Sigma: Steiheim, Germany) (Gabella, 1969). After incubation the segments were opened and placed in buffered formol.

The stomachs were sectioned along the greater and lesser gastric curvatures; then, the aglandular and the glandular regions were split through an incision at the level of the limiting fold. Both parts of the stomach were dissected under stereomicroscope with trans-illumination, by removal of both the mucosa and submucosa. The resultant whole-mounts were dehydrated in ascending series of ethyl alcohol, diaphanized in xilene and mounted on slide with coverslip fixed

with Permount® synthetic resin (FischerChemical, New Jersey, USA).

For the analysis of the cell body profile it was measured the profile of neurons from the regions of the aglandular stomach (central region next to the limiting fold and region next to the greater gastric curvature) and of the glandular stomach (regions next to the greater and lesser gastric curvature), totaling 1,000 neurons in each group. The neurons were visualized with an Olympus BX40 light microscope under 40X objective, and their profiles were measured through a computerized system of image analysis (Image-Pro Plus 3.0.1).

For each region analyzed, it was obtained the mean and SEM of the neuronal size in each group. These values were compared through Student's t text at the significance level of 5%.

Results

The rats from the control group, at the beginning of the experiment, weighted on average 323.8g and after 210 days had a body weight of 508.8g. The alcoholic group weighted 310.2g and reached a final body weight of 407.6g. The alcoholic group had a body weight gain about 19.89% smaller than the rats from the control group.

Through the measuring of the profile of the cell body of the neurons it was observed that the myenteric nervous plexus from both the aglandular and glandular stomachs is composed of nerve cells with differently sized cell bodies, which could be classified as small, medium and large.

In the glandular stomach of the control rats, the cell body of the neurons had 220.05 ± 7.15 μm^2 and 236.56 ± 7.37 μm^2 on the lesser and greater gastric curvatures, respectively; and on the aglandular stomach 285.90 ± 7.34 μm^2 and 211.31 ± 7.63 μm^2 on the limiting fold and greater gastric curvature, respectively.

For each of the four regions analyzed, the mean and its respective standard deviation of the neuronal size of the control values were used to classify the neurons of both groups in small, medium and large, so that small neurons were those smaller than the mean minus its standard deviation, large neurons were those greater than the mean plus its standard deviation, and medium neurons were those intermediate to these values.

The classification of the neurons according to their sizes, on the control group, are presented in Table 1.

Table 1. Variation of the cell body profile (in μm^2) of the small, medium and large myenteric neurons of the glandular stomach, in the regions next to the lesser (A) and greater (B) gastric curvatures; and of the aglandular stomach, in the regions next to the limiting fold (C) and greater gastric curvature (D), of rats pertaining to the control group.

Classification	A	B	C	D
Smaller	77,60 – 105,30	60,16 – 119,80	80,79 – 168,29	60,96 – 90,07
Medium	108,86 – 333,04	120,56 – 352,98	169,93 – 400,24	90,72 – 330,47
Large	333,40 – 589,33	353,42 – 521,37	402,10 – 638,64	335,93 – 572,93

The number of small, medium and large neurons in each region of the control rats is demonstrated in Figure 1.

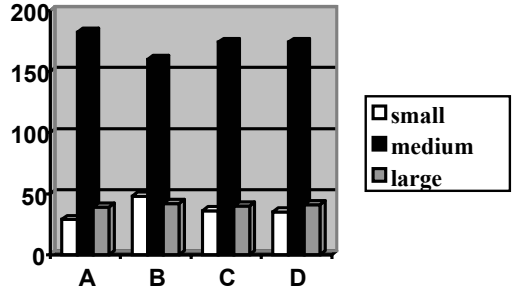

Figure 1. Number of small, medium and large neurons of the glandular stomach in the regions next to the lesser (A) and greater (B) gastric curvatures; and of the aglandular stomach, in the regions next to the limiting fold (C) and greater gastric curvature (D), of rats pertaining to the control group (n=250 per region).

On the glandular stomach of the alcoholic rats the cell body of the neurons had on average 185.29 ± 4.26 μm^2 and 161.89 ± 5.66 μm^2 for the regions next to the lesser and greater gastric curvatures, respectively; on the aglandular stomach they had 215.38 ± 6.01 μm^2 and 186.70 ± 6.49 μm^2 for the regions next to the central portion of the limiting fold and the greater gastric curvature, respectively.

The cell body profile of the neurons of the rats subjected to alcoholism are demonstrated in Table 2.

Table 2. Variation of the cell body profile (in μm^2) of the small, medium and large myenteric neurons of the glandular stomach, in the regions next to the lesser (A) and greater (B) gastric curvatures; and of the aglandular stomach, in the regions next to the limiting fold (C) and greater gastric curvature (D), of rats pertaining to the alcoholic group.

Classification	A	B	C	D
Smaller	40,84 – 106,81	42,65 – 119,90	44,21 – 167,89	44,07 – 88,43
Medium	109,20 – 330,69	120,33 – 341,14	169,82 – 392,82	92,04 – 330,62
Large	341,26 – 476,68	369,22 – 408,15	410,67 – 584,55	332,96 – 599,15

Figure 2 shows the number of small, medium and large neurons, in each region, in the rats of the alcoholic group.

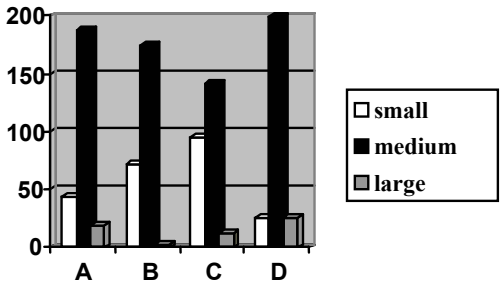

Figure 2. Number of small, medium and large neurons of the glandular stomach in the regions next to the lesser (A) and greater (B) gastric curvatures; and of the aglandular stomach, in the regions next to the limiting fold (C) and greater gastric curvature (D), of rats pertaining to the alcoholic group (n=250 per region).

The mean sizes of the neurons of the four gastric regions of each group were statistically compared. This analysis demonstrated a markedly significant reduction ($p<0.01$) on the size of the cell body of the neurons of the alcoholic animals in all the regions evaluated.

Discussion

The experimental condition of water-diluted alcohol offer during 210 days imposed to the rats a decrease in body weight gain of about 19.89%, demonstrating that the alcohol altered the body development of the rat. The action of experimental alcoholism on body weight was verified also in other investigations (River and Vale, 1983; Pereira, 1997; Pereira *et al*., 2003).

The mean of the cell body profile found in the NADH-d positive neurons of the control rats was 220.05 μm^2 and 236.56 μm^2 in the regions next to the lesser and greater gastric curvatures of the glandular stomach, respectively, and 285.90 μm^2 and 211.31 μm^2 in the regions next to the central portion of the limiting fold and greater gastric curvature of the aglandular stomach, respectively. Research carried out in other organs using a similar method evidenced in the duodenum cell bodies averaging 234.80 μm^2 and 224.50 μm^2 in the antimesenteric and intermediate regions, respectively (Miranda-Neto *et al*., 2000); and in the proximal colon 219.20 μm^2 (Furlan *et al*., 2002). Thus, our data in the stomach are similar to those in other organs of the gastrointestinal tract.

In this investigation the measurement of the cell body profiles of the NADH-d positive neurons of the glandular and aglandular regions of the stomach of control and experimentally alcoholic animals allowed us to observe that alcohol affects the neurons in the sense that it diminishes their cell body size. For instance, when we compare the profile of the smaller cell body of the neurons of the limiting fold of the aglandular stomach of the control rats (80.79 μm^2) with that of the animals subjected to chronic alcoholism (44.21 μm^2), we verify that the latter is almost 55% smaller.

Decreases on the cell body size of NADH-d positive myenteric neurons were seen in rats receiving chow with protein restriction (8%) (Natali and Miranda-Neto, 1996) and in rats subjected to experimental diabetes (Furlan *et al*., 2002).

In all the regions analyzed, medium neurons predominate. When the number of small and large neurons are compared between the groups studied, it is observed a marked reduction in the incidence of large neurons and an increase of small neurons in the alcoholic group. This reinforces the observation that the alcohol treatment causes reduction on the size of the myenteric neurons of the stomach. The data obtained are similar to those found by Pereira *et al*. (2003) in the ileum of rats subjected to chronic alcoholism.

The animals of the alcoholic group had reduced size of the neuronal cell bodies regardless of the region analyzed. These same animals had a body weight gain reduction of about 19.89%. These data show that the treatment impaired both the whole body and the neuronal development.

We believe that the reduction of the neuronal cell body may reflect on metabolic alterations that will affect gastric motility. Our findings reinforce that alcohol is a toxic substance, as highlighted by many other authors, that verified changes in secretion (Alves, 1985; Chacin *et al*., 1991), gastric emptying (Alves, 1985; Pfeiffer *et al*., 1992) and gastric permeability (Keshavarzian *et al*., 1994).

As alcohol can increase gastric secretion and delay gastric emptying, it creates conditions for lesion of the gastric wall that can lead to gastritis, and in some instances small ulcerations or lacerations can cause hemorrhage (Alves, 1985; Mincis *et al*., 1995). Alcohol is capable of disrupting the gastric mucosa and diminish mucus production, mucosa resistance and electric potencial difference. It is also observed inhibition of the active transport of sodium, potassium, hydrogen and chloride ions and protein loss. Alcohol diffuses into the epithelial cells, causing their destruction and changing the

neighboring blood capillaries (Zeitune *et al*., 1991). The hydrogen ions that diffuse through the gastric epithelium cause cellular destruction and lesion in a vicious cycle, contributing to the progressive atrophy of the gastric mucosa and eventually, generalized gastric atrophy. In gastric atrophy, there is loss of the stomach secretions, resulting in achlorhydria and, in some instances, pernicious anemia due to the deficient absorption of vitamin B12 (Guyton and Hall, 1996). Vitamin B12 is essential for the normal metabolism of all cells, especially the gastrointestinal tract, nervous tissue and growth (Pallaoro, 1997).

Because of the gastrointestinal and hepatic complications, alcoholism interferes also with the nutritional state, once it difficults food digestion and nutrient absorption (Lieber, 1988, 1991).

Conclusion

The ingestion of water-diluted alcohol during 210 days had a damaging action on the morphology of the cell body profile of the NADH-diaphorase positive neurons myenteric of the stomach. We observed a significant reduction in the size of the cell bodies, and increase in the number of small neurons and decrease in the number of large neurons.

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