Endocrine cells and nerve ganglia of the small intestine of the Opossum *Didelphis aurita* Wied-Neuwied, 1826 (Mammalia: Didelphidae)

GLÁUCIA M. FREITAS-RIBEIRO[1*], **CLÁUDIO C. FONSECA**[2], **SIRLENE S.R. SARTORI**[4], **ALAN LOURES-RIBEIRO**[3] and **CLÓVIS A. NEVES**[5]

[1]Departamento de Biologia Molecular, CCEN, Cidade Universitária, Castelo Branco,
Universidade Federal da Paraíba, 58051-900 João Pessoa, PB, Brasil

[2]Departamento de Veterinária, Campus Universitário, Universidade Federal de Viçosa,
Avenida Peter Henry Rolfs, s/n, 36570-000 Viçosa, MG, Brasil

[3]Departamento de Sistemática e Ecologia, CCEN, Cidade Universitária, Castelo Branco,
Universidade Federal da Paraíba, 58051-900 João Pessoa, PB, Brasil

[4]Departamento de Biologia Animal, Campus Universitário, Universidade Federal de Viçosa,
Avenida Peter Henry Rolfs, s/n, 36570-000 Viçosa, MG, Brasil

[5]Departamento de Biologia Geral, Campus Universitário, Universidade Federal de Viçosa,
Avenida Peter Henry Rolfs, s/n, 36570-000 Viçosa, MG, Brasil

ABSTRACT

The nervous and endocrine systems jointly control intestinal movements, secretions of their glands and also participate of the processes of nutrient digestion and absorption. Therefore, the central objective of this study was to verify the existence of a possible relationship between the number of nervous cells and ganglia of the submucosal and myenteric plexuses and the number of endocrine cells in the small intestine of adult *D. aurita*. The utilized staining techniques were Grimelius, modified Masson-Fontana, direct immunoperoxidase and H-E. Argyrophillic, argentaffin and insulin immunoreactive endocrine cells do not numerically vary between the initial, mid and final regions of the duodenum, jejunum and ileum ($P>0.05$), except for argyrophillic cells in the jejunum ($P>0.05$). No numerical relationship has yet been verified between the number of nerve ganglia and endocrine cells, and also between nervous and endocrine cells. We recommended the use of new immunohistochemical techniques to confirm the numerical correlation between the nervous and endocrine systems in the small intestine. The morphology and distribution of endocrine cells and the nerve ganglia studied were similar to those encountered in eutherian mammals.

Key words: argyrophillic cells, argentaffin cells, endocrine cells, ganglia, insulin.

INTRODUCTION

Marsupials of the genus *Didelphis* have already been utilized as a model in studies of enteroendocrine cells, aiding in aspects of their identification,

distribution, quantification (Krause et al. 1985, Barbosa et al. 1987, Takagi et al. 1990) and ontogenesis (Krause et al. 1989, Fonseca et al. 2002).

Enteroendocrine cells can be classified as argyrophillic or argentaffin by their capacity to retain and reduce silver salts in both open and closed cells, in accordance with the apical communication

with the lumen and in hormone producing cells (insulin, secretin, somatostatin and others), principally based on the characteristics and content of their secreting granules (Grimelius and Wilander 1980, Polak et al. 1993, Santos and Zucoloto 1996).

Insulin producing endocrine cells have been immunolocalized in extrapancreatic regions, as in the prostate, nephron, central nervous system, retina and intestine (Coutinho et al. 1985, Stahler et al. 1988, Bendayan and Park 1991, Devaskar et al. 2002, Meimaridis et al. 2003). It is speculated that in the intestine, insulin may precipitate glucose not absorbed from the digestive residue, reducing peristalsis to provide sufficient time for a more efficient absorption as well as control secretion of other peptides of the same cell or neighboring cells, mortality and intestinal absorption (Kendzierski et al. 2000).

In addition to the endocrine control of the gastrointestinal functions performed by the enteroendocrine cells, there is also the neural control performed by nerve cells present on the wall of the digestive tube, pancreas and biliary system, constituting the enteric nervous system (Furness 2000). This system contains two ganglion plexuses in the intestine, the myenteric and submucosal plexuses, where the majority of intrinsic nerve cells reside. Neurons in the enteric nervous system of the small intestine have been identified in terms of their morphologies, projections, primary neurotransmitters and physiological identifications. In this region there are 14 functionally defined neuron types, each with a characteristic combination of morphological, neurochemical and biophysical properties. Most neurons are characterized as sensorials (afferent), motors (efferent) and interneurons (Furness 2000). The nervous and endocrine systems jointly control intestinal movements, secretion of its glands and participate indirectly in the processes of nutrient digestion and absorption (Rodrigues et al. 2005).

The enteric and endocrine nervous systems are involved in many physiological processes and even in pathological processes of the digestive tract,

which are essential to normal life (De Giorgio et al. 2000). Therefore, it is necessary to better understand the distribution of ganglions and endocrine cells dispersed along the small intestine of mammals.

Based on these factors, the objective of the present work was to quantify argyrophillic, argentaffin and insulin immunoreactive endocrine cells in the initial, mid and final regions of the duodenum, jejunum and ileum of opossums (*Didelphis aurita*) weighing more than 400 g (adults), and to quantify the ganglia of the submucosal and myenteric plexuses and nervous cells of ganglia in segments of the small intestine. Since regulation of events linked to digestion is performed by the integrated action of the nervous and endocrine systems, we verified that endocrine and nervous cells are somehow related to ganglia. Finally, we performed a descriptive analysis of several morphometric parameters of the mucous, submucous and muscle layers of the small intestine.

METHODS

Ten adult opossums, both male and female, from the species *D. aurita* were used in this experiment. The animals were captured between January and June 2007 at Viçosa, Minas Gerais State, Brazil. Hook traps were placed to capture the opossums using bates composed of pineapple and cotton impregnated with cod liver oil. The animals weighing more than 400 g (adults) were euthanized with a general anesthetic (sodium pentabarbital), followed by the administration of potassium chloride. The capture of these animals was authorized by IBAMA (license n. 10168-1) and the experiment was evaluated by the Ethics Commission of the Veterinary Department of the Federal University of Viçosa (process n. 56/2007).

Two fragments (1 cm^2) were collected from the initial, mid and final regions of each intestinal segment per animal. They were fixed for 24h in Bouin's liquid for staining by the Grimelius

technique (Grimelius 1968), the direct immuno-peroxidase technique (Sternberger 1979) and the H-E techniques (Bancroft and Stevens 1996), and in 10% buffered formalin for the modified Masson-Fontana technique (Barbosa et al. 1984). The fragments were then dehydrated, diaphanized, embedded in paraffin and sectioned 5 μm width with the assistance of a manual rotating microtome (model Leica, RM2155). Each slide had four sections of the same fragment, each one being placed 30 μm far from each other during microtomy. It produced a total of six slides for each region. Therefore, a total of 24 sections were analyzed. After removing the paraffin, the histological sections were hydrated and stained. The staining techniques aimed to identify and quantify argyrophillic cells (Grimelius), argentaffin cells (modified Masson-Fontana), insulin immunoreactive (IR) cells (direct immunoperoxidase), submucosal and myenteric ganglia and nervous cells (H-E techniques).

In the argyrophillic reaction (Grimelius technique), the silver salts in ammoniacal, aqueous or alcoholic solution bond to the cytoplasmic granules, being then reduced to silver metal by the exposure to an exogenous reducing substance. In the argentaffin reaction (modified Masson-Fontana technique), a reduction in the ammoniacal silver nitrate is a result of the reducing capacity of its cellular components (Rodrigues et al. 2005).

The antibodies used in the immunohisto-chemical technique were produced by the Bethyl laboratory, lot n. A90-117P-4, and the opossum pancreas was used as a positive control. Processing of the material was performed at the Laboratory of Structural Biology of the General Biology Department, Federal University of Viçosa.

Quantification of the argentaffin, argyrophillic and insulin immunoreactive cells was performed in six random fields of the mucosa sections, which were defined by the extension of the ocular micrometric scale coupled to a 10X ocular, equivalent to 300 μm in extension and with an objective of 40X. Likewise

we counted the nervous cells in the submucous and myenteric ganglia. The area of the mucosa was obtained from the average thickness multiplied by the extension of the micrometric scale. The average number of endocrine cells was registered for each mm^2 of the mucosa layer, and likewise the average number of nervous cells in muscular and submucous layers. The mucous, submucous and muscle areas were obtained from its average thickness multiplied by the length of the micrometric scale.

For the quantification of submucosal and myenteric ganglia, a 10X objective was used, where six fields of submucous and muscular layers were studied, respectively. These fields were delimited by the extension of the ocular micrometer scale equivalent to 1000 μm in extension. An average number of endocrine cells per mm^2 of the mucous layer area was registered, as well as the number of submucosal and myenteric ganglia for each mm^2 of submucous and muscular layers, respectively. The mucous, submucous and muscular areas were obtained by multiplying the average width by micrometric scale.

To verify differences in terms of the number of endocrine cells in the initial, mid and final regions of each segments, and the number of submucosal and myenteric ganglia between the intestinal segments (duodenum, jejunum and ileum), the Kruskal-Wallis test (H) was employed. The possible relationship between the number of endocrine cells and submucosal and myenteric ganglia, and the number of endocrine and nervous cells was tested by a regression analysis ($P < 0.05$) where the *rs* described in the tables represent the Spearman Coefficient.

RESULTS

The mucous layer of the small intestine of *D. aurita* generally consists of digitiform villosities in all three segments (Figure 1). In the mucous layer of the duodene there are intestinal glands or Brünner glands, and still a muscular layer. Several villi with multiple

heads were found in all segments. The submucous layer with a connective tissue of mucous showed a lot of neurons, forming the Meissner or submucous plexus (Figure 2). The muscular layer also showed neurons of myenteric plexus (Auerbach plexus) (Figure 3). This layer was constituted by two layers of smooth muscles, an inner circular layer and other external longitudinal layer. The neurons of the myenteric plexus were located between these two layers.

Regarding the morphometry of the intestinal tube (Figure 1), the mucous layer had an average thickness of 508.93 ± 177.69 μm; the average
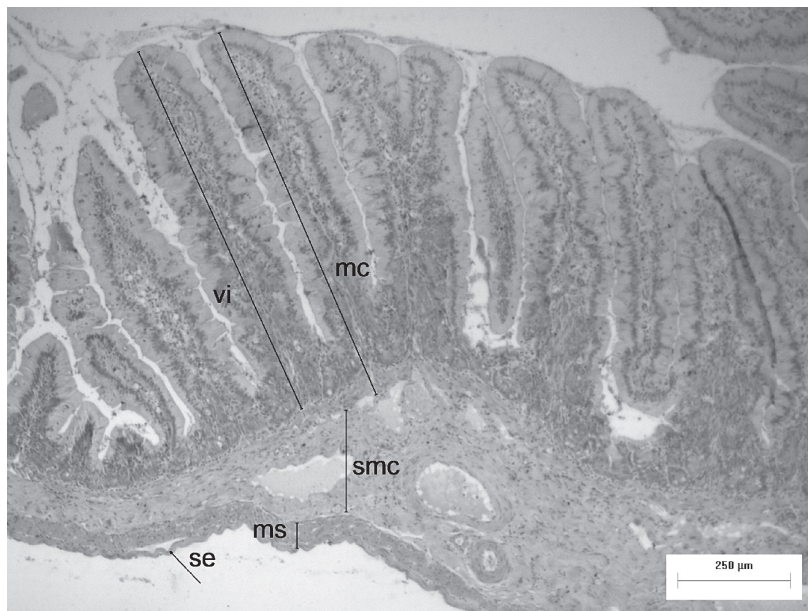

Figure 1 - Tube of the small intestine of *D. aurita*. vi-villus; mc-mucous layer; smc-submucous layer; ms-muscular; se-serous (H-E).
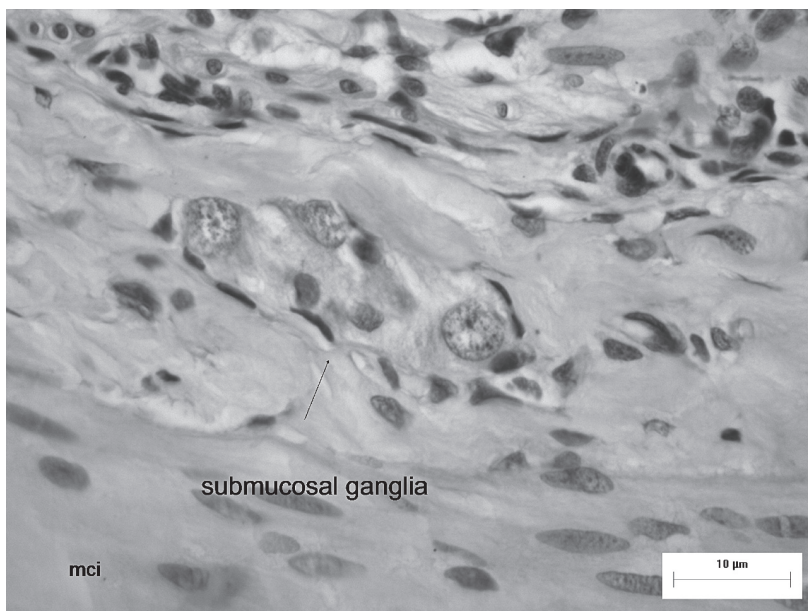

Figure 2 - Submucous nervous plexus in the small intestine of *D. aurita*. mci-inner circular muscular layer (H-E).

height of villi was 395.40 ± 170.59 μm; inner and external muscular layers had 146.47 ± 78.05 μm; and the submucous layer 102.03 ± 74.20μm.

The endocrine cells (Figures 4 and 5) distributed along the digestive tube of *D. aurita* were located in the epithelium lining of the mucous and in the glandular epithelium interspersed with exocrine cells. They had a varied morphology despite most them seeming to be oval in shape. Two cell types were observed, one in which the cells reached the luminal surface (the open type), and the other presenting no continuity with the lumen (the closed type).
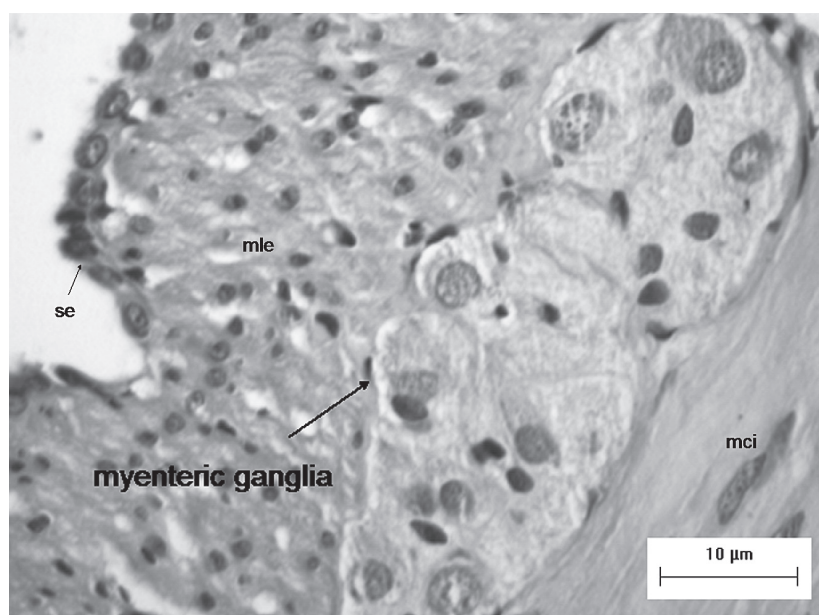

Figure 3 - Myenteric nervous plexus in the small intestine of *D. aurita*. mci-inner circular muscular layer; mle-external longitudinal muscular layer; se-serous (H-E).
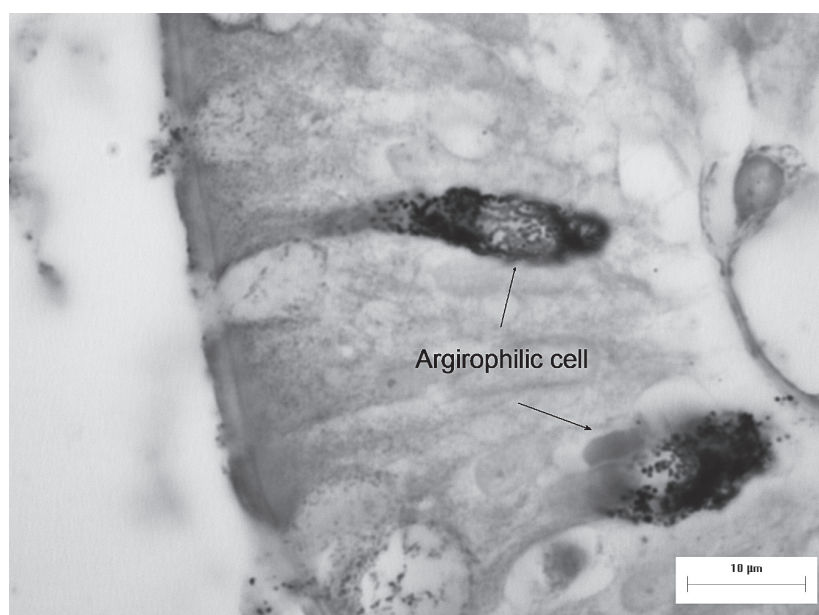

Figure 4 - Argirophilic endocrine cells in the mucous layer of the ileum of *D. aurita* (Grimelius technique).
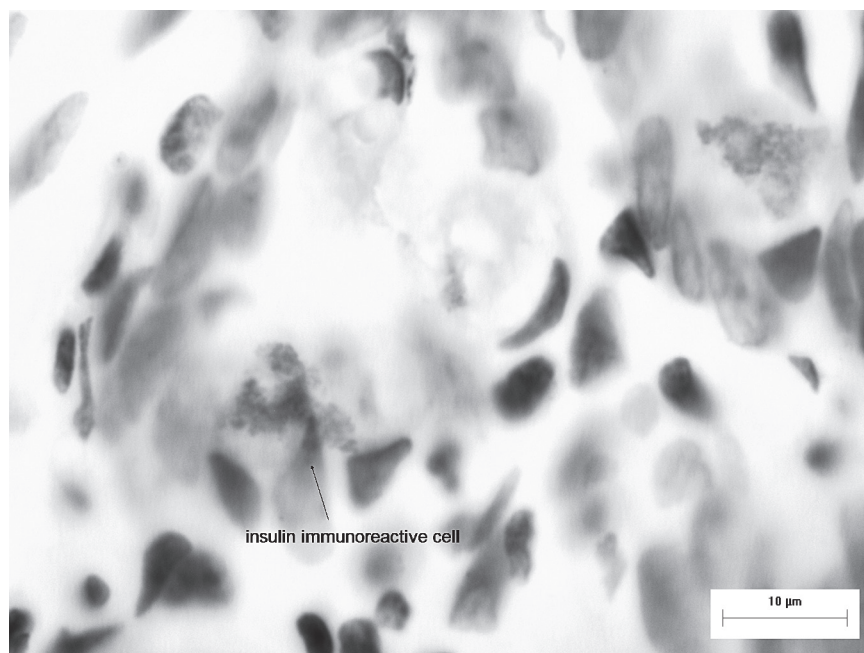
Figure 5 - Immunoreactive endocrine cells to insulin in the mucous layer of the ileum of *D. aurita* (Imunoperoxidase technique).

TABLE I

Number of endocrine and nervous cells and the submucosal

and myenteric nervous ganglia in the small intestine of adult opossums.

1[a]	argyrophillic	argentaffin	Insulin I R	2[b]	Submucosal ganglia	Myenteric ganglia	Nervous Cells
D$_i$	61.11±13.38[a]	17.63±7.14[a]	0.86±0.51[a]				
D$_m$	65.82±16.93[a]	15.36±7.13[a]	0.56±0.42[a]	D	5.49±2.60[a]	5.79±1.42 [a]	29.36±10.06[ab]
D$_f$	68.02±11.40[a]	14.81±6.06[a]	0.72±0.68[a]				
J$_i$	48.41±7.58[ab]	10.18±4.26[a]	0.71±0.65[a]				
J$_m$	54.71±11.56[a]	10.78±3.68[a]	0.49±0.36[a]	J	5.21±2.19[a]	8.03±3.13 [a]	25.40±10.54[a]
J$_f$	40.20±13.40[b]	7.19±4.43[a]	0.62±0.67[a]				
I$_i$	54.11±15.56[a]	6.92±2.54[a]	1.46±1.45[a]				
I$_m$	45.56±11.72[a]	7.61±4.08[a]	1.34±1.27[a]	I	6.59±3.80[a]	9.95±5.54 [a]	35.09±15.54[b]
I$_f$	51.36±18.57[a]	5.98±4.43[a]	0.96±1.04[a]				

Endocrine and nervous cells per mm^2 (average ± standard deviation) and the submucosal and myenteric ganglia (average ± standard deviation) per mm^2 of the submucous and muscular layers. (1[a]: regions) **D**, **J** and **I** represent the duodenum, jejunum and ileum segments, respectively. (2[b]: segments) Lower case letters **i**, **m** and **f** represent, in this order, the initial, mid and final regions of each segment. The average followed by the same letter, in the same column, does not differ among themselves by the Kruskal-Wallis test at the significance level of 5% (n = 10).

TABLE II

Comparative analysis of the number of endocrine cells between the regions
of the mucous layers in the duodenum, jejunum and ileum of adult opossums.

	Initial, Mid and Final Duodenum	Initial, Mid and Final Jejunum	Initial, Mid and Final Ileum
Argyrophillic	$H = 1.0; P = 0.59$	$H = 6.82; P = 0.03$	$H = 1.40; P = 0.49$
Argentaffin	$H = 0.96; P = 0.61$	$H = 3.83; P = 0.14$	$H = 1.89; P = 0.38$
Insulin IR	$H = 2.04; P = 0.36$	$H = 0.23; P = 0.88$	$H = 0.95; P = 0.62$

Kruskal-Wallis test. $gl = 2$. The adopted significance level was 5% (n=10).

TABLE III

Relationship between the number of enteroendocrine cells and submucous and myenteric ganglia,
and endocrine and nervous cells in the duodenum, jejunum and ileum of adult opossums.

	Argyrophillic Cells	Argentaffin Cells	Insulin IR Cells
Submucosal ganglia	$rs = 0.12; P = 0.51$	$rs = 0.20; P = 0.27$	$rs = 0.19; P = 0.29$
Myenteric ganglia	$rs = -0.34; P = 0.06$	$rs = -0.16; P = 0.37$	$rs = -0.19; P = 0.30$
Nervous cells	$rs = 0.03; P = 0.79$	$rs = 0.07; P = 0.53$	$rs = 0.20; P = 0.05$

Regression Analysis. The adopted significance level was 5% (n=10).

No variation was observed between different endocrine cells in the initial, mid and final regions of the segments, except for argyrophillic cells in the jejunum (Tables I and II). Most of them presented an oval shape. Open and closed types of endocrine cells were observed interspersed with exocrine cells.

Nerve cells were observed in groups and also isolated, both in the submucous layer and between the muscular layers. The number of submucosal ($H = 0.54$; df = 2; $P = 0.76$) and myenteric ($H = 5.44$; df = 2; $P = 0.06$) ganglia did not vary between all small intestine segments of *D. aurita* (Table I). The number of nervous cells varied only between jejunum and ileum ($H = 8.70$; df = 2; $P = 0.01$) (Table I). In average, 17.29 submucosal ganglia/mm^2 were encountered in the submucous, and 23.77 myenteric ganglia/mm^2 in the muscular region. The myenteric plexus was apparently greater than that of the submucous.

There is no relationship between the number of ganglia and nervous cells, and endocrine cells in different segments of the small intestine of *D. aurita* ($P > 0.05$) (Table III).

DISCUSSION

The difference among duodenum, jejunum and ileum of *D. aurita* was similar to that described for rodents (Altmann and Leblond 1970, Allogninouwa et al. 1996), pigs (Mitjans and Ferrer 2004) and capybaras (Velásquez et al. 2003, Rodrigues et al. 2006). The duodenum was highlighted by the presence of Brünner glands in the submucous layer. These glands were not observed in the submucous layer of the jejunum and ileum. The ileum was identified by the presence of associated lymph nodes (Peyer dishes) for all mucous layer, as well by submucous layer.

In descending order, the argyrophillic cells were more numerous than argentaffin cells, and argentaffin than insulin immunoreactive cells. Such results can be explained by the employed technique since argentaffin reactions appears only in enterochromaffin cells, while the argyrophillic reaction occurs in nearly all enteroendocrine cells, except in cholecystokinin, somatostatin and insulin-producing cells (Polak et al. 1993). The

immunohistochemical technique used to stain the insulin immunoreactive cells is more specific than those used for other cells. This is due to the fact that the employment of monoclonal antibodies increases the sensibility of this method.

In our work, insulin immunoreactive cells were found insolated in intestinal crypts and villi. Kendzierski et al. (2000) encountered intracellular immunoreactivity to insulin in glandular cells of the stomach and the colon of rats, but not in the small intestine. Contrarily, Coutinho et al. (1984) observed a positive insulin staining in the brush border and in some cells isolated in segments near the small intestine of adult opossums (*Didelphis albiventris*), although no evidence was observed in the mid and distal segments. Likewise, Bendayan and Park (1991) located extrapancreatic islets between the duodenum crypts and the muscle layer of the mucosa in rats. These islets were restricted to the duodenum region and crossed by the final portion of the bile duct before its opening in the intestinal lumen. The islets were surrounded by conjunctive tissue and exhibited no direct contact with the epithelial cells of the ducts and crypts. Ito et al. (1988) also encountered a small number of insulin immunoreactive cells in the pyloric antrum and duodenum of pigs between 32 and 41 days old.

Pancreatic insulin reduces glucose in blood, increases the deposit of glycogen in the muscles and the metabolic use of the glucose, while enteral insulin controls the intestinal motility (Kendzierski et al. 2000).

Studies with endocrine cells distributed along the digestive tube of mammals generally refer only to those located in one of the segment regions, therefore disconsidering the initial, mid and final regions of each segment (Krause et al. 1989, Takagi et al. 1990, Fonseca et al. 2002). It was believed that differences in the number of endocrine cells can be encountered between regions of the small intestine segments of *D. aurita*. However, this fact was not statistically proven in this work.

Ganglia of the submucosal plexus were concentrated near the muscularis mucosa and myenteric ganglia located between the circular internal layer and the longitudinal external layers together with nerve clusters as related to eutherian mammals (Young et al. 1993, Brehmer et al. 1994, Liberti et al. 1994, 1998). The plexuses were prominent, being the myenteric one apparently greater than that of submucous, that is, with a greater population of neuron bodies, similar to those encountered in the small intestine of the ginea-pig (Liberti et al. 1994). According to Bressan et al. (2004), the greater size of the myenteric ganglia reflects its importance in the intestinal motility control. The propulsion and mixing movements of the small intestine include an assembly of neurons in the plexus. In the small intestine of pigs, the contraction of a single 10 mm unit includes roughly 6,500 intrinsic primary afferent neurons in the myenteric ganglion, 1,200 ascending interneurons, 3,000 descending interneurons, 4,000 inhibitory motor neurons of the circular musculature and 3,000 excitatory motor neurons for the circular muscle (Clerc et al. 1998, Kunze and Furness 1999).

The clusters formed by the neuron bodies had varying sizes and forms in the two plexuses. Nerve cells were observed isolated, not grouping together in both plexuses. Leaming and Cauna (1961) also encountered isolated nerve cells and grouped nerve cells in the duodenum and jejunum of cats, being that the myenteric plexus of the jejunum contains more nerve cells forming groups than the myenteric plexus of the duodenum, with an average of 37 cells/mm^2 in the jejunum and 12.17 cells/mm2 in the duodenum.

Myenteric and submucosal plexuses can differ between organs regarding the function of their motility standards, as well as the shape and number of neurons within the same organ, and differ between species (Tafuri and Brenner 1967, Christensen 1988). For example, the small intestine of sheep contains 11 times more

myenteric neurons than the one of pigs, and approximately 80 times more than that of mice (Gabella 1987).

Our data indicate a greater quantity of myenteric ganglia (23.77 ganglia/mm^2) when compared to those of submucous (17.29 ganglia/mm^2) in the small intestine of *D. aurita*. Rodrigues (unpublished data) also obtained this relation, in which in the small intestine of capybaras, 2.29 myenteric ganglia were found in the muscular layer and 0.69 submucosal ganglia in the submucous layer, both with 1.2 mm length. There was no difference between the quantity of myenteric and submucosal ganglia in the segments in the small intestine of capybaras. However, it seems that a greater quantity of ganglia was found in the ileum. According to this author, since the number of ganglia is more frequent in portions with greater motility, it is possible that there exists no significant difference among the duodenum, jejunum and ileum of capybaras, as well as the capacity of muscle peristalsis. The myenteric ganglia control peristaltic movements of the intestine and the submucous, as well as participate in the innervation of the muscle layer, regulate the gastrointestinal secretion and blood flow (Hens et al. 2002).

Neuro-histological studies have been carried out on the myenteric plexus of the duodenum and jejunum of adult cats using silver method (Leaming and Cauna 1961). In this study, these authors found 122 and 30.7 cells/mm^2 in the duodenum and jejunum, respectively. In our work, we found 29.36 and 25.40 cells/mm^2 in the duodenum and jejunum, respectively. These differences probably occurred in the number of plexuses analyzed. We investigated two plexuses (submucosal and myenteric), while Leaming and Cauna (1961) have analyzed only the myenteric plexus.

Polak et al. (1993) reported some similarities between endocrine cells and enteric nerve cells, such as the presence of morphologically similar secretion granules, the presence of a sensorial and a transmitter part that liberates granules to the synaptosomal area and vascular pole. However, the peptides liberated by the nerve cells do not integrally meet the criteria for conceptual harmony, therefore employing the peptide-regulator term. A large portion of these peptides present a paracrine signaling effect locally stimulating neighboring cells, although they can interact with receptors and transform intracellular signals in other ones similar to those produced by circulating hormones (Santos and Zucoloto 1996). In the intestine, regulatory peptides can be encountered in the ganglia cells, nerve fibers and endocrine cells.

Immunohistochemical and molecular biology studies have revealed functional interactions between endocrine and neuronal cells (Delellis and Dayal 1992). However, the present study did not demonstrate this relationship in the morphometric field. We did not identify any relationship between the number of endocrine cells and submucous nervous and myenteric ganglia, and between endocrine and nervous cells of the opossum's small intestine. Bressan et al. (2004) who did not find any relationship between the number of endocrine cells and submucous and myenteric nervous ganglia of the caecum of capybara. Various techniques can be used in the identification of endocrine and ganglion cells, including histochemical (Masson-Fontana, Grimelius, Servier-Munger, Hellerstrom-Hellman) and immunocytochemical utilizing humoral and non-humoral markers (membrane proteins, enzymes and acid glycoproteins). Therefore, other techniques should probably be employed to confirm the quantitative interrelationship between the endocrine and nerve cells studied in this work.

RESUMO

Os sistemas nervoso e endócrino controlam integradamente os movimentos intestinais, a secreção de suas glândulas e também participam dos processos de digestão e absorção de nutrientes. Portanto, o objetivo central deste estudo foi verificar a existência de uma possível relação entre o número de células nervosas e gânglios dos plexos submucosos e mioentéricos e o número de células endócrinas no intestino delgado de adultos de *D. aurita*. As técnicas de coloração utilizadas foram Grimelius, Masson-Fontana modificada, imunoperoxidase direta e H-E. As células endócrinas argirófilas, argentafins e imunorreativas à insulina não variaram numericamente entre as regiões inicial, média e final do duodeno, jejuno e íleo (*P*>0,05), exceto as células argirófilas no jejuno (*P*<0,05). Nenhuma relação numérica foi verificada entre o número de gânglios nervosos e células endócrinas, e também entre células nervosas e endócrinas. Nós recomendamos o emprego de novas técnicas imunohistoquímicas para confirmar a correlação numérica entre os sistemas nervoso e endócrino no intestino delgado. A morfologia e a distribuição das células endócrinas e dos gânglios nervosos estudados foram similares àqueles encontrados em mamíferos eutérios.

Palavras-chave: células argirófilas, células argentafins, células endócrinas, gânglios, insulina.

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