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Histopathologic features of the vagus nerve after electrical stimulation in swine

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Summary. This paper describes the histological features of the vagus nerve after its stimulation with an electrostimulation system that is being developed for morbid obesity treatment.

An electrostimulation system was implanted laparoscopically around the ventral vagal trunk of five Large White female pigs (49.63±1.94kg.). Vagal nerve stimulation was performed by continuous constant voltage current pulses. Thoracic samples of both ventral and dorsal vagal trunks were obtained thoracoscopically one month after implantation. Animals were sacrificed one month after thoracoscopic vaguectomy. Tissue samples were then harvested from the vagal nerve at the implantation site, 1cm cranial to it, thoracic portion of ventral and dorsal vagal trunks, sub-diaphragmatic dorsal vagal trunk, left and right vagus nerves. Specimens were analysed with light microscope. The severity of the lesions was graded from 0 to 4 (0: no lesion, 1: mild, 2: moderate, 3: severe and 4: extremely severe), taking into account fibrosis, vascularization, necrosis, fiber degeneration and inflammation.

Electrode implantation resulted in thickened epineurium and endoneural connective tissue. The greatest lesion score was evidenced at the leads implantation site in the ventral vagal trunk, followed by, in order of decreasing lesion severity, left vagus nerve, thoracic portion of ventral vagal trunk, subdiaphragmatic dorsal vagal trunk, thoracic portion of dorsal vagal trunk and right vagus nerve.

The stimulation device used in this study caused connective tissue growth, greatest in the samples located closer to the implantation site. However, there was no sign of altered vascularization in any studied specimen.

Key words: Ventral vagal trunk, Electrical stimulation, Histopathologic features

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Introduction

Despite clinical applications of Vagal Nerve Stimulation (VNS) becoming more and more widespread in medical practice, there has been relatively little documentation of the effects of electrical stimulation of nerves in animal studies. VNS is currently being used to treat medically intractable seizures (Ben-Menachem et al., 1999).

The vagus nerve is mainly composed of afferent non-myelinated fibers. 90% of the total abdominal vagus nerve fibers are afferents (Agostoni et al., 1957) and 50% of the cervical vagus afferent fibers come from the abdomen (Asala and Bower, 1986). Prechtl and Powley (1990) reported that only about a 0.6% of the total vagus nerve fibers are myelinated, with the greatest majority of fibers being non-myelinated.

The vagus nerve connects the central nervous system and the gastrointestinal tract. Based on this strategic anatomical location, it could be theoretically possible to develop a stimulator that, by acting on the ventral vagal trunk, promotes early satiety. The afferent vagal fibers connect to satiety-related CNS nuclei, such as the hypothalamus (Berthoud et al., 1990), the parabrachial nucleus (Ricardo and Koh, 1978) and the central nucleus of the amygdala (Higgins and Schwaber, 1983).

Based on our early experience in this field (Sobocki et al., 2001), the development of such a device has been attempted and is now undergoing animal testing. The fact that the electric stimulation of the vagus nerve can affect daily food intake has been previously demonstrated (Krolczyk et al., 2001; Sobocki et al., 2001; Laskiewicz et al., 2003). However, data on the vagus nerve response to the electrical and mechanical action of the electrostimulator are still lacking.

This study reports our preliminary results, describing the histological features of the porcine vagus nerve after its stimulation with an electrostimulator undergoing preclinical testing for future clinical application in the treatment of morbid obesity. The aim of this study was

to assess the histopathologic changes caused by both mechanical and electrical effects of the electro-stimulation on the vagus nerve.

Material and methods

The study protocol was conducted in compliance with the Guide for the Care and Use of Laboratory Animals, and it was approved by the Institutional Ethical Committee for Animal Research. Every effort was made to minimize the number of animals used.

Surgical protocol

Five female Large White pigs, aged approximately 6 months and with a mean weight of 15.42±2.43 kg. were used for this study. All the procedures were performed under general anesthesia. The first procedure consisted of laparoscopic functional stimulation implantation. Two leads were implanted in the ventral vagal trunk in all animals. For this purpose, the lesser omentum was incised to expose the ventral vagal trunk, and then the leads were carefully attached to the ventral vagal trunk using a plastic cylinder (Fig. 1) The lesser omentum was subsequently sutured. Finally, the impulse generator was placed in a serosal pocket created on the gastric surface.

Electrostimulator characteristics

The device consisted of an impulse generator, which continuously delivered a square constant-voltage current of 0.5 V, 0.5 Hz, with a 10 milliseconds pulse width (Krolczyk et al., 2001) all thorough the postoperative period. The system transmitted these impulses to the target nerve through the above described leads and plastic cylinder. A fixed 10 mm inter-electrode distance was maintained in all cases. The internal lumen of the cylinder was slightly larger than the cross-sectional area of the nerve in order to avoid nervous compression.

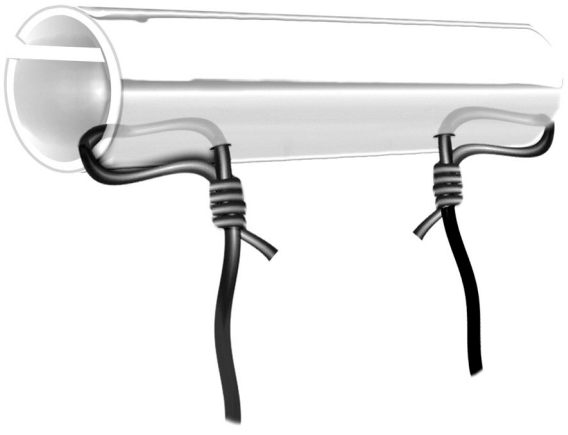

Fig. 1. Leads implantation system for the ventral vagal trunk.

Vagus nerve samples

One month after electrostimulator implantation dorsal and ventral vagus nerve vagotomy were carried out by thoracoscopic approach, severing the vagus nerve from the central nervous system. During this procedure 1 cm of each nerve was resected caudally to the vagus nerve esophagic plexus. These specimens were prepared for light microscopic examination.

Finally, animals were sacrificed 30 days after thoracoscopic vaguectomy. A postmortem study was performed immediately after euthanasia, harvesting nerve tissue samples from the following sites: implantation site, 1cm cranial to it, subdiaphragmatic dorsal vagal trunk, left vagus and right vagus.

All samples were fixed in 4% buffered formaldehyde and routinely processed and embedded in paraffin wax. The specimens were cut into 4 µm thick sections and stained with Hematoxylin/Eosin and Masson's Trichrome. An experienced pathologist who was not aware of the nature of the experiment examined the histological sections.

The severity of the lesions was graded from 0 to 4, taking into account fibrosis, vascularization, necrosis, fiber degeneration and inflammation (0: no lesion, 1: mild, 2: moderate, 3: severe and 4: very severe). In order to provide the pathologist with normal histological images of the vagus nerve, two samples were obtained from healthy animals, labeled accordingly and submitted along with the rest of the studied samples.

Results

The plastic cylinder remained in place, completely surrounding the ventral vagal trunk, in all animals. At gross examination considerable connective tissue growth could be seen around the leads implantation site. Light microscopy at this area supported macroscopical findings, confirming the existence of complete encapsulation of the nerve by connective tissue (Fig. 2). No signs of necrosis were seen in any of the studied samples.

Connective tissue growth was also noted at the inner layers (Fig. 3A,B). This was evidenced by increased uptake of connective tissue specific stain in Masson's Trichome stained sections obtained from the implantation site on the ventral vagal trunk (Fig. 3B) -as opposed to stains from intact vagus nerve (Fig. 3A).

An inflammatory reaction was observed predominantly on subdiaphragmatic muscle fibers and ganglionar tissue, whereas nervous tissue showed almost no evidence of inflammation. In all cases, the type of inflammatory reaction present was lymphoplasmocytic.

With regard to the samples obtained from thoracoscopic vaguectomy, the lesions observed 30 days after electrostimulator implantation in the thoracic portions of the dorsal vagal trunk were slight. One sample presented mild inflammation and other showed signs of fibrosis. No lesion was seen in the remaining

three samples. The lesions observed in the thoracic portion of the ventral vagal trunk, whilst also mild, were more evident than those present in the dorsal one. Only one sample of the thoracic portion of the ventral vagal trunk did not present any lesion.

The overall score of severity of the samples obtained from thoracoscopic vaguectomy was higher in the ventral vagal trunk compared to the dorsal trunk.

30 days after thoracoscopic vaguectomy inflammation was detected in two of the samples of the dorsal vagal trunk. A third sample presented only a mild decrease in the amount of nervous fibers and the two remaining samples did not present any lesion.

Four of the samples obtained from the left vagus nerve showed inflammation, fibrosis and a decrease in the number of fibers. Three of the left vagus nerve samples presented slightly altered vascularization. No lesion was observed in the rest of the samples obtained from the left vagus nerve nor in the four samples of the right vagus nerve specimens. Higher inflammation, fiber degeneration and overall lesion scores were therefore obtained in left vagus nerve samples (Table 1).

Observed lesions were greater in the samples obtained from the implantation site. Mild fibrosis and inflammation ranging from severe (2 samples) to very severe (3 samples) was evidenced at this area. Significant differences were seen between lesions score at the implantation sites when compared to specimens obtained from the subdiaphragmatic dorsal vagal trunk. These differences were evidenced in all studied parameters except for vascularization and overall score (Table 1).

Discussion

VNS is used nowadays for the management of medically intractable epilepsy (Murphy, 1999). On the basis of the vagus nerve anatomical disposition, vagal

Table 1. Lesion score of vagus nerve samples.

	LESIONS SCORE OF VAGUS NERVE SAMPLES				
	I	F	V	Fi	T
Thoracic portion of dorsal vagal trunk	0.20±0.45	0.20±0.45	0±0	0±0	0.10±0.3
Thoracic portion of ventral vagal trunk	0.60±0.55	0.60±0.89	0±0	0.60±0.55	0.45±0.60
Implantation site	3.40±0.55	2±0	0.40±0.57	3.00±1.41	2.20±1.34
Dorsal vagal trunk caudal to vaguectomy	0.80±1.30	0.20±0.45	0±0	0.20±0.45	0.30±0.73
Left vagus nerve	1.60±0.90	1.20±0.84	0.60±0.55	1.00±0.70	1.10±0.79
Right vagus nerve	0±0	0±0	0.20±0.45	0±0	5.10^{-2}±0.22

Assessed lesions: I, Inflamation; F, Fibrosis; V, Vascularization; Fi, Nervous fibers degeneration; T, Total lesions.

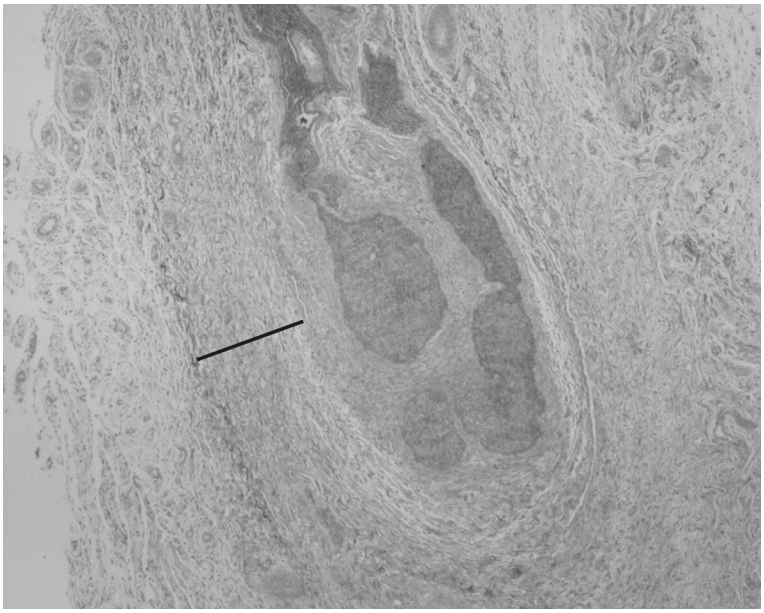

Fig. 2. Transverse section of the whole ventral vagal trunk at the implantation site. Connective tissue proliferation in the epineurium layer can be observed (line). x 10

nerve stimulation could be applied in the treatment of some other conditions, such as depression (Rush et al., 2000) or pain (Ness et al., 2000), and it may even play a role for memory enhancement (Clark et al., 1999). Similarly, and according to previous reports of studies performed in rats (Krolczyk et al., 2001) and in rabbits (Sobocki et al., 2001), the stimulation of vagus nerve could be applied as a new therapeutic option for morbid obesity management.

In our opinion, before these techniques could be considered as an option for daily practice, and in order to optimize the results of vagal stimulation it is mandatory to determine the short term structural responses of living tissues to the implanted device, especially as regards the effect that these structural changes may have on the electrode-neural interface.

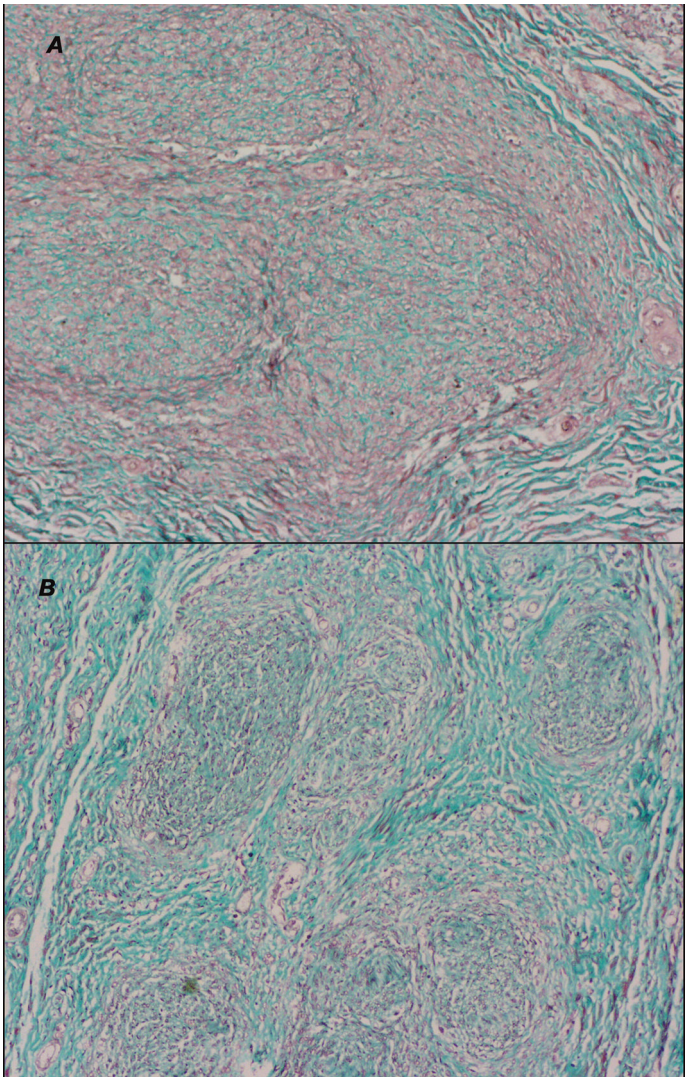

Fig. 3. Cross-section of intact ventral vagal trunk **(A)** and leads implantation site in the ventral vagal trunk **(B)**. Connective tissue growth in the inner layers of the operated nerve is evidenced by the increased uptake of connective tissue specific stain (green) **(B)** compared with intact ventral vagal trunk (**A**). Masson's Trichome, x 25

Firstly, we studied whether the implantation system caused nervous degeneration secondary to compression. It has been previously reported that the larger the diameter of the nervous fiber, the more susceptible it is to compressive lesions (Strain and Olson, 1975; Mackinnon et al., 1984; Krarup et al., 1989; Larsen et al., 1998). Therefore, although this study did not examine nerve fascicle diameter, it may be inferred that the lesions were present in these larger fibers. If that is the case, it must be considered that loss of these fibers could be associated with an increase in the stimulation threshold (Stein et al., 1977), which would be followed by changes in the effects exerted by nerve stimulation on food intake. Subsequently, in order to maintain an equivalent stimulation, it would be necessary to increase the administered current.

Our implantation system is similar to the H type electrodes previously described by Yuen et al. (1984). Despite their cylinder having larger diameter than the nerve, these authors described nervous compression signs. In their opinion, this difference in diameter should have been enough to accommodate volume changes secondary to postoperative edema. The same principle and diameter mismatch was applied in our study, but we did not see any sign of compression in any of the studied samples. On the contrary, the fascicles affected the most were those located deep in the nerve. In our opinion, changes in these fascicles were secondary to electrical stimulation rather than to compression, as the constriction should have affected peripheral fascicles more. Moreover, the implantation system did not have much effect on vascularization, either at a superficial or at a deeper level. Taking into consideration both facts, it is our opinion that the implantation system used in this study did not compress the nerve.

The implantation system used in the present work was similar to the one described by Yuen et al. (1984), who do report nervous compression in their study. The difference could be attributed to the method used to fix the plastic cylinder. The above mentioned authors closed the plastic cuff itself around the nerve, whereas in our study the cylinder was left open, and fixed by suturing the surrounding tissue. In our opinion, this allows for increases in diameter without the nerve being compressed by the plastic cylinder. One disadvantage of this technique could be that the nerve may become dislodged from the cylinder. This was not seen in any of the animals included in this study, where all ventral vagal trunks were surrounded by the plastic cylinder at the time of explantation.

One of the most important hystopathologic and macroscopic features seen in our study is a significant increase on the amount of connective tissue, observed mainly at the implantation site. This is probably secondary to the injuries caused by the implantation system mechanical effect. Mechanically induced

damages include thickened epineurium due to accumulation of connective tissue beneath the electrode and increased amount of perineural and endoneural connective tissue (Agnew and McCreery, 1990). In order to determine which kind of nerve fibers were affected by the electrical stimulation a electron microscopy study could have been performed.

Although the functional significance of this fibrous tissue growth has not been fully clarified, in our opinion, as previously reported by Andrew et al. (1990a), it is an important issue, as it means that the nerve impedance varies as time passes. Consequently the effect of the stimulation on the nerve also varies, because the stimulus amplitude and frequency are the parameters which discriminate which kind of fibers are going to be stimulated (Banzett et al., 1999; Krahl et al., 2001). In this study, the stimulus frequency was kept constant. As previously stated by Rodriguez et al (2000), connective tissue growth could cause an increase in the stimulation threshold. These authors reported that 35-45 days after implantation the mean threshold increased by 11-18% when compared to the implantation day.

A decrease in the amount of nervous fibers was observed, especially in the leads implantation site. This may be attributed to degeneration of the afferent fibers - which represent 90% of the total amount of fibers (Agostoni, 1957)-, secondary to the performed vaguectomy. However, the fact that the degeneration was minimum in the subdiaphragmatic dorsal vagal trunk suggests that the etiology could be different.

Nervous stimulation may provoke degeneration by nervous fibers hyperactivity (Agnew et al., 1990), by the elution of toxic substances into the endoneural fluid or by depletion of vital substances (Agnew and McCreery 1990; McCreery et al., 1992). The neuronal hyperactivity can lead to metabolic stress resulting in a loss of homeostasis and eventually cell death (Yaroswky Ingvar, 1981). In order to minimize the neural damage we would need to change to an intermittent stimulation protocol, as in the VNS, because neural damage appears to be closely associated with hyperactivity of fibers.

The different severity of lesions found in the abdominal and thoracic portions of the vagal trunk could be attributed to the period of stimulation. Some authors believe that there is no correlation between duration of the stimulation and lesion severity. In the study performed by Yuen et al. (1984), where the electrostimulation device was implanted in the sacral ventral root S2 for 7 months, nervous degeneration was seen after 5 months of stimulation, but no lesions were observed after 7 months. However, these data can not be extrapolated to our study, because our period of study is shorter. In view of the results reported by Yuen et al. (1984), it may be inferred that, in their study, the evolution of alterations brought over by the stimulation reached a plateau by month 5.

Lesions were also observed in the thoracic portion of the dorsal vagal trunk, probably because the impulse is also transmitted from one trunk to another through communicating branches between both trunks (Sauter et al., 1983; Fitzakerley and Lucier, 1988).

Our results indicate that ventral vagal trunk stimulation had a much greater effect on the left vagus nerve than on the right one. Considering that the left vagus nerve is primarily composed of afferent fibers from the ventral vagal trunk, it is only logical to think that ventral vagal trunk stimulation has more influence on the left vagus nerve than on the right nerve (Qian et al., 1999).

As expected, alterations were more marked at the implantation site. These lesions were characterized by a foreign body reaction and connective tissue proliferation within all three nervous layers (endoneurium, perineurium and epineurium). There were no signs of necrosis in any animal, nor even in the samples that presented the most severe fibrosis. This finding can be attributed to the mononuclear characteristic of the inflammation response which, in contrast to the supurative reaction, rarely induces tissue degeneration.

Although the implantation system seems to be appropriate, mainly because its manual construction is easy and reliable, it can be done using simple tools and its implantation requires a minimal amount of manipulation of the nerves, thereby reducing the chance of surgical trauma. Further studies are needed, especially in the long term, to study the evolution of histopathological features induced by this system. Similarly, nervous fibers degeneration is a parameter that can be subjected to errors due to operator's subjective assessment, which may render it difficult to determine objectively. For this reason, it should be necessary to study the histopathological appearance of the stimulated vagus nerve with electron microscopy, which can assess both myelinic degeneration and number of fibers objectively.

In conclusion, stimulator implantation in this study caused connective tissue growth, especially in the area where the leads were implanted, despite no vascular compression signs being observed. Further studies to modify the design in order to decrease electrode-dependant tissue growth are under way.

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