NADPH-diaphorase positive cells (mast cells) around and within the autonomic nerves in the periphery of subglandular connective tissue layer of dog paranal sinus (*Sinus paranalis*)

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**SUMMARY**

The present study aimed to investigate the enzyme histochemical expression of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) in mast cells and in autonomic nerves in the wall of the paranal sinus in 8 adult dogs (4 males and 4 females). The NADPH-d histochemical expression was investigated according to the method of Sherer-Singler and metachromasia evidenced by toluidine blue staining on frozen and paraffin serial sections was used for confirming the cell type. Positive NADPH-d cells (mild to strong reactivity in cytoplasmic granules) were found next and within autonomic nerves located in the periphery of subglandular connective tissue layer of paranal sinus and metachromasia of mast cells was observed in the same localization. NADPH-d reactivity was also evidenced in autonomic nerves. These results suggest that NADPH-d positive cells (mast cells) and autonomic nitricergic nerves may produce nitric oxide and may be together involved as a structural and functional unit in the sinus function.

**Keywords:** Paranal sinus, dog, NADPH-diaphorase, mast cells, autonomic nitricergic nerves, nitric oxide.

Introduction

Since 1987, when the endothelium-derived relaxing factor was identified as nitric oxide (NO) [9, 13], numerous reports have indicated that this small gaseous molecule, nitric oxide, is a ubiquitous mediator involved in many different biological processes, such as vasodilatation [15], neurotransmission [4, 6], macrophage-mediated cytotoxicity [12], gastrointestinal smooth muscle relaxation [4] and bronchodilatation [7], through a variety of downstream pathways. The NOS (NO synthase) is an enzyme family responsible for NO biosynthesis. Three different NOS isoenzymes, with corresponding genes located on different chromosomes, are identified: NOS 1 (neuronal, nNOS), NOS 2 (inducible, iNOS), and NOS 3 (endothelial, eNOS) [3]. These enzymes share a common structure, i.e. an oxidase/reductase domain and a calmodulin binding site, with 51–57% of homology in the primary amino-acid sequence [3]. The C-terminal reductase domain has FMN, FAD, and NADPH binding sites and is linked to the oxidase domain through a calmodulin binding site and nicotinamide adenine dinucleotide phosphate (NADPH) is one of the cofactors required for NO formation [1, 3]. Because NO cannot be stored, its signalling specificity must be controlled at the level of synthesis. Indeed, the members of the NOS family are among the most highly regulated of the known enzymes.

The aim of this study was to investigate the localisation of the nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) positive mast cells in the vicinity of nitricergic autonomic nerves in the wall of dog paranal sinus in order to suggest that there is a relationship between mast cells and nerves in this organ.

**Material and Methods**

**ANIMALS**

Samples of 1 cm³ were collected from different parts of the paranal sinus wall from 8 adult dogs (4 males and 4 females, 1-9 years old, from 4 Rottweilers, 1 Golden Retriever, 1...
mixed-breed dog, 1 Pit-bull and 1 Drahthaar), immediately after death of the animals in the clinics of the Faculty of Veterinary Medicine due to diseases that had not affected the paranal sinus.

HISTOCHEMICAL DETECTION OF NADPH DIAPHORASE

Part of the samples was immediately immersed in 4% paraformaldehyde (Sigma Aldrich Chimie, Switzerland) in phosphate-buffered saline (PBS), pH 6.9, for 24 hours at 4°C. Sections of 10-20 µm thickness were prepared by means of a freezing microtome (Slee, Mainz, Germany). The free-floating sections were further processed according to the protocol of SHERER-SINGLER et al. [18] by incubation in a solution containing nitro blue tetrazolium (0.2 mg/mL, Sigma Aldrich Chimie GmbH, Germany), β-NADPH (Santa Cruz Biotech, Santa Cruz, CA, USA) (12.5 mg) and Triton X-100 (0.5%) (Merck Belgalabo, Overisje, Belgium) in PBS (0.1 M, pH 7.4) for 1-2 hours at 37°C. Microscopic assessment of the reaction was scored as absent (0), weak (+), medium (++), and strong (+++) in 40 microscopic fields at X 400 magnification.

HISTOCHEMICAL DETECTION OF METACHROMASIA IN MAST CELLS

The other part of the samples were fixed for 1 or 2 hours in Carnoy’s fixative at room temperature and further processed for serial cryostat (10–20 µm) and paraffin (5–7 µm) sections that were stained with 0.1% solution of toluidine blue in Mc Ilvane’s buffer, pH 3 [14] for assessment of metachromasia of the mast cells.

STATISTICAL ANALYSIS

The density of NADPH-d positive cells (number / 0.1 mm²) in the nerves was determined by light microscope (ZEISS Primo Star, Germany), camera (Progres, Capture 2.6 - JENOPTIK) and software analysis programme (Soft Imaging Sistem GmbH). Statistical analysis was done using Data Analysis tool and T-test by means of the StatMost for Windows software. Data for cell density are expressed as mean ± standard deviation.

Results

The predominant part of NADPH-d reactive cells were observed in the perineurium of autonomic nerves which are localized in the periphery of subglandular connective tissue layer of canine paranal sinus. They showed medium to strong reactivity in granules, surrounding the negative nucleus (figure 1). Single NADPH-d positive cells were also found into the autonomic nerves. The mean number of observed NADPH-d-reactive cells in perineurium and into autonomic nerves were n = 4.3 ± 0.75 in males and n = 4.5 ± 0.76 in females. These data show that there was not statistically significant difference (P > 0.05) between the sexes. In parallel, the identity of mast cells with similar localisation in the same areas was confirmed by metachromasia after toluidine blue staining of both paraffin and frozen sections (figure 2).

Autonomic nerves also showed strong to medium NADPH-d reactivity (figure 1).

Moreover, the localisation of NADPH-d positive cells was similar in the 8 dog samples investigated here.

Discussion

In the present investigation, the presence of NADPH-d positive cells around and within the autonomic nerves in the periphery of subglandular connective tissue layer of canine paranal sinus is described for the first time. The data from toluidine blue staining indicate that observed NADPH-d positive cells are most probably mast cells. This colocalisation, which
could be further corroborated by confocal microscopy, corresponds with similar findings in porcine kidneys [19]. Others have demonstrated that mast cells and nerve fibres in other species and organ systems have an intimate relationship and that nerve fibre innervation and function change in response to inflammation [5]. As it is known, signalling molecules that mediate the exchange of information between these cells include cytokines, neurotransmitters and neurophic factors [17]. Histamine, prostaglandins and leukotrienes are paracrine signals in the communication pathway from mast cells to the small intestinal enteric nervous system and histamine anaphylactic effects involve excitation of neurones in the small intestine via H3 and H2 receptors [10]. That is the reason why it was supposed that mast cells and nerve fibres in the peri- phery of subglandular connective tissue layer of canine paranal sinus have such an intimate relationship.

According to GILCHRIST et al. [7] human mast cell lines produce NO in both cytoplasmic and nuclear compartments, and endogenously produced NO can regulate leukotriene production by mast cells. A special attention, by our opinion, should be paid to the localization of NADPH-d-reactive mast cell within autonomic nerves with the same reactivity. Obviously, the NADPH-d reactive axons, observed in the periphery of sinus subglandular layer, belong to nitricergic (or NANC, i.e. non-adrenergic and non-cholinergic) nerves, which are known to release NO as their neurotransmitter substance [1, 11], confirming in this way the study of BIENENSTOCK [2] about the presence of relationships between mast cells and the nervous system. According to this author, there is a bi-directional mast cell communication with nerves both in vitro and in vivo.

This type cell communication is already described in the skin, lung, intestine and urinary bladder both in vitro and in vivo [2]. This communication appears to be important in a variety of situations relating to emotion, behaviour, stress or allergic and other hypersensitivity reactions [2], and these interactions appear to be important also in host resistance and responses to toxins [2]. It is reasonable to speculate that mast cells and nerves can form a homeostatic unit, in which mast cells act in part as relays of environmental information to the nervous system, and in turn are involved in the regulation of immune and inflammatory responses. They can act as a switchboard in this latter condition, or as a regulatory gateway in control of the release of hormones from the hypothalamic-pituitary-adrenal axis. Mast cells are multifunctional cells and when associated with nerves, these functions are further amplified, and in this sense they can act both as conductors of the orchestra as well as players within it [2]. HOFMEISTER et al. [8] showed that the number of mast cells and nerve fibres play an important role in the diagnosis of interstitial cystitis. The relationship between the nerve fibres and mast cells in interstitial cystitis may provide additional avenues of research into possible aetiologies and pathogenic mechanisms of cystitis. From such studies, new treatments may be targeted not only at the symptoms but at the cause of the disease. The NADPH-d-reactivity observed in the mast cells in the dog and related changes in the vessels in any condition of the body could be affected by both endogenous and exogenous NO. According to SALVEMINI et al. [16], mast cell reactivity can be suppressed by exogenous NO supply through direct effect of mast cell induced leukocyte / endothelial interactions and via altered permeability of vessels from the microcirculatory bed during inflammation. Based on these studies, it can be assumed that the reactivity of the NADPH-d mast cells observed in the canine paranal sinus wall as well as the related vascular changes observed in disorders of this organ could be influenced by both endogenous and exogenous NO. The localization of mast cells around and within autonomic nitricergic nerves observed in this organ indicates that these nerves regulate the function of mast cells by nitric oxide.

Since the dog paranal sinus inflammation are common, we assume that knowledge of the already established contacts between mast cells and autonomic nerves would help to clarify not only the normal development of the body but also the pathogenesis, prevention and cure of this place.

As a conclusion, present findings suggesting that NADPH-d positive mast cells are present next to/or in the autonomic nerves as well as the autonomic nitricergic nerves in the periphery of subglandular connective tissue layer of canine paranal sinus have a well-expressed NADPH-d reactivity. Obviously, further studies are needed for precise evaluation of interactions between mast cells and nerves in one hand and of NO roles on paranal sinus function on the other hand.

References


