ENZYME HISTOCHEMICAL EXPRESSION OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-DIAPHORASE (NADPH-D) IN PARANAL SINUS, EXTERNAL AND INTERNAL ANAL SPHINCTERS IN DOGS

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Summary

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The present study showed the sites of histochemical expression of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) in the different parts of canine paranal sinus. Keratinocytes in the basal and spinous layers exhibited a moderate to strong reaction whereas those in the upper layers – a weak enzyme histochemical reaction. No enzyme reaction was observed in stratum corneum. NADPH-d expression in secretory and myoepithelial cells of apocrine glands was moderate to strong. Sebaceous glands' reaction was moderate to strong in peripheral sebocytes and weak to absent in central ones. The endothelium of blood vessels located in the subepithelial and subglandular connective tissue of the paranal sinus wall showed a moderate degree of enzyme histochemical activity. The longitudinal cross section of the external anal sphincter revealed areas of muscle fibres sarcolemma and perinuclear cytoplasmic zones with moderate to strong enzyme reaction. Strong enzyme activity of nitrergic nerve fibres was detected in the internal anal sphincter. This investigation was the first to show a marked NADPH-d activity in the various structures of canine paranal sinus that was probably involved in nitric oxide synthesis related to physiological and pathological events in this organ.

Key words: dog, NADPH-d, paranal sinus

INTRODUCTION

NADPH-d is a marker of the enzyme nitric oxide synthase (NOS) (Shimosegawa & Toyota, 1994). Intracellular NOS catalyzes the synthesis of nitric oxide (NO) from L-arginine that yields equal parts of citrulline and NO (Palmer *et al.*, 1987).

Three forms of NOS are known: NOS1, also called neuronal or nNOS; NOS2 (inducible, iNOS) and NOS3 (endothelial; eNOS). They are homodimers with a molecular weight of 130–160 kDa and require cofactors such as flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin, reduced nicotinamide adenine dinucleotide phosphate. Endogenously produced nitric oxide plays a variety of biological functions, and is involved in neurotransmission, smooth muscle relaxation and response to immunogens (Cals-Grierson & Ormerod, 2004).

The expression of NADPH-d is detected histochemically in various organs in men and animals. May *et al.* (2000) have observed NADPH-d expression in ciliary nerves and choroidal endothelial

cells of porcine eye. NADPH-d positive reaction was also observed in perivascular nerve fibres near the arteries and was probably related to nitrergic vasodilative innervation of choroidal blood vessels.

NADPH-d and NOS expression was demonstrated by Kawamoto *et al.* (1998) in the epithelium of human nasal mucosa, submucosal glands, nerve fibres and the endothelium. Kobzik *et al.* (1994) have established immunohistochemically the expression of two NOS isoforms in skeletal muscles: neuronal (NOS1) and endothelial (NOS3). Regular physical exercise has been shown to enhance NOS1 and NOS3 expression in homogenates of rat soleus muscles (Balon & Nadler, 1997).

The lack of literature data about the expression of NADPH-d and the related nitric oxide synthesis in the wall of the paranal sinus, as well as in external and internal anal sphincters in dogs has motivated the present investigation on enzyme histochemical expression of NADPH-d in the different structures of the organ in animals from both genders.

MATERIALS AND METHODS

The material for this investigation was obtained according to the requirements of Bulgarian legislation from the wall of paranal sinuses of 8 adult dogs from the following breeds: 4 Rottweilers (at the age of 1, 2, 5 and 9 years), one Golden Retriever (aged 1.7 years), one mixed-breed dog (aged 9 years), one Pitbull aged (6 years), one Drathaar (aged 1.5 years) obtained after the death of animals at the clinics of the Faculty of Veterinary Medicine on occasion of diseases that did not involve the paranal sinus.

Pieces of 1 cm^3 were obtained from different parts of the organs and put in

4% paraformaldehyde (Sigma Aldrich Chemie, Switzerland) in phosphate-buffered saline (PBS), pH 6.9, for 24 h at 4°C. The fixation stage was followed by washing with 0.01M PBS, pH 7.2. Cross sections of 10–20 μm were prepared on a freezing microtome (Mainz, Germany). Then, free-floating sections were processed by the NADPH-d histochemical technique of Sherer-Singler *et al.* (1983) – incubation in a solution containing 0.2 mg/mL, nitro blue tetrazolium (NBT, Sigma Aldrich Chemie Switzerland), 1 mg/mL β-NADPH Sigma Aldrich Chemie) 0.5% and Triton X-100 (0.5%) (Merck Belgalabo, Overisje, Belgium) for 1 h at 37 °C. After colour development, sections were washed twice: first in 0.1 M Tris HCL and finally, in 0.01 M PBS.

The reaction was scored as weak (+), moderate (++) and strong (+++). The lack of reaction was designated with (0).

RESULTS

Light microscopy showed a various degree of NADPH-d activity in each structure of the paranal sinus (Table 1).

Keratinocytes in the basal layer and the spinous layer exhibited moderate to strong NADPH-d activity. In outer layers, the enzyme activity of keratinocytes was weak. No enzyme activity was detected in the stratum corneum (Fig. 1).

The expression of the enzyme in secretory and myoepithelial cells of apocrine glands was moderate to strong (Fig. 2). Moderate to strong enzyme reaction was detected in peripheral sebocytes in sebaceous glands, whereas central sebocytes' reaction was weak, less frequently moderate to absent.

Table 1. NADPH-d expression in the wall of the paranal sinus, internal and external anal sphincters in dogs

	NADPH-d expression
Paranal sinus	
Stratified squamous epithelium	
basal and medium layers	+/++/+++
stratum corneum	0
Subepithelial connective tissue: blood vessels	++/+++
Subglandular connective tissue: blood vessels	++/+++
Apocrine glands	
secretory cells	++/+++
myoepithelial cells	++/+++
Sebaceous glands	
central sebocytes	0/+/++
peripheral sebocytes	++/+++
External anal sphincter	+/++
Internal anal sphincter	
smooth muscle cells	0
autonomous nerve fibres	+++

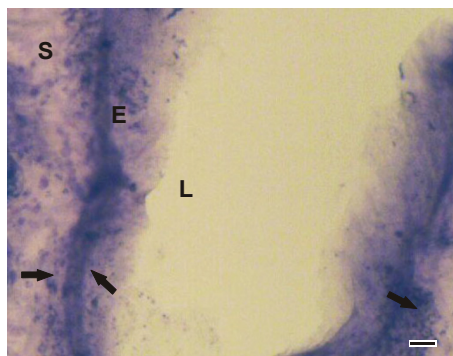absent (0), weak (+), medium (++), strong (+++) expression.

Fig. 1. Moderate to strong NADPH-d activity (arrows) in keratinocytes of basal and spinous layers of the stratified squamous cornified epithelium (E). S – subepithelial connective tissue; L – sinus lumen. Bar=20 μm.

The endothelium of blood vessels in both subepithelial and subglandular connective tissue and in the interstitium among tubules of apocrine glands in paranal sinus' wall exhibited a moderate enzyme histochemical activity (Fig. 2). There were no NADPH-d positive nerve fibres in paranal sinus wall.
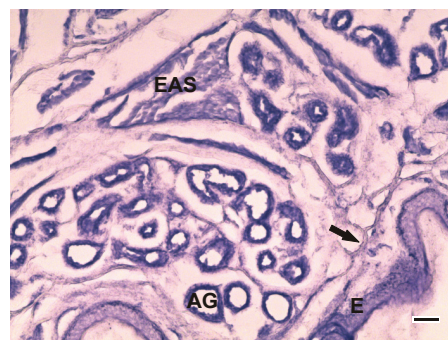

Fig. 2. Moderate enzyme histochemical activity (arrows) in the endothelium of microcirculatory vascular bed in subepithelial connective tissue of the sinus. Moderate to strong NADPH-d expression in secretory and myoepithelial cells of apocrine glands (AG); EAS – external anal sphincter (transverse and oblique cross sections); E – stratified squamous cornified epithelium. Bar=40 μm.

Muscle cells in the external anal sphincter exhibited either moderate to strong or weak enzyme histochemical reaction (Fig. 2). There were areas from the sarcolemma and perinuclear cytoplasmic zones with moderate to strong reaction. The nuclei of muscle cells remained unstained. Muscle cells with weak reaction were located among muscle fibres with strong NADPH-d reaction.

On external anal sphincter cross- sections, muscle cells also showed a variable degree of enzyme activity. Single areas of the sarcoplasm near to the sarcolemma, as well the sarcolemma of some cells itself, reacted strongly for NADPH-d.
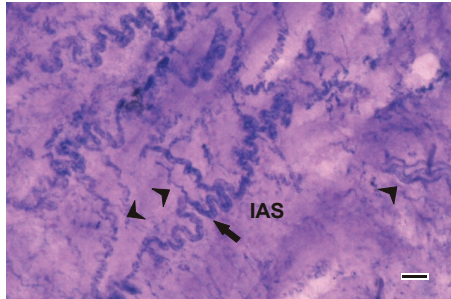
Fig. 3. Strong enzyme activity (arrow) of nit-rergic nerve fibres located among the smooth muscle cells along the longitudinal axis of the muscle. Some of nerve fibres (arrowheads) cross transversely the internal anal sphincter (IAS). Bar=20 μm.

In the internal anal sphincter, a strong enzyme activity of nitrergic nerve fibres located among smooth muscle cells along the longitudinal axis of the muscle was observed (Fig. 3). Some of them crossed transversely the internal anal sphincter (Fig. 3).

The blood vessels from the microcirculatory bed were NADPH-d positive only in endothelial cells' cytoplasm.

DISCUSSION

The stratified squamous cornified epithelium, stroma, apocrine and sebaceous glands in paranal sinus wall showed a NADPH-d activity similar to that reported by Meyer & Neurand (1976) in different skin layers in pigs. All layers of the epidermis in domestic pigs, except for stratum corneum have shown a moderate to strong NADPH-d activity. In the view of Weller *et al.* (1996) the differentiation of keratinocytes was probably locally controlled by nitric oxide. Nitric oxide production of the normal human skin was shown to be independent from NO-synthesizing enzymes. NO production depended upon skin surface pH and sweat nitrite concentrations resulting from degradation of nitrates by bacterial nitrate reductase. Nitric oxide is also believed to influence cutaneous blood circulation, the differentiation of keratinocytes and cellular immune response. Thus, the assumed physiological role of NO was defense against skin pathogens.

NADPH-d expression in vascular endothelium of the paranal sinus allowed us to agree with the conclusions of Bull *et al.* (1996) about the effect of nitric oxide on skin blood vessels. The authors have detected eNOS in the microvascular endothelium of the human skin and provided evidence that these cells released NO. The latter was believed to maintain the vascular tone of the microcirculatory vascular bed as well as to assist in the neurogenic vasodilatation in response of vasodilator neuropeptides such as substance P. According to researchers, substance P induces NO release from the dermal microvascular epithelium. The low rate of dermal NO synthesis was presumably important for maintaining the barrier function and circulatory regulation in microcirculatory vascular bed. The increased NOS activity after skin injury is important for infiltration of leukocytes and initiation of inflammation. The higher rates of NO synthesis are accompanied by production of proinflammatory cytokines in the skin of guinea pigs (Teixeira *et al.*, 1993). The authors concluded that NO inhibitors could also act as anti-inflammatory substances. The antimicrobial effect of NO in human skin was also reported by Cals-Grierson & Ormerod (2004).

In this study, no NADPH-d positive nerve fibres were observed in the subepithelial, subglandular connective tissue and paranal sinus' interstitium and thus,

we confirmed the findings of Bull *et al.* (1996) in human skin.

The moderate diaphorase activity in peripheral sebocytes and weak to absent expression of the enzyme in central sebocytes, observed in this study, corresponds to findings about diaphorase activity in dermal sebaceous glands in deer (Barling & Shirlei, 1999) and domestic pigs (Meyer & Neurand, 1976). According to Barling & Shirley (1999) NADPH-d activity in sebaceous glands was related to lipid synthesis and further, to the development of sebaceous acini.

The strong NADPH-d activity, demonstrated in secretory and myoepithelial cells of apocrine glands confirmed what was reported by Meyer & Neurand (1976) in the skin of domestic pigs. The inhibition of NOS has resulted in considerable reduction of sweat and increased body temperature of a horse during exercise (Mills *et al.*, 1997). This effect could be attributed to vasoconstriction, but authors suggested a possible modulation via NO upon central and/or peripheral sympathetic control of sweating.

In rats, mammary gland apocrine cells, smooth muscle cells, sebaceous glands, vascular endothelium and the epidermis reacted positively for NADPH-d and NOS (NOS1 and NOS3) (Iizuka *et al.*, 1998). The detection of NADPH-d and NOS-positive cells in rat mammary gland suggested nitric oxide synthesis in this organ. The authors assumed that nitric oxide was involved in milk secretion as well. On the basis of these findings, it could be anticipated that nitric oxide could influence the secretory processes in canine paranal sinus too.

Our results for NADPH-d expression in the external anal sphincter of the dog are similar to those of Rothe *et al.* (2005) about enzyme activity in rat skeletal muscles. The longitudinal cross-section of

canine external anal sphincter revealed a strong enzyme reaction in several areas of the sarcolemma and perinuclear cytoplasmic zones. The nuclei of muscle cells were not stained. Muscle fibres with a weak reaction were distributed among strongly reacting muscle fibres. Transverse cross section of the external anal sphincter also demonstrated muscle cells with variable degree of enzyme activity. Some areas of the sarcoplasm adjacent to the sarcolemma, and muscle cells sarcolemma itself were strongly NADPH-d-positive. By electron microscopy, Rothe *et al.* (2005) have observed the expression of NADPH-d and NOS1 near to the endoplasmic reticulum and mitochondria or bound to membranes in rats. By light microscopy, the authors established NADPH-d expression in the sarcolemma, in some areas of perinuclear cytoplasm but not in the nucleus itself. Areas of sarcolemma that have shown a strong reaction were attributed to the contact between nerve ends and muscle fibres in the zone of neuromuscular apparatus in a simultaneous enzyme histochemical study for NADPH-d and acetyl cholinesterase. Balon & Nadler (1997) outlined that regular exercise increased the expression of NOS1 and NOS3 in soleus muscle homogenates in rats. In their view, NO enhanced glucose transport in skeletal muscles. On the basis of these facts it could be speculated that NO has a similar role in canine external anal sphincter.

The strong enzyme activity of nitrergic nerve fibres among the smooth muscle cells along the longitudinal axis of the internal anal sphincter supports the results obtained by Lalatta-Costerbosa *et al.* (2007) with regard to NADPH-d and NOS expression in intestinal wall. Some of them crossed transversely the internal anal sphincter. The authors concluded that nitrergic nerve fibres and ganglia were

related to smooth muscle cell relaxation and therefore, that nitric oxide influenced the function of the internal anal sphincter whose contractions are known to facilitate the discharge of secretion from the paranal sinus.

According to Wink *et al.* (1998), the role of nitric oxide for carcinogenesis is multifaceted. Tissue exposed to high NO concentrations over a long period of time, i.e. affected by chronic inflammation or under environmental NO influence, could accumulate mutations due to NO itself or to other genotoxic agents. After the tumour begins to grow, macrophages and killer cells use NO produced by iNOS to destroy tumour cells. During progression of the tumour, NO enhances capillary permeability, supports angiogenesis and limits the infiltration of leukocytes. Furthermore, NO could confine metastases and induce tumour cells apoptosis.

Provided evidence about the expression of NADPH-d imply a possible nitric oxide synthesis in the different structures of the canine paranal sinus and suggest a role of this compound in neoplastic and inflammatory conditions, that are commonly seen in dogs (Meuten *et al.* 1982, Van Duijkeren, 1995).

CONCLUSION

The NADPH-d expression in stratified squamous cornified epithelium of canine paranal sinus, in apocrine glandular and myoepithelial cells and sebaceous glands observed in the present investigation was probably related to nitric oxide synthesis in these structures. Furthermore, nitric oxide may be involved in the generation and release of paranal sinus secretion. NADPH-d-positive nerves in the internal anal sphincter as well as NADPH-d-positive skeletal muscle cells of the exter-

nal anal sphincter confirmed the importance of NO for the function of these muscles and canine paranal sinus. The enzyme activity in the endothelium of microcirculatory vascular bed allowed affirming that nitric oxide was not only synthesized in the vascular wall, but could have an effect in vascular tone as well. Nitric oxide inhibitors could then have an anti-inflammatory effect in canine paranal sinus, where inflammations are common. Further research is necessary for elucidating the role of nitric oxide in canine paranal sinus function.

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