Coll. Antropol. **26** (2002) 2: 681–688
UDC 616.314.18-002.2:616-074
Original scientific paper

Nicotinamide Adenine Dinucleotide Phosphate-Diaphorase (NADPH-d) Histochemistry Detecting NOS in Healthy and Chronically Inflamed Pulp

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A B S T R A C T

The aim of this study was to examine the expression of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity in human dental pulps and determine whether there are changes of the activity in chronically inflamed pulp tissue. Nineteen pulps with clinical diagnosis of chronic pulpitis were collected during endodontic treatment. The healthy controls were obtained from teeth extracted for orthodontic therapy. The clinical diagnosis was confirmed by histological analysis. Healthy pulps showed stratified odontoblasts in peripheral parts, while in central area there was normal connective tissue. Chronically inflamed pulps showed less expressed stratification of odontoblasts and infiltration of lymphocytes, polymorphonuclear leukocytes, plasma cells and mastocytes. NADPH-d granular reactivity was assessed semi quantitatively under the light microscope by a single observer and scored on an intensity scale from negative reaction to very strong reaction. In healthy human pulps, NADPH-d activity was strong to very strong in odontoblastic layer. Endothelial cells and Schwann cells showed strong NADPH-d reactivity, while the other parts of central area were weakly positive. Similar distribution of reactivity was expressed also in chronically inflamed pulp; moderate to strong reaction was observed in stromal area as result of positive reaction in inflammatory cells and endothelial cells of abundant newly formed capillaries.

Received for publication October 18, 2002

Introduction

The endogenous production of nitric oxide (NO) plays a role in the regulation of the physiological processes such as blood vessel tone and neurotransmission, as well as in host defence and immunity[1,2]. The finding of nitric oxide synthase expression in human cancers indicates its role in pathophysiology of carcinogenesis[3]. Nitric oxide is synthesized via oxidation of arginine by a family of nitric oxide synthases (NOS). There are three isoforms of NOS. Two of them are dependent on elevated intracellular Ca^{2+} and because of their constitutive appearance are termed cNOS. These isoforms are NOS1 (also called nNOS or ncNOS) which is found in neurons and NOS3 (eNOS or ecNOS) which originated from endothelial cells. The NOS2 cloned from macrophages is independent of elevated intracellular Ca^{2+}. Its expression is inducible (iNOS) by cytokines and bacterial lipopolysaccharide[4]. However, all NOS are flavoproteins that require nicotinamide adenine dinucleotid phosphate (NADPH) and tetrahydrobiopterin as co-factors[5]. Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) has been found to be highly resistant to many protein fixatives and hence visualisation of NADPH-d activity by histochemical reaction of tissue sections has become a viable technique for localising NOS activity[6,7].

In previous research NADPH-d histochemistry of dental pulp has been done on rats and cats[8]. The expression of NADPH-d activity has been never examined in human pulp tissue yet, neither the changes of its activity in chronically inflamed pulp. In this study we tried to localize NADPH-d activity in human dental pulp and to find out whether there are changes of the NADPH-d activity in chronically inflamed pulp tissue.

Material and Methods

Nineteen samples of human inflamed pulp tissue were obtained from teeth during endodontic treatment with clinical diagnosis of chronic pulpitis. The patients were between 20 and 45 years old. Nine pulp samples were obtained from female and ten from male patients. The pulp tissue was extirpated by Hödstrom files (Maillefer, Ballaigues, Switzerland) after anaesthesia and preparing the access cavity. The tissue was removed gently from the endodontic file by tweezers and placed in aluminium foil, compressed to evacuate the air and frozen in fluid nitrogen. Fifteen healthy controls (seven female and eight male) were obtained from teeth extracted because of orthodontic reasons. Extracted teeth were grooved by high-speed diamond drill with water coolant to one half of the thickness of dentine along the tooth axis. They were split by sharp extraction lever and hammer. The pulp tissue was extirpated and processed as described. The samples were frozen in cryostat at –25 C, and 8 m-thick sections of the tissue were cut.

The incubation media for NADPH-d histochemistry was used according the method described by Pearce[9]. Prepared incubation media was put on tissue cuts mounted on glass slides and left 20 min at 37 C. Then the glasses were washed once in formol saline, twice in distilled water and enclosed in gelatine. Also adjacent sections were stained with haematoxylin and eosin.

All samples were examined under the light microscope. The reaction of NADPH-d is granular in the cells. Reactivity was assessed by a single observer and scored by examination of five randomly chosen fields on an intensity scale from 0 to ++++, with 0 indicating negative reactivity; + slight reactivity; ++, moderate reactivity; +++ strong reactivity; and ++++ very strong reactivity e.g. dense granular reaction. To

enable statistic analysis intensity scale was transformed to numbers: + – 1, ++ – 2, +++ – 3, ++++ – 4. Statistical analysis was done by descriptive statistic for population ($p < 0.05$).

Results

The sections of the healthy pulp showed peripheral area with more or less conserved palisade arrangement of odontoblasts with subodontoblastic layer that is lacking the cell nucleus. Cell rich zone was underlying peripheral part. Central part consists of fibroblasts, blood vessels, nerves and connective fibbers (Figure 1). Chronically inflamed pulp tissue showed abundance of lymphocytes, polymorphonuclear leukocytes, plasma cells and mastocytes. Odontoblasts had irregular stratification. Mineralizations were often found (Figure 2).

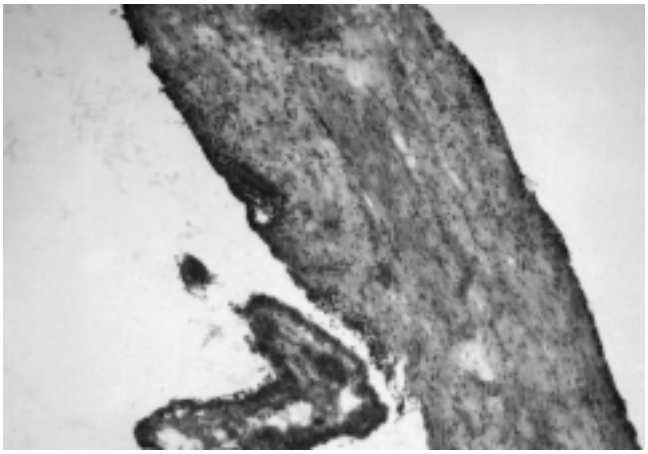

Fig. 1. Healthy pulp (HE, original magnification 40).

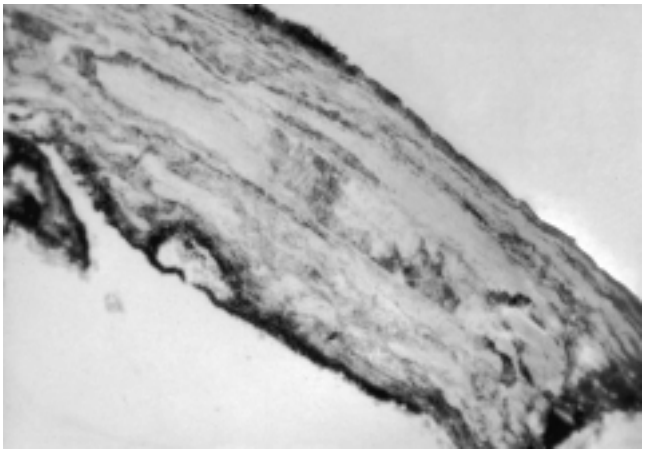

Fig. 2. Inflamed pulp with disarrangement of odontoblasts, abundance of capillaries and oedema (HE, original magnification 100).

In human healthy pulps, NADPH-d activity was strong (+++) to very strong (++++) and observed predominantly in odontoblastic cells. Endothelial cells and Schwann cells showed strong reactivity. In the other cells of the stromal area the activity was slight (+) (Figure 3).

Strong (+++) to very strong (++++) NADPH-d activity was obtained in odontoblasts of chronically inflamed dental pulp. Strong reactivity (+++) was observed in stromal part of the pulp tissue due to strong activity expressed in polymorphonuclear leukocytes and lymphocytes, and plenty of new-born capillaries with NADPH-d positive endothelial cells (Figure 4).

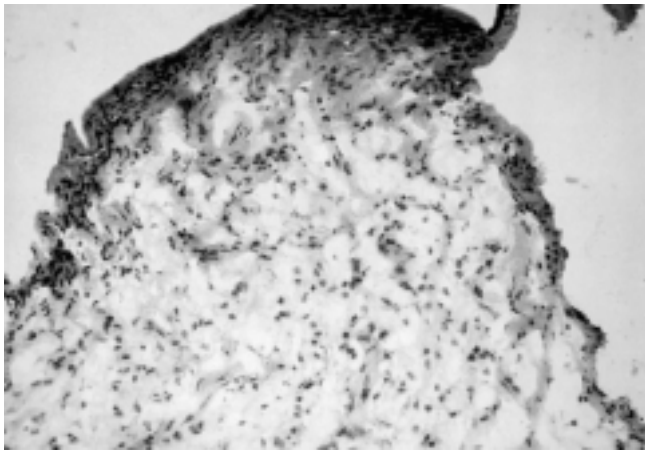

Fig. 3. NADPH-d immunohistochemistry of healthy pulp showing positive odontoblastic and subodontoblastic layer, endothelial cells and cells surrounding nerve (original magnification 40).

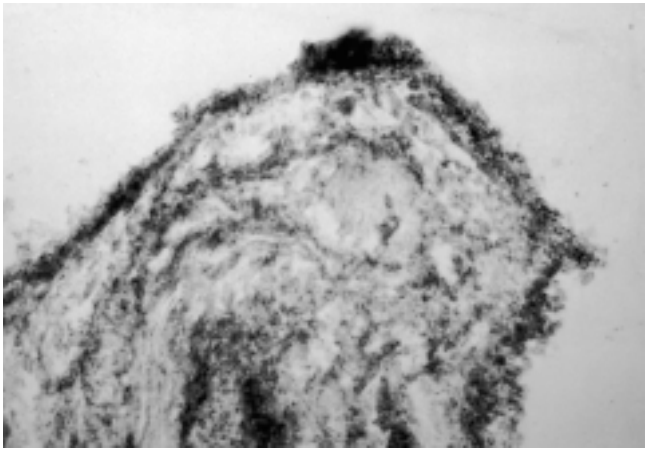

Fig. 4. NADPH-d immunohistochemistry of chronically inflamed pulp (original magnification 100).

TABLE 1
ACTIVITY OF NADPH-D IN HEALTHY AND CHRONICALLY INFLAMED HUMAN PULP

	Healthy pulp	Chron. inflamed pulp	t	F	p	Healthy pulp	Chron. inflamed pulp
	X	X				N	N
Odontoblasts	3.33	3.34	–0.04	32	0.97	15	19
Endothelial cells	2.87	2.89	–0.11	32	0.91	15	19
Schwann cells	3.13	3.58	–1.62	32	0.12	15	19
Stromal part	1.27	2.45	–4.93	32	0.00	15	19

The difference between healthy and inflamed pulp was statistically significant only in the central parts of the pulp (Table 1).

Discussion

In this study the NADPH-d activity was examined in healthy and diseased human dental pulp. We found prominent NADPH-d activity in the odontoblasts, endothelial cells and Schwann cells. Although, there is evidence of interspecies differences in the expression of NADPH-d/NOS reactivity, and NADPH-d histochemistry is not always coincident with NOS[10], the localization of NADPH-d in the human pulp, found in this study is in accordance with studies that examined other mammalian species. The localization of NADPH-d activity in the dental pulp of the rat incisor and molar teeth, and cat teeth are well documented in 1993 by Kerozoudis et al.[8]. They demonstrated the activity in odontoblastic layer, subodontoblastic layer and endothelial cells of pulpal blood vessels. In the radicular part of incisor pulp, however only cells in subodontoblastic layer showed NADPH-d activity. This author failed to detect NADPH-d positive nerve fibers in contrast to Lohinai et al.[11] who found a small number of NADPH-d positive nerve fibers scattered in the connective tissue of dog's and cat's pulp axons occupying a perivascular location.

In spite of destruction of odontoblastic processes during endodontic treatment with Hödstrom files in this study, distinct expression of NADPH-d could be noticed in the odontoblastic cells. Kerozoudis et al.[8] demineralized tooth hard tissue and preserve cell processes that extend deep in to the dentine and found NADPH-d positive Tomes's processes of odontoblasts in rats. Odontoblasts are cells of mesenchimal origin and they are derived directly from neural crest cells[12]. They are highly specialized cells involved in secretion of dentine matrix, its mineralization, dentine sensitivity and dentine nutrition. The expression of NADPH-d activity as the marker of NOS in odontoblasts confirms that they are unique cells and their role in the pulp physiological and pathological conditions could be even more com0plex than it is assumed[13]. As they are first cells facing various noxa from oral environment, the role of NO releasing could be their local communication with other cells. NO accounts for tonic relaxation of all types of blood vessels and it act as neurotransmitter in central and peripheral nervous system[14]. Also NO is proposed to be a second messenger in the management of dentine hypersensitivity by the agency of K^+ [15]. This view is supported by studies that report analgesia in association with increased NO production through a modulation of nociceptive input and downregulation of sensitized nociceptors. It is assumed as a conse-

quence of ability of NO to activate the soluble guanylic cyclases[16] which produce increase level of intraneural cGMP[17].

Endothelial cells of the human pulp showed marked NADPH-d activity what is in accordance with study of Lohinai et al[11]. who found positive NOS/NADPH-d endothelial cells of dog's and cat's pulps and inferior alveolar artery. The fact that a NOS-basal dependent vasodilatation exists in the dental pulp, as in other tissues, was demonstrated, also by Lohinai et al.[18]. They showed the high responsiveness of dental pulp vasculature to exogenous NO. Therefore, it is presumed that the stimulated release of endogenous NO is involved in vasoregulation of dental pulp.

Although, the presence of small number of NADPH-d positive and NOS immunoreactive axons in the dental pulp of cats and dogs was reported previously[17], our study is the first in the literature that describes occurrence of NADPH-d positive Schwann cells in the pulp. NADPH-d positive perisynaptic Schwann cells were found in the frog neuromuscular junctions and immunohistochemical labeling of neuronal NOS was detected at the membrane and occasionally in the cytoplasm of these cells. The author proposed that NO may act as diffusible glial messenger that modulate synaptic activity and synapse formation in the neuromuscular junction[19]. According that, the role of NOS in Schwann cells of the pulp could be the modulation of signal transmission through the pulp nerve fibers.

Chronically inflamed human pulp exhibited NADPH-d activity in polymorphonuclear leukocytes and lymphocytes. Also endothelial cells of numerous newborn capillaries and disordered odontoblasts contribute to strong activity of NADPH-d central parts of the inflamed pulp. Chronic inflammation is more often related to the carious lesion, and the number of inflammatory cells depends on the progression of the lesion[20]. Also deep cavity preparation results in acute inflammation that progress to chronic, granulomatous inflammation. Results of this study are similar to findings of Law et al.[21] who examined NADPH-d reactivity and NOS immunoreactivity following tooth preparation in rats. They found increased intensity of NADPH-d and macrophage NOS reactivity surrounding the inflamed area of pulp. Also pulp vessels supplying inflamed area showed increased NADPH-d reactivity[21].

Nitric oxide has been found to play an important role in many chronic inflammatory diseases[22,23]. Macrophage NOS produces a large amount of NO with appropriate stimulation and induces substantial tissue damage. The exact nature, positive or negative, of NO is still a meter of scientific debate. Okuda et al.[24] found that experimental allergic encephalomyelitis of mice is characterized by elevation of nitric oxide. The authors suggest that excessive NO via NOS played an important role in eliminating inflammatory cells through apoptotic mechanism. However, researchers have noticed the suppressive effect of activated macrophages on proliferate responses of lymphocytes to mitogens or antigens. Also the leukocyte cell adhesion to endothelial cells is inhibited by NO[25]. Therefore, the modulating role of NO in regulation of inflammation, tissue destruction, and metabolite dysfunction has been proposed.

The presence of iNOS immunoreactivity in radicular cyst which is mostly a consequence of pathological events in pulp, was examined by Takeichi et al.[4]. The immunoreactive iNOS is present in epithelial cells, endothelial cells, fibroblasts, macrophages and polymorph nuclear leucocytes. Interesting finding is that the proximity of blood of vessels influences iNOS production. Unlike PMNL and macrophages adjacent to blood vessels which exhibit positive iNOS production, the dis-

tant cells are iNOS reactivity weak or negative. This is explained by distribution of different types of T-helper cells, which may or may not synthesize iNOS. The iNOS production could be stimulated with bacterial lipopolysaharids (LPS) and inflammatory cytokines, such as interleukin-1 , tumor necrosis factor and interferon- (INF-). Close vicinity of iNOS-producing cells and INF- -producing cells, and appearance of immunoreactivity for both molecules in some cells indicates the interaction of iNOS producing cells and INF- -producing cells in radicular cysts. The interaction of the pulp and periradicular tissue allow as to compare this findings with ours, so we assumed that the stimulation of iNOS by bacterial LPS and inflammatory cytokines plays significant role in pathogenicity of the chronical pulp inflammation. Takeichi et al.[4] proposes that NO inhibitors (N^G-monomethyl-L-arginine) could be used through root canal as a pharmacological therapy for periapical lesions, so the addition of such inhibitors in materials that are used for the pulp capping could prevent the onset of inflammation caused by production of large amounts of NO.

These facts, together with further investigation that should carried on about the distinct type of NOS expressed in specific parts of healthy and inflamed pulp, could clarify the exact role of NO in physiology and pathophysiology of this tissue.

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SINTAZA DUŠIKOVA OKSIDA (NOS) U ZDRAVOJ I KRONIČNO UPALJENOJ ZUBNOJ PULPI PRIKAZANA HISTOKEMIJOM NIKOTINAMID ADENIN DINUKLEOTID FOSFAT – DIJAFORAZE (NADPH-d)

S A Ž E T A K

Svrha istraživanja je bila ispitati ekspresiju koenzima nikotinamid adenin dinukleotid fosfat-dijaforaze (NADPH-d) u ljudskoj pulpi i odrediti postoji li razlika u njegovoj aktivnosti u kronično upaljenoj pulpi. Devetnaest uzoraka je prikupljeno iz zubi koja su bila u postupku endodontskog liječenja s kliničkom dijagnozom kroničnog pulpitisa. Zdravi uzorci su dobiveni iz zubiju ekstrahiranih zbog ortodontskih razloga. Pulpe su ekstirpirane, smrznute i obrađene histokemijskim postupkomza prikazivanje NADPH-d. Uzorci su ispitani pod svjetlosnim mikroskopom. U zdravim uzorcima aktivnost NADPH-d je jaka do vrlo jaka u odontoblastičnom u subodontoblastičnom sloju. Jaka aktivnost je pronađena u endotelnim stanicama krvnih žila i stanicama koje okružuju živčana vlakna, dok su preostali središnjeg dijela pulpe bili jako slabo do slabo pozitivni. Slična raspodjela aktivnosti je pronađena u kronično upaljenoj pulpi, te je umjerena do jaka aktivnost, raspršene rasprostranjenosti, pronađena u središnjem dijelu gdje su prevladavale upalne stanice.