EXPERIMENTAL STUDY

Alterations of the vessel wall innervation during diabetes mellitus

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Abstract

Coronary and valvular heart disease during diabetes mellitus (DM) are major contributors of morbidity and mortality in the diabetic population. Relatively little atention has been given to the study of heart valve nerve structures in different pathological processes. In this study we have demonstrated the presence of possible morphological alterations in vessels of the anterior cusp of the rat mitral valve during 8–12 weeks DM. A histochemical method was used for the detection of NADPH-diaphorase (NADPH-d), which is the indirect NO-synthase marker.
Arterioles and fine capillaries were localized in the attachment zone of the anterior cusp. Perivascular nerve fibres were identified running in the *tunica adventitia*.
A marked dilatation of the vessels was seen in diabetes in comparison with control samples. No NADPH-d positive nerve fibres were observed in the *tunica adventitia*. It can be presumed that metabolic changes in the vessel walls during DM reflect modified neurotransmission of NO by means of their excessive overproduction of NOS (endothelial – eNOS) in endothelial cells. *(Fig. 6, Ref. 32.)*
Key words: mitral valve, vessels, NADPH-d, diabetes mellitus, rat.

After radiation, ischaemia and experimental diabetes, neural tissue and vessels show many functional and structural alterations. These alterations impair, in tissues described above, enzymatic activity (Schmidtová et al, 1994; Mechírová et al, 2001). *Diabetes mellitus* (*DM*) is one of the main causes of many neurovascular abnormalities which affect important systems of living organisms by disturbing their metabolic and enzymatic balance. Diabetes is the activator of sclerotic alterations of the coronary arteries. The high glycaemia and permanent metabolic inbalance in the vessel wall lead to coronary artery regression (Poston and Taylor, 1995). Clinical and animal studies have suggested that cardiac dysfunction could be more closely linked to major abnormalities in carbohydrate and lipid metabolism (Rodrigues and McNeill, 1992).

The mitral valve is nourished in the zone of *anulus fibrosus* by the terminal branches of coronary arteries (Halpern, 1957; Jew et al, 1996). The protective mechanism of endothelial cells (ECs) against oxygen free radicals, reactive oxygen species (ROS), is impaired. ROS, if not scavenged, then damage the vascular endothelium and neutralize the NO. Oxidative stress contributes to the development of neural and vascular complications in experimental diabetes (Cameron and Cotter, 1996; Pop-Busui et al, 2001).

Nitric oxide (NO) acts as a gaseous free radical neurotransmitter, and an effective cardiovascular modulator. NO participates in the regulation of coronary blood flow and tension of vessel wall. The regulators of NO production are the physical and chemical stimulations transmitted by the vessel wall.

The NO production is conditioned by the activity of three basic NO synthases (isoforms). In mammalian heart tissues two constitutive synthases are present permanently: neuronal NO synthase, nNOS (NOS I), which is present in cells of peripheral nervous system (in cardiac intrinsic ganglia), in heart nerve fibres, and also is expressed in vascular smooth muscle cells (Boulanger et al, 1998). Endothelial NO synthase, eNOS (NOS III), is produced by ECs in two types: the cytosol and membraneous types. NOS III is expressed in endothelial cells, endocardial cells, and in cardiomyocytes (Klimaschewski et al, 1992; Nathan and Xie, 1994; Štvrtinová et al, 1998; Drexler, 1999).

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Inducible NOS (iNOS, macrophage NOS, NOS II) is not present in the cells permanently. In disease states associated with infection, inflammation, or cytokine activation, the expression of iNOS is clearly demonstrated in the heart, including in the cardiomyocytes. This NO synthase is produced by macrophages, neutrophiles, cells stimulated by *E. coli*, cytokines as well as by smooth muscle cells (SMCs) and by ECs of vessel wall (Nathan and Xie, 1994; Balligand et al, 1995; Depre et al, 1999). NOS II may be included in the pathophysiology of diabetic cardiomyopathy, ischaemic heart and valvular heart diseases and appeares as a response to permanent activation by cytokines and bacterial products (Drexler, 1999; Muller et al, 2000). Induction of the iNOS in the vascular and cardiac tissue by several inflammatory stimuli may result in the production of large amounts of NO (Muller et al, 2000).

The valve heart system reacts to many of the above mentioned impacts. The aim of this study was therefore to characterize the possible morphological alterations of vessel structures of the anterior cusp of the mitral valve in experimental *DM*.

In this study we also investigated the possibility that vessels, as well as nerve structures, may be a source of nitric oxide in the diabetic heart. The study of Dawson et al (1991) showed, that under conditions of tissue fixation the NADPH-diaphorase is identical with the NO synthase, which corresponds to our laboratory conditions (the tissue – cusps of valves were fixed by paraformaldehyde).

Material and methods

Groups of animals used in experiment

Twenty male white *Wistar* rats, aged 3 months, with a mean weight of 250–350 g were used in this study. All animals were allowed food and water *ad libitum* and during the experiment rats were kept on *Larsen's* diet. *Diabetes mellitus* was induced in a group of 10 animals, i.p. administration of streptozotocin (STZ) in two doses 45 mg/kg and 20 mg/kg, while rats were under light ether anaesthesia. The control group consisted of 10 rats. Control rats were injected with citrate buffer only. The diabetic animals were observed without insulin from 8 to 12 weeks after induction of experimental diabetes. During the period of observation the level of blood glucose and weight were determined.

All experiments on laboratory animals were performed in accordance with:

1. The Act on protection of animals No. 115/1995 Coll., as amended,

2. Decree of the Ministry of Agriculture of the Slovak republic No. 231/1998 Coll.,

On breeding of domestic animals, wild animals, dangerous animals and on protection of experimental animals.

Samples of tissue and fixation

The rats of both groups were anaesthetized with pentobarbital (50 mg/kg). A midline abdominal incision, followed by two lateral costal margin incisions exposed the diaphragm which was then incised. The thorac cavity was opened and the heart was perfused with a saline solution and 4 % paraformaldehyde with 0.1 % glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The heart was removed, the left ventricle was rapidly opened and the valves were cut from the atrioventricular ring. The sample material was placed in the same fixative for 2 hours. The fixed tissue was transfered overnight into 30 % saccharose in the same PB at 4 °C.

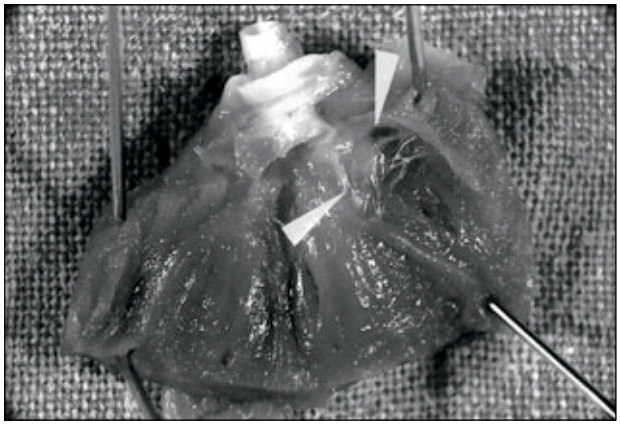

Fig. 1. The anterior cusp of the mitral valve of the rat heart. The picture shows the zone adjacent to the fibromuscular (atrioventricular) ring (arrow on the right), the free moving zone and chordae tendineae (arrow on the left).

NADPH-diaphorase histochemistry

The investigation of NADPH-diaphorase activity vessels of the anterior cusp of the mitral valve was carried out using an histochemical technique according to Scherer-Singler et al (1983) and modified by Kluchová et al (2002). The valves were incubated for 1 h at 37 °C in a solution containing 1.5 mM nitro blue tetrazolium, 1.0 ß-NADPH, 10 mM L-malic acid (all Sigma Chemicals) and 0.5 % Triton X-100. After incubation, the valves were rinsed in 0.1 M PB (pH 7.4) and processed by a *whole mount stretch* technique in which they were mounted on glass slides, dried overnight and covered with Entellan (Merck, Germeny). The preparations were viewed under a light microscope with photo *Labophot-2*, HFX-DX, NIKON.

The valves of small laboratory animals (mainly rats) are suitable to use, because of their optimal size, as a pattern for the study of valves. Examine structures were often observed already with the basic objective magnification (x4). The zone adjacent to the fibromuscular – atrioventricular ring (*anulus fibrosus*), (Fig. 1) was that part of the anterior cusp of the mitral valve where possible morphological alterations were examined.

Results

NADPH-d activity under physiological condition

In varying intensities, ECs of the endocardium (Figs 3 and 4), vessels and fine nerve fibres (Figs 2, 3 and 4) in the attachment part of the anterior cusp were stained as blue shadows and dark-blue up to black reaction product as evidence of the reduction of nitro blue tetrazolium (NBT) to the formazan insoluble in water.

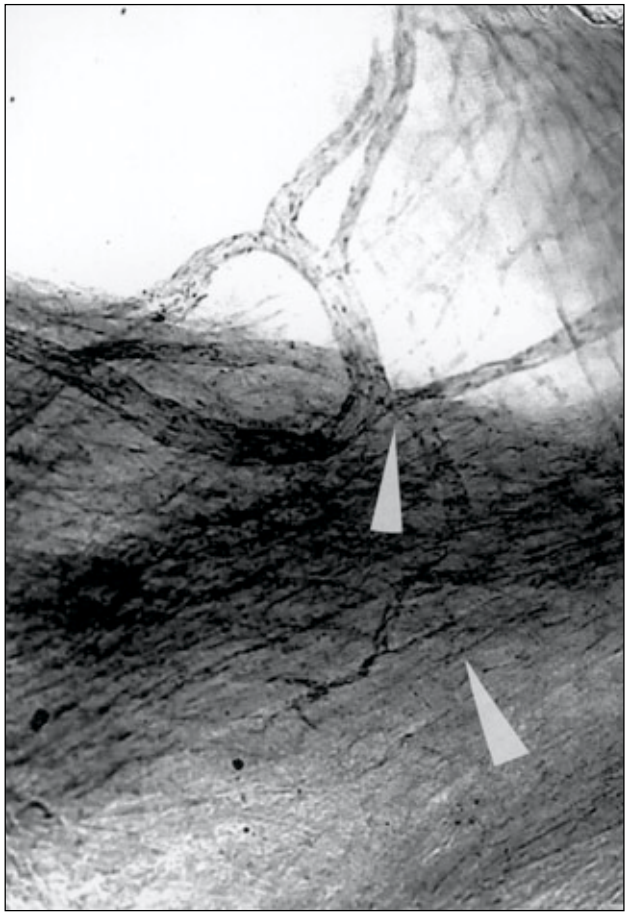

Fig. 2. Control group: NADPH-d positive vessels (capillaries) and a dense network of fine nerve fibres in the attachment zone of the valve cusp (arrows).

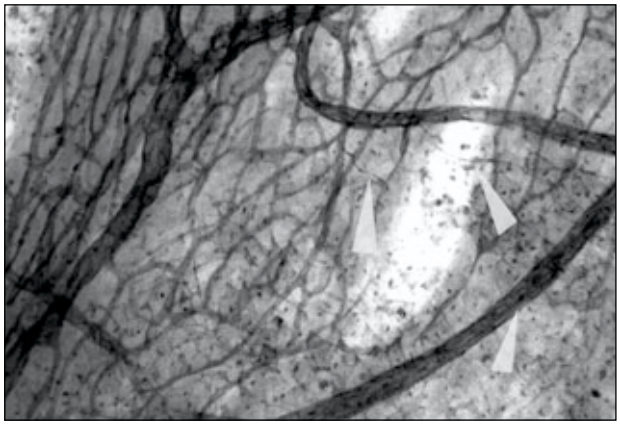

Fig. 3. Control group: NADPH-d positive nerve fibres in the attachment zone running along the capillaries and producing adventitial plexuses (arrows).

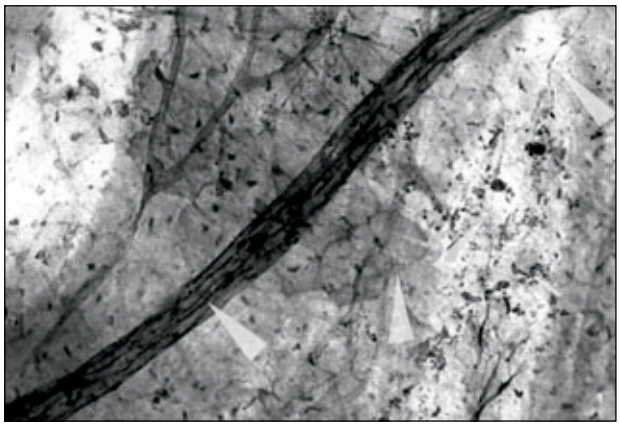

Fig. 4. Control group: Detail of the NADPH-d positive fine blood vessel with dark stained nerve fibres in the tunica adventitia and in the perivascular localization. In the back, endothelial cells (ECs) are seen (arrows).

The expressive NADPH-diaphorase activity was manifested in peripheral cell borders and in the perinuclear cytoplasm as well (Fig. 4). Multi-edge ECs were arranged surfacially with the borders located close to each other and with separate processes to each other formed the interdigitations. The cytoplasm of ECs showed colour intensity from medium dark up to very light. ECs did not show any signs of compression or elongation (Fig. 4). The nerve fibres which ran perivascularily in the *tunica adventitia* were stained expressively (Figs 3 and 4). NADPHd-positive nerve fibres often ran along myocardial ventricular fibres, close to vessels (Fig. 2).

NADPH-d activity during experimentaly induced diabetes mellitus

During the 5-week investigation of the rats involved in the experiment, the glycaemia values of this group were about 21.5 mmol/l on average. The glycaemia values of the control group were about 5.2 mmol/l. The glucose levels of the experimental group increased almost by four times and weight of this group was reduced proportionally to the glycaemia increase by approximately 10 %.

The NADPH-d activity was retained in the structures investigated in the period from 8 to 12 weeks after induction of diabetes. Vessels located in the cusp attachment part manifested signs of expansion (i.e. possible dilatation). Through the vessel wall only contoured ECs were seen. Nerve fibres were situated in the perivascular location in limited range and their presence was not seen in the *tunica adventitia* of the vessels (Figs 5 and 6).

By means of the modified histochemical method, the NADPH-d activity in the endocardial ECs of the anterior cusp of the mitral valve, in endothelium of vessels presented in it and in the nerve fibres (in limited range also in diabetic tissue) was confirmed by indirect determination of the activity of the eNOS and nNOS synthases.

Discussion

NO produced by nNOS co-operates (when maintaining the homoeostatic condition of the inner space) with NO, which is produced in endothelium by the eNOS and participates in the vessel dilatation by SMCs activation. NO produced by ECs, to-

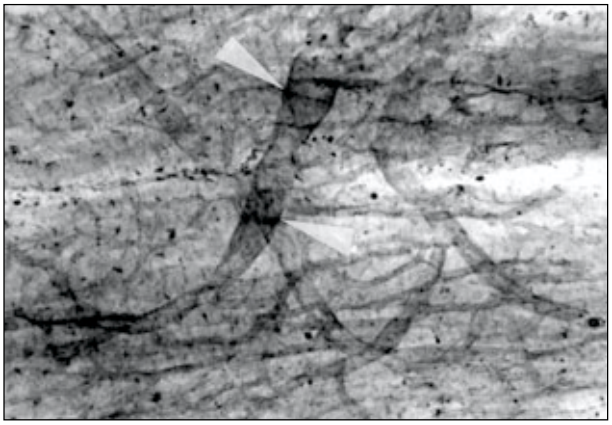

Fig. 5. Diabetic group: NADPH-d positive vessels are dilated in the attachment zone of the diabetic mitral valve (arrows).

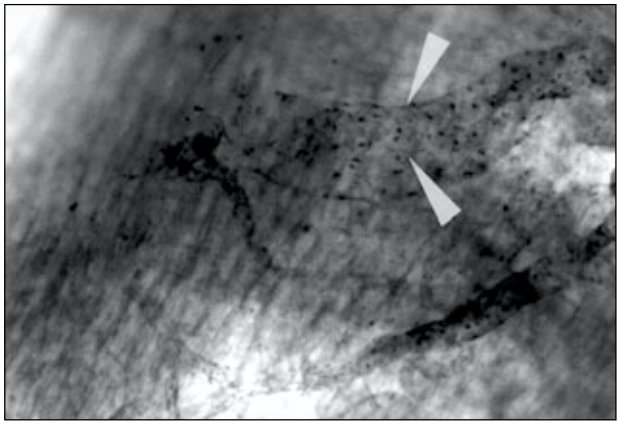

Fig. 6. Diabetic group: One of many dilated blood vessels. No nerve fibres are seen in the tunica adventitia. In the vessel wall only ECs are seen (arrows).

gether with NO, which is produced by diaphorase (nitrergic) nerve fibres, represents the main endogenous vasodilatory system (Bernátová and Pecháňová, 1998). So we expect that with possible damage of the *tunica intima,* when no damage of the *tunica media* has occurred, under influence of metabolic diabetic alterations, the NO molecules may act on the SMCs after release from plexuses of nerve fibres in the *tunica adventitia*. These plexuses are formed mainly by sympathetic, adrenergic postganglionic and also efferent cholinergic nerve fibres. These nerve fibres represent axone bundles which are unmyelinized and typically varicose (Williams et al, 1995). A small group is represented also by nitrergic fibres (NADPH-d positive, NOS-immunoreactive), which are branched, anastomosed and which form plexuses around the blood vessels (Ceccatelli et al, 1994; Sawada et al, 1997). These fibres are considered to be parasympathetic postganglionic fibres with vasomotor function (Jew et al, 1996; Rösen et al, 1991). The above is confirmed by the statement, that small branches of coronary arteries (arterioles and fine capillaries) are innervated by means of *n. vagus* (Williams et al, 1995). The evidence of perivascularily (adventitially) located NADPH-d positive nerve fibres showed that the NO fulfils the vasodilatory function not only from the vessel inside – by release from ECs (by means of eNOS, by diffusion into SMCs), but also under the influence of the *tunica adventitia*. The role and function of NO, mainly vasodilatation, are very important for clinical practice in specifical areas of the coronary blood supply, especially in valves.

The existence of NADPH-d activity in the terminal branches of coronary arteries (arterioles) in the zone adjacent to the atrioventricular ring is the evidence of blood supply in this part of the anterior cusp of the mitral valve. Klimaschewski et al (1992), Ursell and Mayes (1995) and Andries et al (1998) stated that there was strong activity of NADPH-d in the ECs of coronary arteries and arterioles and generally in the vessel endothelium.

The pathological atherosclerotic process may impact the fine vessels as a consequence of developed *DM*. High glucose levels increase the expression of eNOS in ECs of capillaries (Bernátová and Pecháňová, 1998) and NO production. A non-desired event which causes the origin of many cardiovascular diseases is therefore also the over-production of NO (Shah, 1996; Štvrtinová et al, 1998). It is possible to expect that partial or total damage – axonal degeneration of nervous sympathetic as well as parasympathetic fibres (Rösen et al, 1991) occurrs as a consequence of the increased production of NO (under the influence of metabolic diabetic alterations) into the vessel lumen and under influence of its cytotoxic activity that is manifested by a reduction of nerve fibres or their eventual absence. We suppose, that NO neurotransmission is probably locked by its own excessive production participating in substantial vessel vasodilatation under influence of SMCs as seen in this experiment study.

Rösen et al (1991) described the morphometric alterations of fine capillaries in the myocardium of diabetic *Bio-Breeding* (*BB)* rats. Capilaries are manifested by enlargement of diameter and volume density under influence of their reconstruction. This author suggests that the vasodilatation of vessels is caused metabolically, presumably by the enhanced release of lactate and adenosine. The diabetic heart has a limited capacity for oxygen supply, and the ability of the diabetic heart to adapt to an accelerated metabolic demand is limited. These findings were confirmed by Pieper (1998) who found that the increase in tissue blood flow in the early stages of diabetes may be due to hypoxic vasodilatation (by decrease of ATP concentration). Alternatively, increased blood flow may be a direct response to acute or short-term hyperglycaemia.

The occurrence of iNOS in vessels activated by lipopolysaccharides (LPS) or some cytokines suggests the excessive NO production with pathological consequences results in vasodilatation and hypotension. Takeda et al (2001) found that NOS is associated with clinical vascular dysfunction in the early stages of diabetes. Abnormal production of NO could contribute to the development of diabetic vasculopathy and retinopathy. Cytotoxic function of NO is also manifested by excessive concentration of glutamate which is a very important activator of receptors and neurons producing NO which release lethal amounts of NO causing the degeneration of target neurons (Bergendi and Ferenčík, 1999). It may be also manifested by locking the above mentioned transmission by means of NO. It may be also its own form of protection, which may become pathological after reaching a cer-

tain critical state (Bergendi and Ferenčík, 1999). NO produced by adventitial cells, which are activated by iNOS, according to Muller et al (2000), does not activate SMCs immediately but by gradual release may assure a long-term protection influence in the *tunica media*. It is possible, that iNOS derived NO likely plays a role in the induction of cytoprotective mechanisms against oxidative stress. However, whether high concentrations of NO produced by iNOS during sepsis or chronically in heart failure still have beneficial effects or may even be damaging, is much less clear (Zanzinger, 1999).

From the above it is clear that the NO overproduction may play a destructive or protective role in blood vessels. The role of NO in diabetic vasculopathy is still not clear (Chan et al, 2000).

This experimental study demonstrates that the induction of *diabetes mellitus* in the rat by use of streptozotocin is accompanied with marked changes in the morphology of valvular vessel and nerve structures.

References

Andries LJ, Brutsaert DL, Sys SU. Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium. Circulat Res 1998; 82: 195—203.

Balligand JL, Ungureanu-Longrois D, Simmons WW, Kobzik L, Lowenstein CJ, Lamas S, Kelly RA, Smith TW, Michel T. Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1ß and IFN-•. Amer J Physiol 1995; 268: H1293—H1303.

Bernátová I, Pecháňová O. NO-synthase: biochemical characterization and physiological implications. Bratisl Lek Listy 1998; 99: 474—482.

Bergendi Ľ, Ferenčík M. Oxid dusnatý (NO) — tvorba, fyziologické a patofyziologické funkcie. 39—73. In: Durackova Z, Bergendi L, Carsky J (Eds). Voľné radikály a antioxidanty v medicíne. Bratislava, SAP 1999.

Boulanger CM, Heymes C, Benessiano J, Geske RS, Lévy BI, Vanhoutte PM. Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells. Activation by angiotensin II in hypertension. Circulat Res 1998; 83: 1271—1278.

Cameron NE, Cotter MA. Metabolic and vascular factors in the pathogenesis of diabetic neuropathy. Diabetes 1996; 46 (Suppl 2): S31—S36.

Ceccateli S, Lundberg JM, Zhang X, Aman K, Hökfelt T. Immunohistochemical demonstration of nitric oxide synthase in the peripheral autonomic nervous system. Brain Res 1994; 656: 381—395.

Dawson T, Bredt TS, Fotuhi M, Hwang PM, Snyder SH. Nitric oxide synthase and neuronal NADPH-diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci USA 1991; 88: 7797—7801.

Depre C, Havaux X, Renkin J, Vanoverschelde JLJ, Wijns W. Expression of inducible nitric oxide synthase in human coronary atherosclerotic plaque. Cardiovasc Res 1999; 41: 465—472.

Drexler H. Nitric oxide and coronary endothelial dysfunction in humans. Cardiovasc Res 1999; 43: 572—579.

Halpern MH. The dual blood supply of the rat heart. Amer J Anat 1957; 101: 1—16.

Chan NN, Vallance P, Colhoun HM. Nitric oxide and vascular responses in type I diabetes. Diabetologia 2000; 43: 137—147.

Jew JY, Fink CA, Williams TH. Tyrosine hydroxylase-and nitric oxide synthase-immunoreactive nerve fibers in mitral valve of young adult and aged Fischer 344 rats. J Autonom Nerv Syst 1996; 58: 35—43.

Klimaschewski L, Kummer W, Mayer B, Couraud JY, Preissler U, Philippin B, Heim Ch. Nitric oxide synthase in cardiac nerve fibers and neurons of rat and guinea pig heart. Circulat Res 1992; 71: 1533—1537.

Kluchová D, Klimčík R, Kľoc P. Neuronal nitric oxide synthase in the rabbit spinal cord visualised by histochemical NADPH-diaphorase and immunohistochemical NOS methods. Gen Physiol Biophys 2002; 21: 163—174.

Mechírová E, Zachariáš L, Domoráková I, Gdovinová Z. Repetitive ischemia-induced injury in microcirculation of the rabbit spinal cord. Sbor Lék 2001; 102: 161—165.

Muller B, Kleschyov AL, Gyorgy K, Stoclet JC. Inducible NO synthase activity blood vessels and heart: New insight into cell origin and consequences. Physiol Res 2000; 49: 19—29.

Nathan C, Xie Q. Nitric oxide synthases: Roles, toolls, and controls. Cell 1994; 78: 915—918.

Pieper GM. Review of alterations in endothelial nitric oxide production in diabetes. Protective role of arginine on endothelial dysfunction. Hypertension 1998; 31: 1047—1060.

Pop-Busui R, Raffael D, Marinescu V, Stevens MJ. Oxidative stress is associated with abnormal myocardial blood flow regulation in subjects with type 1 diabetes and microangiopathy. Diabetologia 2001; 44: A292.

Poston L, Taylor PD. Endothelium-mediated vascular function in insulin-dependent diabetes mellitus. Clin Sci 1995; 88: 245—255.

Rodrigues B, McNeill JH. The diabetic heart: metabolic causes for the development of a cardiomyopathy. Cardiovasc Res 1992; 26: 912—922.

Rösen P, Kiesel U, Reinauer H, Boy C, Addicks K. Cardiopathy in the spontaneously diabetic (BB) rat: Evidence for microangiopathy and autonomic neuropathy in the diabetic heart. 145—157. In: Nagano M, Dhalla NS (Eds). The Diabetic Heart. New York, Raven Press, Ltd., 1991.

Sawada K, Kondo T, Chang J, Inokuchi T, Aoyagi S. Distribution and neuropeptide content of nitric oxide synthase-containing nerve fibers in arteries and conduction system of the rat heart. Acta Anat 1997; 160: 239—247.

Shah AM. Paracrine modulation of heart cell function by endothelial cells. Cardiovasc Res 1996; 31: 847—867.

Scherer-Singler U, Vincent SR, Kimura H, McGeer EG. Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. J Neurosci Methods 1983; 9: 229—234.

Schmidtová K, Bánovská E, Kočišová M, Gomboš A. Effect of irradiation on distribution of the acetylcholinesterase (AChE)-positive nerve fibres in the spleen of the rats. Func Develop Morphol 1994; 4: 261—262.

Štvrtinová V, Ferenčík M, Hulín I, Jahnová E. Vascular endothelium as a connecting operator in the information transfer between the cardiovascular and immune system. Bratisl Lek Listy 1998; 99: 5—19.

Takeda M, Mori F, Yoshida A, Takamiya A, Nakagomi S, Sato E, Kiyama H. Constitutive nitric oxide synthase is associated with retinal vascular permeability in early diabetic rats. Diabetologia 2001; 44: 1043—1050.

Ursell P, Mayes M. Anatomic distribution of nitric oxide synthase in the heart. Cardiovasc Res 1995; 50: 217—223.

Williams PL, Bannister LH, Berry MM, Collins P, Dyson M, Dussek JE, Ferguson MVJ. 2092. In: Williams PL (Ed). Gray's Anatomy. London, Churchill Livingstone 1995.

Zanzinger J. Role of nitric oxide in the neural control of cardiovascular function. Cardiovasc Res 1999; 43: 639—649.

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