

Cardiac Troponin T Following Repeated Administration of Pyridoxal Isonicotinoyl Hydrazone in Rabbits

M. ADAMCOVÁ, J. MACHÁČKOVÁ, V. GERŠL, V. PELOUCH¹, T. ŠIMŮNEK²,
I. KLIMTOVÁ², R. HRDINA², P. POŇKA³

Charles University in Prague, Faculty of Medicine in Hradec Králové, ²Faculty of Pharmacy in Hradec Králové, ¹Second Faculty of Medicine in Prague, Czech Republic, ³McGill University, Montreal, Canada

Received April 6, 2001

Accepted April 5, 2002

Summary

Pyridoxal isonicotinoyl hydrazone (PIH) is a new tridentate Fe-chelating agent that should be very promising in many pathological states resulting from both an iron-overload and formation of free radicals. The aim of our study was to investigate the effect of PIH on the cardiovascular system focusing to the regulatory protein – cardiac troponin T (cTnT). The study was carried out in two groups of Chinchilla male rabbits: 1) PIH (50 mg/kg dissolved in 10 % Cremophor i.p., once a week, 10 administrations, n=8) and 2) Cremophor (2 ml/kg i.p. in the same schedule, n=7). Plasma concentrations of cTnT (as a marker of myocardial damage) were measured using a commercial kit (Roche). cTnT was within the physiological range (i.e. < 0.1 µg/l) during the whole experiment in the Cremophor group. In the PIH group, the cTnT levels were not significantly increased when compared with the control group or with the initial values (except with those before the 5th administration). Furthermore, we analyzed the cytosolic and myofibrillar fraction of cTnT in the left ventricular myocardium. Using SDS-PAGE and Western blot we resolved three isoforms. The profiling of TnT did not differ significantly between the PIH-treated group and the Cremophor-treated group. Our data concerning cTnT support the opinion that the possible cardiotoxicity of PIH is very low.

Key words

Iron chelators • Cardiotoxicity • Cardiac troponin T • Protein profile • Rabbits

Introduction

Pyridoxal isonicotinoyl hydrazone (PIH) is a new tridentate Fe-chelating agent, formed by condensation of pyridoxal hydrochloride and isonicotinic acid hydrazide (Poňka *et al.* 1979). Iron chelators have been primarily used for the management of iron overload, but they could be very useful for the treatment of a wide variety of diseases including cancer or malaria, and the

chelation of iron may also attenuate a number of states involved in free radical-mediated damage. Moreover, the preliminary studies of cardiotoxicity of PIH are very promising. The LD₅₀ in both mice and rats were 5 g/kg when given orally and 1 g/kg intraperitoneally, respectively (Sookvanichsilp *et al.* 1991). The aim of our study was to evaluate the cardiotoxicity of repeated i.p. administration of PIH in rabbits *in vivo*. No significant changes were usually found in the biochemical and

hematological parameters followed. Furthermore, no significant changes of functional parameters of the heart (including non-invasive polygraphic recordings of systolic time intervals and invasive hemodynamic measurements of dp/dt_{max}) were described. These data have been published in our previous papers (Klimtová *et al.* 2001, Šimůnek *et al.* 2001). An integral part of the

investigation of cardiotoxicity was the evaluation of cardiac troponin T (cTnT) as a new marker of myocardial damage. Our paper is focused on the study of the effects of a new chelator PIH on plasma cTnT levels and detection of cTnT in myocardial tissues as well as on the significance of this new methodological approach in pre-clinical studies of new drugs.

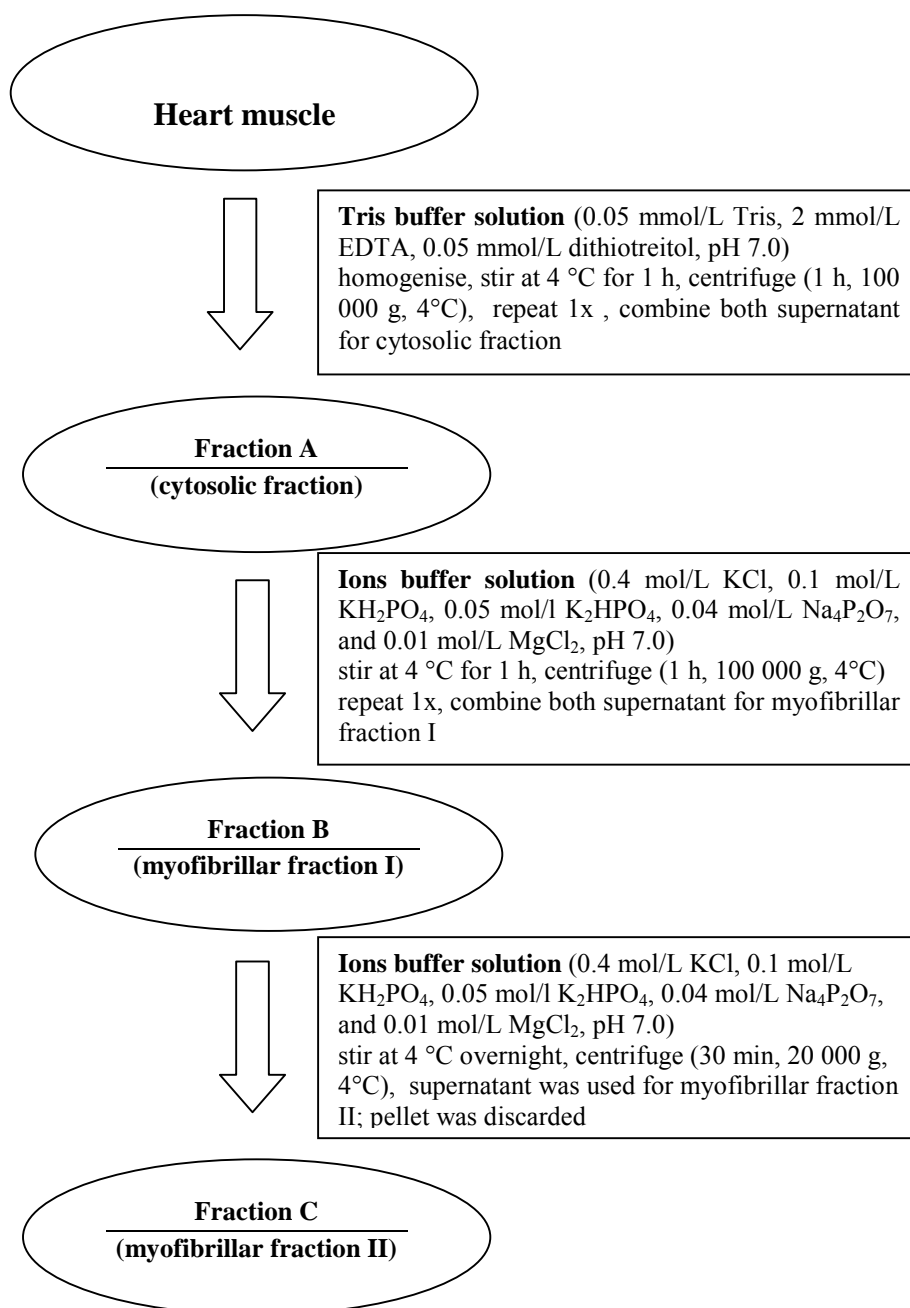


Fig. 1. Scheme of isolation of both cytosolic and myofibrillar fraction of troponin T from heart muscle.

Methods

The study was carried out on two groups of Chinchilla male rabbits (average weight 3 kg at the

beginning of the experiment): 1) PIH (pyridoxal isonicotinoyl hydrazone 50 mg/kg dissolved in 10 % Cremophor i.p., once a week, 10 administrations, n=8) and 2) control group (Cremophor EL = a derivative of

castor oil and ethylene oxide, Sigma-Aldrich, Czech Republic – 2 ml/kg i.p. in the same schedule, n=7).

Determination of plasma concentrations of cTnT

The plasma concentration of cardiac troponin T was measured using Elecsys Troponin T STAT Immunoassay (Roche) on the Elecsys 2010 immunoassay analyzer (Roche) with the detection limit < 0.010 µg/l.

Determination of cTnT in both cytosolic and myofibrillar fractions in the myocardium

Both the cytosolic and myofibrillar fractions of cardiac troponin T (cTnT) were isolated by stepwise extraction (Bleier *et al.* 1998) from the left ventricular musculature (Fig. 1). The concentration of proteins was measured using Coomassie Plus Protein Reagent Kit (Pierce).

After isolation, one-dimensional SDS polyacrylamide gel electrophoresis was carried out according to a modified version of the method of Laemmli (1970), using a 12 % separating gel and a 4 %

stacking gel. In some cases after SDS-PAGE, proteins were electroblotted onto a nitrocellulose membrane for immunoblotting and analyzed using the JLT-12 monoclonal antibodies (Sigma Chemicals). Immune complexes were detected on blots with horseradish peroxidase-linked goat anti-mouse immunoglobulin (Bio Rad Laboratories). Visualization was performed with 4-chloro-1-naphthol (Bio Rad Laboratories). The Western blots were scanned and evaluated using the software Elfoman 2.0.

Results

Determination of plasma concentration of cTnT

Plasma concentrations of cTnT were within the physiological range (i.e. < 0.1 µg/l) during the whole experiment in the control and Cremophor groups. In the PIH group, the cTnT levels were not significantly increased when compared with the control group or with the initial values, except for those before the 5th administration (Fig. 2).

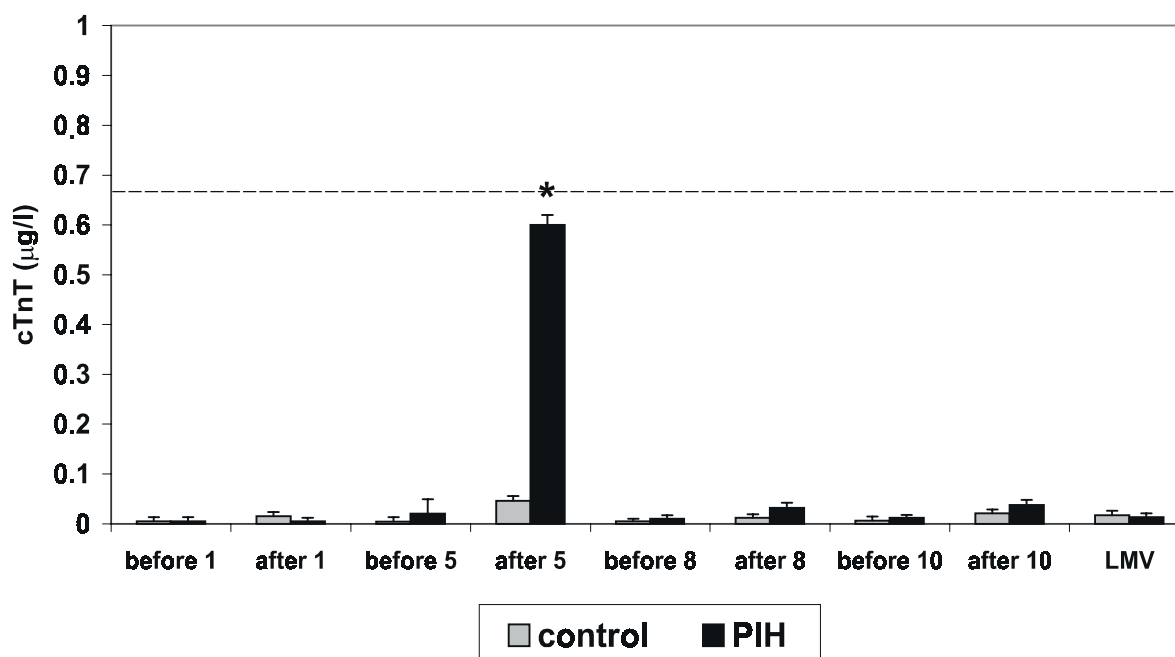


Fig. 2. Plasma concentration of cardiac troponin T following repeated administration of PIH. The venipunctures for biochemical examination were performed in the following time intervals of the experiment: (1) the initial control value before the 1st (weekly) administration of the drug, (2) 24 h after the 1st administration, (3) before the 5th administration, (4) 24 h after the 5th administration, (5) before the 8th administration, (6) 24 h after the 8th administration, (7) before the 10th administration, (8) 24 h after the 10th administration, (9) LMV interval (the last measured value) i.e. 5-7 days after the last administration of the drug. PIH – pyridoxal isonicotinoyl hydrazone dissolved in 10 % Cremophor; control group – Cremophor.

Determination of cTnT in both cytosolic and myofibrillar fractions in the myocardium

The myofibrillar fractions I and II were extracted in the same buffer but they differed in the duration of incubation. The myofibrillar fraction I obtained by the short-term extraction primarily extracted the proteins of the A-zone of myofibrils. Overnight incubation (i.e. myofibrillar fraction II) led to the extraction of proteins from both I- and A-zones.

No significant changes in myofibrillar and cytosolic fractions of cTnT were found in the PIH treated group in comparison with the control group. The representative Western blot and densitometric scan illustrate these findings (Fig. 3). The proportion of troponin T isoforms does not differ significantly between the PIH treated group and the control group (Fig. 4).

Discussion

Troponin T (TnT) is one of the three components of troponin – the regulatory protein located on the actin filament. Recently, cardiac troponin has been considered to be one of the most sensitive and specific markers of myocardial damage in laboratory animals (O'Brien *et al.* 1997). In addition, our previous data support the recent opinion that troponins are very useful biochemical markers that should be included in the evaluation of cardiotoxicity of new drugs (Adamcová *et al.* 1998, 1999, Wu 1999, Macháčková *et al.* 2001). The repeated administration of PIH did not lead to significant changes of cTnT levels compared with the Cremophor group, which confirms the very low cardiotoxicity of PIH.

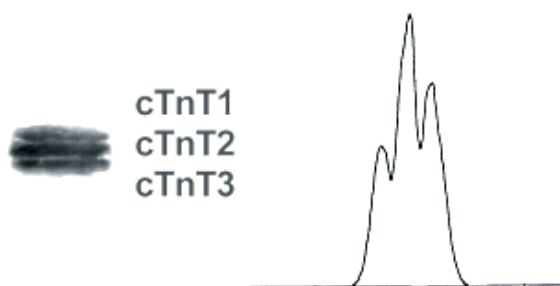


Fig. 3. Western blot of cardiac troponin T after repeated administration of PIH with corresponding densitometric scan. PIH – Pyridoxal isonicotinoyl hydrazone dissolved in 10 % Cremophor; control group – Cremophor

Furthermore, we analyzed the intracellular compartmentation of cTnT in the left ventricular myocardium. There are two pools of troponin T: the greater pool is myofibril-bound in the thin filaments, the lesser pool is found as a soluble, cytosolic pool, which probably serves as a precursor pool for the synthesis of the troponin complex. The cytosolic fraction of cardiac TnT (cTnT) is estimated at 6 % of total TnT (Katus *et al.* 1991). Several authors suppose that the proportion of the cytosolic and myofibrillar fraction can be affected by various pathological states but, on the other hand, also can result in different release kinetics of cTnT during myocardial damage (Voss *et al.* 1995, Ricchiuti *et al.* 1997, Bleier *et al.* 1998) and more extensive studies are needed. The mammalian ventricular myocardium has recently been found to contain more than one isoform of TnT (Anderson *et al.* 1991, Adamcová and Pelouch 1999). The various isoforms are the products of alternative splicing of a primary RNA transcript. Expression of the multiple isoforms has been reported to be developmentally regulated and has also changed under various physiological and pathological conditions. Although the exact relations between cardiac function and TnT isoforms are not yet clear, data are available showing their functional significance, i.e. cTnT isoforms expression correlated with ATPase activity of myofibrils, the sensitivity of myofilaments to calcium and also binding capacity of myofilaments for calcium ions (Anderson *et al.* 1993, Saba *et al.* 1996). The functional

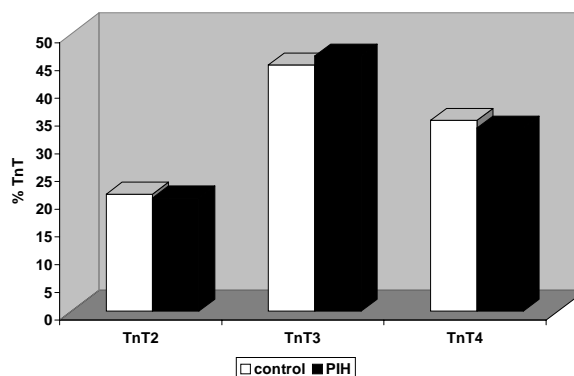


Fig. 4. The proportion of troponin T isoforms after repeated administration of PIH. The isoforms TnT2, TnT3 and TnT4 were found in rabbit left ventricular myocardium; PIH - Pyridoxal isonicotinoyl hydrazone dissolved in 10 % Cremophor; control group – Cremophor

consequences of TnT isoform variation are of particular interest. Recent reports described the re-expression of a fetal cTnT isoform in the myocardium from humans with heart failure (Anderson *et al.* 1991, 1995) and in a guinea pig model of severe pressure overload hypertrophy/failure (Gulati *et al.* 1994).

Anderson *et al.* (1988) showed that TnT from the rabbit ventricular myocardium was separated by SDS-PAGE into five isoforms. These isoforms migrated faster than actin and slower than tropomyosin and TnI, with TnT₁ having the slowest mobility and TnT₅ the fastest. Although M_r of rabbit cardiac TnT was estimated by gel electrophoresis between 38 500 and 39 500, the M_r of rabbit cardiac TnT obtained from the amino acid sequence was 32 881 daltons (Pearlstone *et al.* 1986). Monoclonal antibody, MAb JLT-12, which recognizes a highly conserved epitope in cardiac muscle TnT, recognized its determinant with all five isoforms of the purified cardiac troponin T (Anderson and Oakeley 1989). The expression of cardiac troponin changed significantly with maturation and the isoforms TnT₂, TnT₃ and TnT₄ became dominant in rabbits in 99-day-old rabbits (Anderson *et al.* 1988). In agreement with this

study we were able to distinguish three dominant isoforms of cardiac troponin T in the left ventricular myocardium of rabbits that was designated according to Anderson *et al.* (1988) as TnT₂, TnT₃ and TnT₄. The proportion of troponin T isoforms was not affected by PIH administration.

To sum up, our data about cTnT support the opinion that the possible cardiotoxicity of PIH is very low which is important from the viewpoint of possible clinical use of this agent. The above described method of determination of cTnT in both cytosolic and myofibrillar fractions can be used for the study of intracellular compartmentation and the isoform expression of regulatory protein troponin T. That is why it could be very useful because the data about cTnT alterations in various pathological states are not still sufficient.

Acknowledgements

We thank Mrs. L. Koželuhová and Mrs. J. Hofmanová for their skilful technical assistance. The study was supported by the grant of the Grant Agency of the Czech Republic No. 305/00/0365 and Research Project CEZ: J13/98: 111500001.

References

- ADAMCOVÁ M, PELOUCH V: Isoforms of troponin in normal and diseased myocardium. *Physiol Res* **48**: 235-247, 1999.
- ADAMCOVÁ M, GERŠL V, HRDINA R, PALIČKA V, MAZUROVÁ Y: Contribution to prediction and pathogenesis of daunorubicin-induced cardiomyopathy in rabbits. *Exp Clin Cardiol* **3**: 179-183, 1998.
- ADAMCOVÁ M, GERŠL V, HRDINA R, MĚLKA M, MAZUROVÁ Y, VÁVROVÁ J, PALIČKA V, KOKŠTEIN Z: Cardiac troponin T as a marker of myocardial damage caused by antineoplastic drugs in rabbits. *J Cancer Res Clin Oncol* **125**: 268-274, 1999.
- ANDERSON PAW, OAKELEY AE: Immunological identification of five troponin I isoforms reveals an elaborate maturational troponin T profile in rabbit myocardium. *Circ Res* **65**: 1087-1093, 1989.
- ANDERSON PAW, MOORE GE, NASSAR RN: Development changes in the expression of rabbit left ventricular troponin T. *Circ Res* **63**: 742-747, 1988.
- ANDERSON PAW, MALOUF NN, OAKELEY AE, PAGANI ED, ALLEN PD: Troponin T isoforms expression in humans: a comparison among normal and failing adult heart, fetal heart, and adult and fetal skeletal muscle. *Circ Res* **69**: 1226-1233, 1991.
- ANDERSON PAW, HENDERSON PM, NASSAR R: The calcium transient in cardiac myocytes increases with development. *Circulation* **88** (Suppl I): I-436, 1993.
- ANDERSON PAW, GREIG A, MARK TM, MALOUF NN, OAKELEY AE, UNGERLEIDER RM, ALLEN PD, KAY BK: Molecular basis of human cardiac troponin T isoforms expressed in the developing, adult and failing heart. *Circ Res* **76**: 681-686, 1995.
- BLEIER J, VORDERWINKLER KP, FALKENSAMMER J, MAIR P, DAPUNT O, PUSCHENDORF B, MAIR J: Different intracellular compartmentations of cardiac troponins and myosin heavy chains: a causal connection to their different early release after myocardial damage. *Clin Chem* **44**: 1912-1918, 1998.
- GULATI J, AKELLA AB, NIKOLIC SD, STARC V, SIRI F: Shifts in contractile regulatory protein subunits troponin T and troponin I in cardiac hypertrophy. *Biochem Biophys Res Commun* **202**: 384-390, 1994.

- KATUS HA, REMPIS A, SCHEFFOLD T, DIEDERICH KH, KUBLER W: Intracellular compartmentation of cardiac troponin T and its release kinetics in patients with reperfused and nonreperfused myocardial infarction. *Am J Cardiol* **67**: 1360-1367, 1991.
- KLIMTOVÁ I, ŠIMŮNEK T, GERŠL V, HRDINA R, MACHÁČKOVÁ J, MAZUROVÁ Y, POŇKA P, ADAMCOVÁ M, PALIČKA V: Biochemical and haematological changes following repeated pyridoxal isonicotinoyl hydrazone administration in rabbits. *Biomarkers Environ* **4** (Suppl 1): 70-75, 2001.
- LAEMMLI UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685, 1970.
- MACHÁČKOVÁ J, ADAMCOVÁ M, GERŠL V, HRDINA R, MAZUROVÁ Y, NOBILIS M: Evaluation of cardiac effects of the new antineoplastic drug – dimethoxybenfluron – on a rabbit model. *Physiol Res* **50**: 491-499, 2001.
- O'BRIEN PJ, DAMERON GW, BECK ML, KANG YJ, ERICKSON BK, DI BATTISTA TH, MILLER KE, JACKSON KN, MITTELSTADT S: Cardiac troponin T is a sensitive, specific biomarker of cardiac injury in laboratory animals. *Lab Anim Science* **47**: 486-495, 1997.
- PEARLSTONE J, CARPENTER M, SMILLIE L: Amino acid sequences of rabbit cardiac troponin T. *J Biol Chem* **261**: 16795-16810, 1986.
- POŇKA P, BORO VÁ J, NEUWIRT J, FUCHS, O: Mobilization of iron from reticulocytes. Identification of pyridoxal isonicotinoyl hydrazone as a new iron chelating agent. *FEBS Lett* **97**: 317-321, 1979.
- RICCHIUTI V, ZHANG J, APPLE FS: Cardiac troponin I and T alterations in hearts with severe left ventricular remodeling. *Clin Chem* **43**: 990-995, 1997.
- SOOKVANICHSILP N, NAKORNCHAI S, WEERAPRADIST W: Toxicological study of pyridoxal isonicotinoyl hydrazone: acute and subchronic toxicity. *Drug Chem Toxicol* **14**: 395-403, 1991.
- SABA Z, NASSAR R, UNGERLEIDER RM, OAKELEY AE, ANDERSON PAW: Cardiac troponin T isoform expression correlates with pathological descriptors in patients who underwent corrective surgery for congenital heart disease. *Circulation* **94**: 472-476, 1996.
- ŠIMŮNEK T, KLIMTOVÁ I, HRDINA R, GERŠL V, MAZUROVÁ Y, MACHÁČKOVÁ J, POŇKA P, HOLEČKOVÁ M: The effect of repeated administration of pyridoxal isonicotinoyl hydrazone on cardiovascular system in rabbits. *Biomarkers Environ* **4** (Suppl 1): 93-97, 2001.
- VOSS EM, SHARKEY SW, GERNERT AE, MURAKAMI MM, JOHNSTON RB, HSIEH CC, APPLE FS: Human and canine cardiac troponin T and creatine kinase-MB distribution in normal and diseased myocardium. *Arch Pathol Lab Med* **119**: 799-806, 1995.
- WU AHB: Role of serum biochemical markers in clinical trials. In: *Myocardial Damage*. JC KASI, DW HOLT (eds), *Developments in Cardiovascular Medicine* (vol. 205). Kluwer Academic Publishers 1998, pp 189-198.

Reprint requests

MUDr. M. Adamcová, CSc., Department of Physiology, Faculty of Medicine of Charles University, Šimkova 870, CZ-50001 Hradec Králové. E-mail: adamcova@lfhk.cuni.cz