Effect of Pentoxifylline on Endothelaemia and Hypothalamic-Pituitary-Adrenocortical Axis Activation in Female Rats Under Stress Exposure

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Abstract. Endothelial dysfunction may belong to negative consequences of stress exposure accompanied by activation of several stress systems including the hypothalamic-pituitary-adrenocortical (HPA) axis. The present experiments were aimed at testing the hypotheses that i) immobilization (IMO) stress results in sustained increase in endothelaemia for 24 h and that ii) pentoxifylline, a drug with endothelium protective properties, attenuates the rise in endothelaemia and HPA axis activation in female rats as shown previously in males. Circulating endothelial cells increased immediately after the IMO for 2 h, returned back to control levels at 12 h and increased again at 24 h. Stress-induced rise in adrenocorticotropic hormone (ACTH) and corticosterone levels was particularly high immediately after the IMO. Pretreatment with pentoxifylline (20 mg/kg subcutaneously for 7 days) attenuated the rise in endothelaemia and adrenal corticosterone measured at 24 h following IMO. Plasma levels of ACTH and proopiomelanocortin gene expression in the anterior pituitary were not affected by pentoxifylline treatment. The present results indicate that IMO stress in female rats induces a biphasic rise in endothelaemia early at the time of stress exposure and than 24 h thereafter. Based on these data and our previous study we can conclude that intensive stress has a negative influence on endothelial cells in both sexes and no gender differences seem to be present in the protective action of pentoxifylline.

Key words: Endothelium — Stress hormones — Pentoxifylline — Gender differences

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Introduction

Chronic or intensive stress exposure is inducing endocrine, metabolic, immune and behavioural changes contributing to the development of different pathological states including cardiovascular diseases (de Kloet 2003; Carney et al. 2005; Jezova 2005). A crucial role in the homeostasis of the cardiovascular system plays the endothelium. Several cardiovascular disorders (e.g. hypertension, coronary artery disorders, stroke) are associated with a dysfunction or damage of endothelial lining. Endothelial dysfunction may belong to negative consequences of stress exposure as suggested by results of limited number of studies using indirect techniques in humans (Arbogast et al. 1994; Sherwood et al. 1999; Ghiadoni et al. 2000) and primates (Strawn et al. 1991). Recently, we have provided an evidence of stress-induced endothelial cell damage by demonstrating increased endothelaemia and von Willebrand factor concentrations in male rats subjected to immobilization (IMO) stress (Jezova et al. 2003).

New approaches in the pharmacotherapy of cardiovascular disorders involve the utilization of several groups of drugs with protective effect on vascular endothelium, e.g. angiotensin-converting enzyme inhibitors, statins and antioxidants (Spieker et al. 2001). Pentoxifylline, a methylxanthine derivative, is classified as a hemorrheological agent with potential endothelium-protective properties. Beneficial effects of pentoxifylline have been demonstrated in various models of vascular injury, e.g. sepsis, hemorrhagic and endotoxic shock (Doherty et al. 1991; Graninger and Wenisch 1995; Sikora 2000). We have shown a positive influence of pentoxifylline treatment on signs of endothelial injury induced by immobilization stress in male rats (Jezova et al. 2003). Moreover, pre-treatment with pentoxifylline reduced the activation of hypothalamic-pituitary-adrenocortical (HPA) axis, which represents one of the main characteristics of neuroendocrine response during stress.

In the study mentioned above (Jezova et al. 2003), circulating endothelial cells were measured in male rats at two time intervals, namely 20 min and 24 h after a 2 h lasting IMO. The present experiments were aimed at testing the hypotheses that i) this intensive stressor results in sustained increase in endothelaemia by performing repeated measurements up to 24 h, ii) there are no gender differences in the negative influence of intensive stress on endothelial cells by using female rats throughout the studies and iii) pentoxifylline attenuates the rise in endothelaemia and HPA axis activation also in female rats by evaluating the effects of one week pentoxifylline treatment. The extent of endothelium lining alterations in response to stress stimuli and effect of pretreatment with pentoxifylline was assessed by endothelial cell counts in peripheral blood. Plasma levels of corticosterone, adrenocorticotropic hormone (ACTH) and gene expression of its precursor proopiomelanocortin (POMC) in the anterior pituitary served as indicators of HPA axis activity.

Materials and Methods

Animals

Adult female Sprague Dawley rats weighing 300–350 g purchased from Charles River Wiga (Silzfelg, Germany) were used. The animals were housed four *per* cage in a room with controlled temperature 22 ± 2 ℃ and light/dark cycle (6:00–18:00 h light). Food and water were available ad libitum. Principles of laboratory animal care and all procedures were approved by the Animal Care Committee of the Slovak Academy of Sciences.

IMO stress and drug treatments

Rats ($n = 6$–7 *per* group) were immobilized for 2 h (from 8–10 h in the morning). They were decapitated immediately, 6, 12, or 24 h after IMO. The IMO was performed in a prone position as described previously (Jezova et al. 1998). Before the treatments, animals were repeatedly handled to avoid non-specific stress. Control rats were decapitated before 10:00 h a.m.

Other groups of rats were treated subcutaneously with isotonic saline (0.9 % NaCl) or pentoxifylline (Agapurin inj., Slovakofarma, Slovakia) in the dose of 20 mg/kg once daily for 7 days. 30 min after the last injection, one half of pentoxifylline- and saline-treated animals was exposed to IMO stress for 2 h, while the unstressed controls remained in their cages. 6 h later, all animals received an additional injection of pentoxifylline or saline and were sacrificed 24 h after IMO.

Endothelial cell counting

Endothelaemia was measured by counting the endothelial cells in Burker's chamber after their isolation, cleaning from platelets and an additional treatment with adenosinediphosphate as described previously (Hladovec and Rossmann 1973; Jezova et al. 2003). Average counts from four Burker's chambers were used for final calculation. The results were expressed as number of endothelial cells in 9 μl of plasma.

ACTH and corticosterone measurements

Plasma ACTH was measured by a radioimmunoassay using a double antibody technique to separate free and bound fractions. Plasma corticosterone levels were analyzed by a radioimmunoassay after dichloromethane extraction of the steroids from 10 μl aliquots of plasma (Jezova et al. 1994).

In situ hybridization

Coronar 12 μm thick sections from the pituitary were cut in a cryocut device (Reichert), mounted onto poly-L-lysine (Sigma) coated slides, and hybridized according to the protocol described previously (Skultetyova et al. 1998). The sections were hybridized using ^{35}S-labelled oligonucleotide probe – 48-mer oligonucleotide complementary to the bases corresponding to 102–117 of rat POMC (a gift from Dr. G. Aguilera, NIH, MD, USA), synthesized by Synthecell (Rockville, MD, USA). The

autoradiographic signal was quantified using a computerized image analysis system (Scion Image for Windows 4.0.2, Scion Corp., USA). The results were expressed as arbitrary units. At least 4 sections/animal were analysed.

Statistical analysis

Statistical analysis was performed by two-way analysis of variance (ANOVA) for factors stress and treatment, followed by a *post hoc* Tukey test for pairwise comparisons. In the study without drug treatments, the effects of IMO were calculated by one-way ANOVA. Calculations were made by Jandel SigmaStat statistical software. The probability level was set to 95 % as a limit to reject the null hypothesis. Data are expressed as means ± SEM.

Results

Time course of changes in response to IMO stress

One-way ANOVA indicated significant effect of IMO on endothelaemia ($F_{4,31} = 11.409$, $p < 0.001$). *Post hoc* Tukey test revealed statistically significant rise in circulating endothelial cells immediately after the IMO ($p < 0.05$) and 24 h after the IMO ($p < 0.01$) as shown on Fig. 1.

There was a statistically significant effect of stress on ACTH levels in plasma ($F_{4,30} = 73.879$, $p < 0.001$) with a pronounced increase immediately after the IMO ($p < 0.01$). Hormone levels decreased 6 and 12 h after IMO and they were close to control levels at 24 h (237 *vs.* 156 pg/ml) (Fig. 1).

ANOVA revealed statistically significant effect of the stress stimulus used on plasma corticosterone ($F_{4,31} = 21.712$, $p < 0.001$). Stress-induced rise in plasma corticosterone levels was particularly high immediately after the IMO ($p < 0.01$) and then at 12 h after the IMO ($p < 0.01$) as compared to the levels in controls (Fig. 1).

POMC mRNA levels in the anterior pituitary were found to be significantly increased ($F_{4,24} = 3.816$, $p = 0.015$) but the rise was statistically significant ($p < 0.05$) only at 12 h following stress exposure (Fig. 1).

Pentoxifylline treatment and IMO stress

IMO stress induced a rise in counts of circulating endothelial cells, which was attenuated by pretreatment with pentoxifylline. Two-way ANOVA revealed a significant effect of both stress ($F_{1,15} = 6.372$, $p = 0.023$) and treatment ($F_{1,15} = 14.756$, $p = 0.002$) on endothelaemia but no significant interaction between the stress and treatment (Fig. 2).

A significant rise in plasma corticosterone levels ($F_{1,13} = 16.209$, $p < 0.001$) was found 24 h after the IMO. Hormone response to IMO failed to be affected by pentoxifylline treatment. Stress-induced increase in adrenal corticosterone levels was reduced by the treatment with pentoxifylline. Two-way ANOVA revealed significant effect of stress ($F_{1,12} = 23.324$, $p < 0.001$) and treatment ($F_{1,12} = 5.152$,

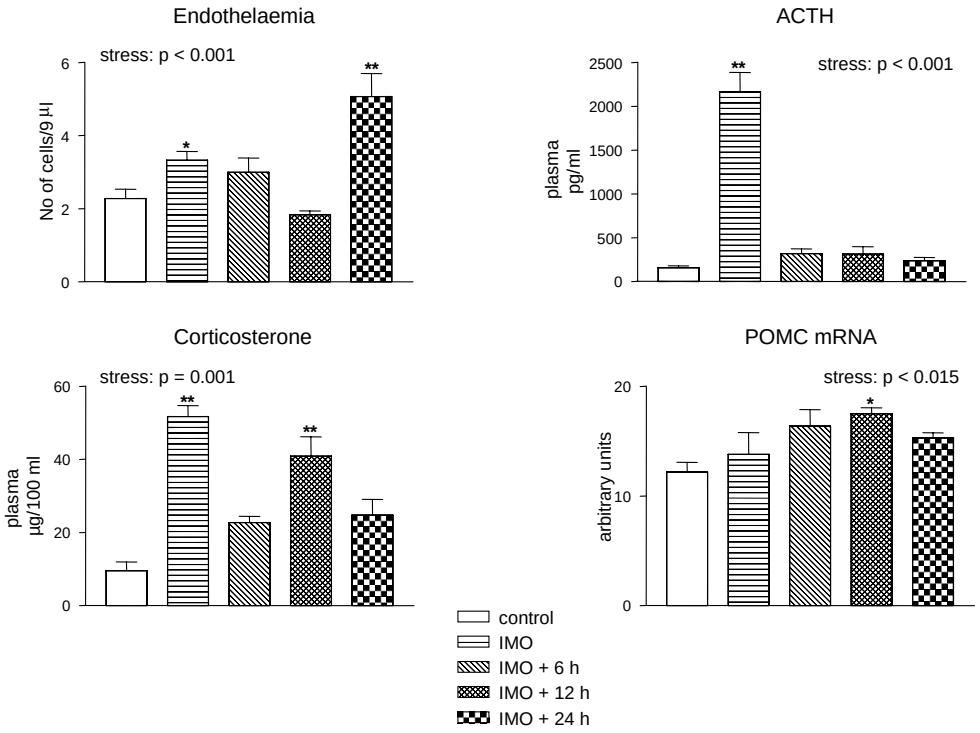

Figure 1. Endothelaemia, POMC mRNA levels in anterior pituitary, plasma ACTH and plasma corticosterone concentrations immediately after and at the later time intervals after exposing rats to 2 h of immobilization (IMO) stress (n = 6–7/group). Data were evaluated by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ *vs.* control.

$p = 0.042$) on corticosterone levels in the adrenals, but not their mutual interaction (Fig. 2).

Plasma levels of ACTH and POMC gene expression in the anterior pituitary were not affected by pentoxifylline treatment 24 h after the IMO (data not shown).

Discussion

The present results indicate that IMO stress in female rats induces a biphasic rise in circulating endothelial cell number immediately after stress exposure and 24 h thereafter. The increase in endothelaemia as well as adrenal corticosterone levels were reduced by pretreatment with pentoxifylline, demonstrating similar effects as those previously observed in male rats (Jezova et al. 2003). These data suggest that stress exposure and pentoxifylline have analogous actions in both genders.

Increased number of endothelial cells in blood after exposure of female rats to IMO stress provides further evidence for the relationship between stress and

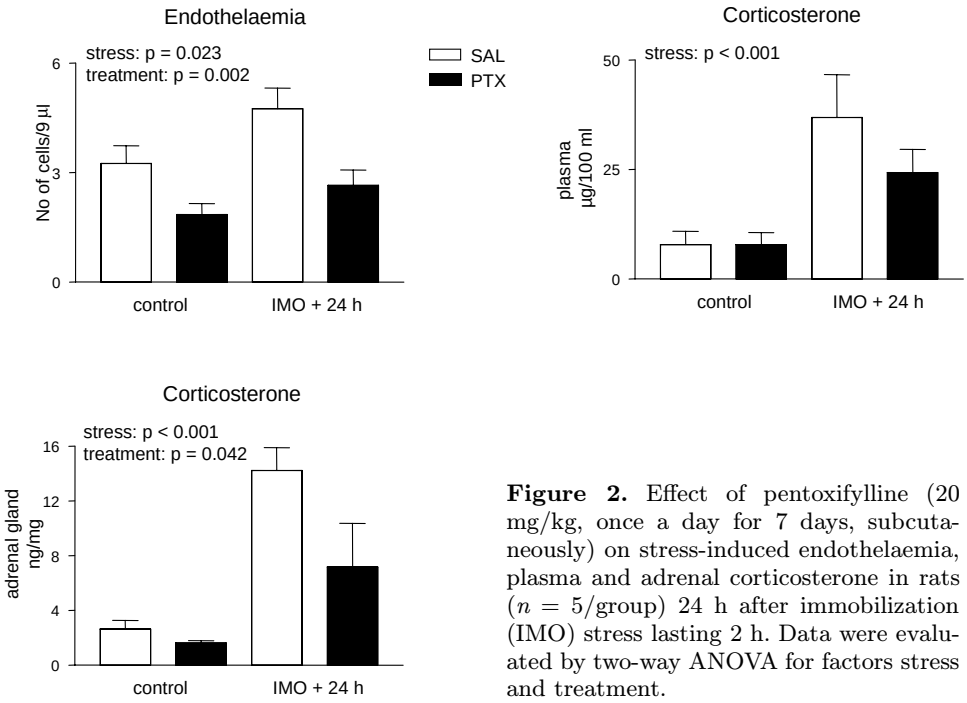

Figure 2. Effect of pentoxifylline (20 mg/kg, once a day for 7 days, subcutaneously) on stress-induced endothelaemia, plasma and adrenal corticosterone in rats (n = 5/group) 24 h after immobilization (IMO) stress lasting 2 h. Data were evaluated by two-way ANOVA for factors stress and treatment.

damage to the endothelium. These data are in agreement with our previous results in male rats (Jezova et al. 2003) using the same model of stress. In contrast to our hypothesis, stress exposure did not induce a sustained increase in endothelaemia for 24 h, but the changes were dependent on time. Endothelaemia increased immediately after the IMO and an improvement seemed to occur thereafter reaching control levels at 12 h. It is possible that the early rise in endothelaemia is related to general stress effects, such as hemodynamic changes or production of free oxygen radicals (Szöcs 2004), on a vulnerable population of endothelial cell. The delayed increase in endothelaemia observed after 24 h could be the result of the development of subsequent changes induced by stress, e.g. altered haemostasis (Hornstein 2004), pro/antiinflammatory cytokine balance (Elenkov and Chrousos 2002) and hormone release (Jezova et al. 2003 and present study).

The present study confirmed well known activation of the HPA axis during stress, namely increased release of corticosterone, ACTH and gene expression of its precursor POMC. Concentrations of plasma corticosterone measured in present experiments reflect the consequences of stress as well as variations in hormone secretion throughout 24 h. Rise in HPA axis activity observed at 12 h following the stress exposure may include also the influence of daily rhythms. Changes in glucocorticoid release may be related to stress-induced endothelial dysfunction. Sup-

portive evidence to this assumption has recently been obtained in a human study (Broadley et al. 2005) where is demonstrated that mental stress-related changes in endothelial function in healthy men could be prevented by blocking cortisol production with metyrapone. Furthermore, observed rise of POMC mRNA levels in the present study draws attention to the importance of changes at the level of gene expression.

Treatment with pentoxifylline in females exerted an inhibitory effect on both endothelaemia and adrenal corticosterone suggesting favorable effects of this drug on the parameters studied. Beneficial effects of estrogens on the control of local blood flow and peripheral resistance are related to increased production of several endothelial mediators (Huang and Kaley 2004). Mentioned sex steroids influence also the activity of the HPA axis, which is higher in females compared to males under both basal and stress conditions (Jezova et al. 1996; Jezova 2004). However, with respect to similar effects of pentoxifylline on endothelial cell counts and hormone release in male rats (Jezova et al. 2003) we suggest that there are no significant gender differences in pentoxifylline action under stress conditions.

Several clinical investigations support the relation between coronary heart disease and psychosocial factors like depression, anxiety and stress. Acute stress has been shown to trigger myocardial ischemia, to increase blood viscosity, and to stimulate platelet function. Recent data indicate that these effects are in part a result of endothelial dysfunction and injury induced by acute stress (Hornstein 2004). Reduction of stress-induced rise of endothelaemia by pentoxifylline treatment suggests a protective effect of pentoxifylline on the endothelium. Pentoxifylline is a drug inducing a variety of pharmacological effects, which may contribute to its protective influence on the vascular wall. The mechanisms to be considered involve stimulation of prostacyclin production in vascular tissues, decrease in leukocyte adhesion to the endothelium or reduction in cytokine production (Sinzinger 1983; Samlaska and Winfield 1994). Hypotensive effects of pentoxifylline are not very likely, but they cannot be excluded. Another option is the reduction of stress hormones, namely glucocorticoid release observed in our studies. However, pentoxifylline treatment induced a more pronounced effect on corticosterone levels in the adrenals than in plasma in both sexes.

In agreement with the presented data, Coe et al. (1997) reported that pentoxifylline prevented endothelial damage in a model of rabbit hindlimb ischemia and reperfusion. Moreover, Kwiecien et al. (2004) demonstrated beneficial effect of pentoxifylline against stress-induced gastric damage. The mechanisms involved in the protective action of pentoxifylline on stress-induced endothelial damage may be similar to those supposed to participate in gastroprotection, such as enhanced nitric oxide activity, attenuation of oxidative metabolism and generation of proinflammatory cytokines. These data allow to suggest that pentoxifylline treatment could have a more general effects ameliorating negative consequence of stress exposure.

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References

Arbogast B. W., Neumann J. K., Arbogast L. Y., Leeper S. C., Kostrzewa R. M. (1994): Transient loss of serum protective activity following short-term stress: a possible biochemical link between stress and atherosclerosis. J. Psychosom. Res. **38**, 871—884

Broadley A. J., Korszun A., Abdelaal E., Moskvina V., Jones C. J., Nash G. B., Ray C., Deanfield J., Frenneaux M. P. (2005): Inhibition of cortisol production with metyrapone prevents mental stress-induced endothelial dysfunction and baroreflex impairment. J. Am. Coll. Cardiol. **46**, 344—350

Carney R. M., Freedland K. E., Veith R. C. (2005): Depression, the autonomic nervous system, and coronary heart disease. Psychosom. Med. **67** (Suppl. 1), S29—33

Coe D. A., Freischlag J. A., Johnson D., Mudaliar J. H., Koscieszа S. A., Traul D. K., Chiang P. C., Cambria R. A., Seabrook G. R., Towne J. B. (1997): Pentoxifylline prevents endothelial damage due to ischemia and reperfusion injury. J. Surg. Res. **67**, 21—25

de Kloet E. R. (2003): Hormones, brain and stress. Endocr. Regul. **37**, 51—68

Doherty G. M., Jensen J. Ch., Alexander H. R., Buresh C. M., Norton J. A. (1991): Pentoxifylline suppression of tumor necrosis factor gene transcription. Surgery **110**, 192—198

Elenkov I. J., Chrousos G. P. (2002): Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. Ann. N. Y. Acad. Sci. **966**, 290—303

Graninger W., Wenisch C. (1995): Pentoxifylline in severe inflammatory response syndrome. J. Cardiovasc. Pharmacol. **25** (Suppl. 2), S134—138

Ghiadoni L., Donald A. E., Cropley M., Mullen M. J., Oakley G., Taylor M., O'Connor G., Betteridge J., Klein N., Steptoe A., Deanfield J. E. (2000): Mental stress induces transient endothelial dysfunction in humans. Circulation **102**, 2473—2478

Hladovec J., Rossmann P. (1973): Circulating endothelial cells isolated together with platelets and the experimental modification of their counts in rats. Thromb. Res. **3**, 665—668

Hornstein C. (2004): Stress, anxiety and cardiovascular disease: an interdisciplinary approach. Vertex **15** (Suppl. 1), S21—31 (in Spanish)

Huang A., Kaley G. (2004): Gender-specific regulation of cardiovascular function: estrogen as key player. Microcirculation **11**, 9—38

Jezova D. (2004): Gender differences in the stress response and in the action of antidepressant drugs. Psychiatrie pro praxi **5**, 136—138 (in Czech)

Jezova D. (2005): Control of ACTH secretion by excitatory amino acids: functional significance and clinical implications. Endocrine **28**, 287—294

Jezova D., Guillaume V., Jurankova E., Carayon P., Oliver C. (1994): Studies on the physiological role of ANF in ACTH regulation. Endocr. Regul. **28**, 163—169

Jezova D., Jurankova E., Mosnarova A., Kriska M., Skultetyova I. (1996): Neuroendocrine response during stress with relation to gender differences. Acta Neurobiol. Exp. (Wars.) **56**, 779—785

Jezova D., Ochedalski T., Kiss A., Aguilera G. (1998): Brain angiotensin II modulates sympathoadrenal and hypothalamic pituitary adrenocortical activation during stress. J. Neuroendocrinol. **10**, 67—72

Jezova D., Kristova V., Slamova J., Mlynarik M., Pirnik Z., Kiss A., Kriska M. (2003): Stress-induced rise in endothelaemia, von Willebrand factor and hypothalamic-pituitary-adrenocortical axis activation is reduced by pretreatment with pentoxifylline. J. Physiol. Pharmacol. **54**, 329—338

Kwiecien S., Brzozowski T., Konturek P. C., Pawlik M. W., Pawlik W. W., Kwiecien N., Konturek S. J. (2004): Gastroprotection by pentoxifylline against stress-induced gastric damage. Role of lipid peroxidation, antioxidizing enzymes and proinflammatory cytokines. J. Physiol. Pharmacol. **55**, 337—355

Samlaska C. P., Winfield E. A. (1994): Pentoxifylline. J. Am. Acad. Dermatol. **30**, 603—621

Sherwood A., Johnson K., Blumenthal J. A., Hinderliter A. L. (1999): Endothelial function and hemodynamic responses during mental stress. Psychosom. Med. **61**, 365—370

Sikora J. P. (2000): The role of cytokines and reactive oxygen species in the pathogenesis of sepsis. Pol. Merkuriusz Lek. **7**, 47—50 (in Polish)

Sinzinger H. (1983): Pentoxifylline enhances formation of prostacyclin from rat vascular and renal tissue. Prostaglandins Leukot. Med. **12**, 217—226

Skultetyova I., Kiss A., Jezova D. (1998): Neurotoxic lesions induced by monosodium glutamate result in increased adenopituitary proopiomelanocortin gene expression and decreased corticosteroneclearance in rats. Neuroendocrinology **67**, 412—420

Szöcs K. (2004): Endothelial dysfunction and reactive oxygen species production in ischemia/reperfusion and nitrate tolerance. Gen. Physiol. Biophys. **23**, 265—295

Spieker L. E., Luscher T. F., Noll G. (2001): Current strategies and perspectives for correcting endothelial dysfunction in atherosclerosis. J. Cardiovasc. Pharmacol. **38** (Suppl. 2), S35—41

Strawn W. B., Bondjers G., Kaplan J. R., Manuck S. B., Schwenke D. C., Hansson G. K., Shively C. A., Clarkson T. B. (1991): Endothelial dysfunction in response to psychosocial stress in monkeys. Circ. Res. **68**, 1270—1279

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