ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Effect of Chronic Immobilization Stress on some selected Physiological, Biochemical and Lipid Parameters in Wistar Albino Rats

Nayanatara AK[1]*, Tripathi Y[2], Nagaraja HS[3], Jeganathan PS[1] Ramaswamy C[4], Ganaraja B[1] Sheila R Pai[1], Asha kamath[5]

[1] Dept. Physiology, Center for Basic Sciences, Kasturba Medical College, Manipal University, Karnataka.
[2] Dept. Physiology, Santosh Medical College, Ghaziabad, Uttarpradesh.
[3] Dept. Human biology, International Medical University, Malaysia.
[4] Dept. Physiology, Saveetha Medical College, Thandalam, Chennai.
[5] Department of Community Medicine, KMC, Manipal University.

ABSTRACT

The stress response is a natural reaction by the body, against potentially harmful stimuli to enhance the chance for survival. Persistent activation of the chronic stress response can cause changes to homeostatic mechanisms. Immobilization/restraint stress is an easy and convenient method to induce both psychological and physical stress. Wistar strain adult albino rats were divided into two groups as non stressed group (n = 10) and stressed group (n = 10). The stressed groups were exposed to 60 days of chronic immobilization stress. At the end of the sixty day the animals were anaesthetized and blood samples were collected through cardiac puncture. The blood samples of both the groups were analyzed for selected biochemical and lipid parameters. The results were analyzed statistically by using student't test. $P < 0.05$ was considered as significant. Our present results showed a significant increase in the various organ weights and a significant decrease in the food intake and body weight. All the biochemical parameters [(serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), blood sugar, tissue malondialdehyde (MDA) and serum lipid profile (cholesterol, serum triglyceride (TG), low-density lipoproteins (LDL)] were significantly increased in the stressed group when compared to the non stressed group. The serum high-density lipoproteins (HDL) level did not show any statistically significant changes. The present data indicate that chronic immobilization stress causes the significant alterations in the physiological, biochemical and lipid parameters. Further the study also confirms the shift of oxidants and antioxidant balance during chronic stress.

Keywords: Chronic Immobilization stress, Lipid profile, lipid peroxidation, free radicals, food intake, organ weight.

Corresponding author
Email:nayanaarun@hotmail.com

ISSN: 0975-8585

INTRODUCTION

Stress is a condition of highly individualized response of an organism to external and internal challenges which one can control with difficulties or cannot control. It is one of the important factor acting upon a large human population in the entire country. It induces the strain upon both emotional and physical endurance which has been considered the basic factor in the etiology of a number of diseases eg: cardiovascular diseases, cancer, diabetes mellitus etc [1, 2]. In response to stressors, a series of behavioral, neurochemical, and immunological changes occur that ought to serve in an adaptive capacity [2, 3]. However, if those systems become overly taxed, the organism may become vulnerable to pathology.

Chronic psychological stress is one of the major factors that contribute to several pathological disorders [4-6]. Immobilization/restraint stress is an easy and convenient method to induce both psychological, an escape reaction and physical stress, a muscle work which leads to restricted mobility and aggression [7, 8]. However, it is well known that intensive stress response results in the creation of reactive oxygen species (ROS) e.g. hydrogen peroxide, hydroxyl radical and superoxide anion radical that cause lipid peroxidation, especially in membranes and can play an important role in tissue injury. It has been suggested that chronic stress and high level of glucocorticoids, the adrenal steroids secreted during stress, affect diverse processes involving ROS and increase ROS by approximately 10% basally [9]. The membrane injury causes disruption of the tissue [10-12].

Brain is the target for different stressors because of its high sensitivity to stress-induced degenerative conditions. Oxygen radicals can attack proteins, nucleic acids and lipid membranes, thereby disrupting cellular functions and integrity. Brain tissue contains large amounts of polyunsaturated fatty acids, which are particularly vulnerable to free radical attacks [13-15]. Lipid peroxidation is one of the main events induced by oxidative stress [16]. Lipid peroxidation can produces a range of enzymatically damaging consequences. Thus, lipid peroxidation is considered as a serious consequence of free radical toxicity leading to profound changes in the membrane structure and function that may cause even cell death [17]. One common method to determine the degree of lipid peroxidation is by measuring the level of malondialdehyde (MDA), a by-product of the lipid peroxidation process [18, 19]. Increased serum level of MDA has been reported in cardiovascular, neurological and other diseases [20, 21]. The biochemical feature which has attracted the most sustained and widespread attention in relation to etiology and prevention of these stress induced diseases is serum cholesterol or its fractions like low density lipoproteins (LDL), high density lipoproteins (HDL). Cholesterol and lipoprotein level correlate well with the risk of cardiovascular diseases [22]. The possibility that these variables would be the sensitive indices for emotional, arousal, elicited by stress also seems to be interest.Cholesterol and lipoprotein levels correlate well with the risk of cardiovascular diseases [5]. As stress related disease and death mount, it becomes increasingly important to characterize it. To our knowledge, there have been no studies correlating the influence of chronic immobilization stress on body weight, food intake, tissue lipid peroxidation level, serum lipid profile, blood glucose level, SGOT and SGPT. This study also tested the

hypothesis that chronic immobilization stress brings about a stress-induced injury in various organs and a significant change in the free radical production

MATERIALS AND METHODS

All experimental procedures and animal maintenance confirmed to the strict guidelines of Institutional Ethics Committee and that of Federal laws for the use of animals in the experiment. Adult albino rats (150 to 250 g) of Wistar strain were used in the present study. The rats were procured from the central animal breeding center at our university. Animals were housed individually in polypropylene cages (29cms x 22cms x 14cms) during the experimental period at 28±2◦ C temperature and 50±5% humidity. The rats were maintained under standard laboratory conditions with 12h light: 12h dark cycle. Animals were fed on laboratory chow (Gold Mohur; Lipton India, Ltd) and tap water in drinking bottles were made available ad libitum The animals were divided into two groups as non stressed group (n =10) and stressed group (n =10).

Body weight: The body weight of the animals was recorded by weighing them in an animal weighing balance before and after the stress period. The fractions of weights were adjusted to the nearest gram unit.

Food intake: Food intake of the animals was studied by keeping weighed food pallets in the food cup every day between 9 to 10 AM during the entire experimental protocol. After 24 hours, the leftover food pellets and spilled rat feed were collected from the tray and weighed. The difference between the food provided and the leftover and spilled food was taken as the food consumed by the rat in 24 hours.

CHRONIC STRESS PROCEDURE

Chronic Immobilization stress: The immobilization chambers used in this study were plastic tubes of varying sizes to accommodate all sizes of rats (15cms long and 4cms diameter, 16cms long and 5cms diameter, 17cms long with 6cms diameter). The tubes had a conical head at one end. The conical head area contained numerous perforations which served as breathing holes. The rat was placed inside the tube with head in the conical end. The rats were totally restrained by packing the rear end of the tube and closing it firmly with a stopper. Rats were exposed to chronic stress in the form of immobilization for 2 hour per day for a period of 60 days.

At the end of experiment, all rats were anaesthetized with sodium pentobarbital (40 mg/kg body weight). About 5 ml were collected from heart puncture with the help of vacutenner) and then liver, kidney, adrenal gland, and brain were quickly removed and weighed from the whole brain, cortex, cerebellum, and hypothalamus were carefully separated [23]. The specimens were stored at -80o C until assay. The wet weight of each organ was expressed as 100 g of body weight and the blood samples. . Blood samples were allowed to clot at room

temperature and serum samples were separated by centrifugation at 3000 rpm. From the serum samples the SGOT, SGPT, blood glucose level, serum lipid profile were analyzed with the help of autoanalyser method [24, 25, 26, 27, 28]. The collected tissues were homogenized with a motor driven glass homogenizer in ice- cold phosphate buffer at 0°C for lipid peroxidation analysis. Lipid peroxidation in liver, kidney, heart, brain was estimated spectrophotometrically by thiobarbituric reactive substance (TBARS) as previously described by Kartha and Krishnamurthy [29].

Statistical analysis

Data were computed for mean values ± standard deviation (SD). Comparisons between control and stress were performed by student t test with a significant criteria of $P<0.05$[30].

Table1: Chronic immobilization stress-induced alterations in body weight, food intake, and organ weights. Values are expressed as mean ± SD.

PARAMETERS	Non-stressed group (n=10)	Stressed group (n=10)
Body weight (g)	215.20 ± 8.36	187.33±2.42**
Food intake (g)	24.66±1.63	10.33± 1.50 **
Heart (g/100g BW)	0.378±0.09	0.451±0.03*
Liver(g/100g BW)	2.533±0.37	3.408±0.14**
Kidneys (g/100g BW)	0.629±0.14	0.748±0.03*
Adrenals (g/100g BW)	0.0288 ± 0.01	0.0494 ±0.04**
Cerebral cortex (g/100 BW)	0.625±0.12	0.762±0.06
Cerebellum (g/100g BW)	0.246±0.03	0.252±0.04
Hypothalamus (g/100g BW)	0.125±0.13	0.138±0.06

n= number of rats; * P<0.05, ** P< 0.001; Stress group compared to Non-Stressed group

Table 2: Chronic immobilization stress induced alterations in SGOT, SGPT, serum lipid profile and blood sugar. Values are expressed as mean ± SD.

Parameters	Non stressed group (n=10)	Stressed group (n=10)
SGOT (U/L)	206.33 ± 11.32	501.33 ± 13.35 **
SGPT (U/L)	187.00 ± 10.04	552.83 ± 7.67**
Cholesterol (mg/dl)	46.67 ± 4.08	67.17 ± 8.78*
Triglyceride (mg/dl)	75.17 ± 6.68	153.00 ± 9.57 **
HDL (mg/dl)	38.20 ± 1.99	36.55 ±6.23
LDL (mg/dl)	8.60 ± 0.490	30.02 ± 0.51**
Blood sugar (mg/dl)	95.38 ± 0.57	134.70 ± 3.56 *

n= number of rats; * P<0.05, ** P< 0.001; Stress group compared to Non- Stressed group

ISSN: 0975-8585

Table3: Chronic immobilization stress –induced alterations in lipid peroxidation (nanomoles of MDA/gm wet tissue) in various organs. Values are expressed as mean ± SD.

Parameters	Non stressed group (n=10)	Stressed group (n=10)
Cerebral Cortex	13.04±2.21	92.01±5.13**
Cerebellum	103.59±3.34	223.91±4.39**
Hypothalamus	102.55±3.60	227.41±8.09**
Liver	31.89±2.09	112.37 ± 5.5 **
Heart	21.24±0.36	98.60±0.38**
Kidneys	24.60±0.20	90.20±0.40**

n= number of rats; ** P< 0.001; Stress group compared to Non- Stressed group

RESULTS

The results of the present study showed that chronic immobilization stress showed a significant decrease (P<0.001) in the body weight, food intake and significant increase in various organ weight [heart (P<0.05), liver (P<0.001), kidney (P<0.05), adrenal, (P<0.001)]. Further, in our study there was a significant increase in the SGOT (P<0.001), SGPT (P<0.001), Blood glucose (P<0.05), tissue MDA level (cerebral cortex, cerebellum, hypothalamus, liver, heart and kidneys; (P<0.001 in all tissues) in the stressed group when compared to non- stressed group. In the lipid parameters analysis except HDL all the other parameters (serum cholesterol; P<0.05, serum LDL; P<0.001, serum triglyceride; P<0.05) were significantly increased in the stressed group.

DISCUSSION

It has been proved through countless study that our mental attitude has powerful Influence on our physical health. As immobilization stress is believed to be the most severe type of stress in rodent models and has a comparative effect in humans, this type of stress was used in the present study. In the present study, the exposure to immobilization stress for 2 hours for 60 days resulted in a significant reduction of body weight. The results of body weight changes are similar to those of other researchers. It is well established that corticotrophin – releasing hormone influences feeding behavior and mediate in behavioral and physiological response to stress. Several investigators have mentioned CRH induced anorexia during stress. This may be due to either activation of serotonin pathways or inhibition of neuropeptide Y release [31-33]. Neuropeptide Y is a potent stimulator of food intake [33]. It has been suggested that restraint stress induces suppression of weight gain occur due to depression and anorexia [33]. Food intake is one of the variables sensitive to stress, and it is particularly interesting in stress research not only because of the impact of food on growth and health but also because it can be measured with minimal disturbance of the animals. The decreased body weight could be due to the decreased food intake in the rats under the influence of stress. In addition to that the decrease in the body weight might also have been presumably associated with chronic stress induced increase in metabolic demands, reduced digestion, and increased adrenal steroid secretion. This observation was in agreement with previously reported studies [34-36] but contradicts others [37-39].

Stress can affect every organ system of the body. In the present study when the rats were subjected to chronic stress for a period of sixty days, there was a significant increase in the weight of heart, liver, kidneys, adrenal glands. The hypertrophy of the cardiac muscle after exposure to stress is well documented [40]. Henry and Stephens have reported cardiac hypertrophy by using mice as experimental animals in their stress study40. Similar observations were reported by Horie et al [41] and Gelsema et al [42]. Since the protein required for repair of wear and tear is greater and the metabolic changes are also more after stress, the liver mass might have significantly increased Changes in the homeostatic mechanism such as increased cardiac output and blood pressure during stress might have contributed to the increased kidney weight after stress [43]. Stress-induced adrenal hypertrophy is a well-established phenomenon. Ever since Selye [44] reported increase in adrenal gland size and weight during stress, now it is considered as an index of assessment of stress and its severity. Infact this appears to be one of the initial responses of the organisms to stress of any kind. Strong stimulation of the adrenal glands during prolonged stress situations is known to cause adrenal hyperplasia and hypertrophy [45, 46]. Adrenal gland is one of the important components of the HPA axis. Release of epinephrine from the adrenal medulla and glucocorticoids from the adrenal cortex initiate the biological responses permitting the organism to cope with adverse psychological, physiological and environmental stressors [47].

Immobilization stress which is widely employed to provoke psychological stress has also been reported to be associated with oxidative damage in rats [48]. The generation of ROS is a primary event under a variety of stress conditions and the consequence of reactive oxygen species formation depends on the intensity of the stress. The present study indicate that repeated immobilization stress for sixty days significantly increased the oxidative damage biomarker of lipid peroxidation in liver, kidneys, heart, and different brain tissues in rats. The increased level of lipid peroxidation is the evidence most frequently cited in support of the involvement of oxidative stress in tissues [49]. Immobilization stress induced increase in the MDA level in various tissues was also observed in previous studies [48, 49] Brain is the target for different stressors because of its high sensitivity to stress induced degenerative conditions. Lipid peroxidation measured by MDA nearly doubled upon stress in the cerebral cortex, hypothalamus, and cerebellum. Lipid peroxidation causes cellular damage and may explain why stress causes neuronal loss in the brain. Our results provide evidence to support the hypothesis of the involvement of oxidants in stress.

Liver is an important organ for metabolism and detoxification. Liver contains considerable amounts of polyunsaturated fatty acids, which are prone to damage by free radicals. SGPT and SGOT are markers of liver function. In the present study, elevated activities of SGPT and SGOT demonstrated liver damage in the rats exposed to chronic immobilization stress. This might be due to alteration in the cell membrane permeability which may permit these enzymes to leak from the cells with intact membrane, when there is stress or any damage to the liver cells, the enzyme escapes into the blood and so the SGPT, SGOT enzymatic activity increases. The above findings are in accordance with the earlier findings [50]. The observed

increase in the blood glucose level may be due to the release of glucocorticoids during stress which will increase the blood glucose level in two ways either by promoting gluconeogenisis in liver from amino acids or by inhibiting glucose uptake and utilization by peripheral cells[50].

Numerous experimental animal studies have provided empirical support for a definite relationship between stress and lipid concentrations. Cholesterol level was significantly higher in stress group which is similar to those reported by others [51]. It has been suggested that various forms of stress raised the cholesterol level by disturbing rate of synthesis and excretion [52]. Various authors have suggested that this change is due to the effect of epinephrine on lipoprotein lipase, hormone sensitive lipase and hepatic lipase [53, 54]. Patterson et al. suggested that psychological stress caused decreased volume, producing hemoconcentration which might be a secondary cause of increased cholesterol level [54]**.** It is well-known that catecholamines activate lipolysis in adipose tissue and increase the free fatty acid flow to the liver where increased triglyceride synthesis and secretion occurs. The observed increased level of triglycerides in this study may also due to the stress induced catecholamine surge.

The LDL is well recognized as a risk factor and HDL as a protective factor against arteriosclerosis [55]. Correlation of lipid profile and lipid peroxidation after the exposure to chronic immobilization stress indicates that increased lipid peroxidation is associated with increased total cholesterol and LDL, the known major risk factors for atherosclerosis and other lipid peroxidation induced diseases. It is well-proven that hyperglycemia is a key causative factor in oxidative stress in tissue sites for diabetic complications. The oxidative stress in diabetes mellitus is characterized by increased production of ROS (reactive oxygen species), sharp reduction in antioxidant defense and altered cellular redox status [56]. These changes occur via multiple mechanisms including glucose glycoxidation, protein glycation and glycooxidation. The hyperglycemia has also been shown to produce increased level of lipid peroxide end products (MDA) and to reduce total plasma free radical trapping activity [57]. Our finding of increased level of lipid peroxide end-product is in agreement with the above mentioned works and may confirm the key role of hyperglycemia in generating the disbalance between pro-oxidative reactions and anti-oxidative defense. In this present study, chronic immobilization stress showed significant alterations in both physiological, biochemical parameters and lipid parameters. In conclusion, exposure to chronic immobilization stress induces increase in glucose, SGOT, SGPT, lipid metabolism and tissue MDA levels are mediated through the increased generation of free radicals. Thus, the present study also confirms the shift of oxidants and antioxidant balance during stress. **.** It appears that stress can stimulate numerous pathways leading to an increased production of oxidants. Thus, stress may add to the oxidant burden associated with normal aerobic metabolism and its consequent damage to lipid that appears to be a one of the major contributor to aging and various diseases. The assessment of the different antioxidants during chronic stress will be an interesting step to identify the mechanisms involved.

ISSN: 0975-8585

REFERENCES

[1] Vogel WH. Human Exp Toxicol 1993; 12:265-271.
[2] Brown GW. Life events and affective disorder: Replications and limitations. Psychosomatic Med 1993; 55:248-259.
[3] Anisman H.. Alcohol Res Health 1999; 23:241-249.
[4] Eliot RS. Stress and the heart. Futura Publishing Company New York 1974.
[5] Nagaraja HS, Jeganathan PS. Biomedicine 1999; 19(2):137-49.
[6] Angela M Gouir and Leslie Matuszewich. Physiology and behavior 2005; 86(1-2):21-31.
[7] Romanova TP, Karpel GG, Brill GF and Markov KM. Pathology Fiziology Expsn Trminol 1994; 3:5-8.
[8] Singh LK, Rang X, Alexacos N and Netqumen R Theoharides. Brain Behv Immunol 1993; 3:225-239.
[9] Kovacs P, Juranek I, Stankovicova T, Svec P. Pharmazie 1996; 51:51-53.
[10] McIntosh LJ and Sapolsky RM. Exp Neurol 1996; 141:201-206.
[11] Begchi D, Carryl OR, Tran MX, Begchi M, Garg A, Milnes MM et al. Mol Cell Biochem 1999; 196:109-116.
[12] Cochrane CG. Mol Aspects Med 1991; 12:137-147.
[13] Gutteridge JM. Clin Chem 1995; 41:1819-1828.
[14] Muller DPR. Neurological disease. In Sies, H. (Ed). Antioxidants in Disease Mechanisms and Therapy. Academic Press, New York 1994; 557-580.
[15] Reiter RJ. FASEB J 1995; 9:526-533.
[16] Kovacheva S and Rebarov SR. Lungs 1995; 173:255-263.
[17] Alessio HM. Med Sci Sports Exerc 1993; 25:218-224.
[18] Millan-Plano, Garcia JJ, Martinez-Ballarin E, Reiter RJ, Ortega-Gutierrez S, Lazaro RM and Escanero JF. J Trace Elem Med Bio 2003; 17:39-44.
[19] Topal T, Oter S, Korkmaz A, Sadir S, Metinyurt G, Korkmazhan ET, Serdar MA, Bilgic H, Reiter RJ. Life Sci 2004; 75:461-467.
[20] Draper HH, McGirr LG and Haldey M. Lipids 1986; 21:305-307.
[21] Mehrotra A, Patniak D and Mishra VN. J Ass Physicians India 1996; 44:944.
[22] Eliot RS. Stress and the heart. Futura Publishing Company, New York 1974.
[23] Culling CFA, Allison RT, Batt WT. Cellular pathology techniques, 14th edition, London: Butter worths, 1985.
[24] Sugiuchi H, Uji Y, Okabe H, Irie T, Uekama K, Kayahara N and Miyauchi K. Clin Chem 1995; 41:717-723.
[25] Matsuzaki Y, Kawaguchi E, Norita Y. J Anal Bio Sci 1996; 19:419-427.
[26] Siedel J, Schmuck R, Staepels J. AACC Meeting. Abstract 34 Clin Chem 1993; 39:1127.
[27] White WL, Erickson MM, Stevens SC, editors. Chemistry for the clinical laboratory. St Louis: The C. V. Mosby Co 1976; 92-7.
[28] Trinder P. Annl Clin Biochem 1969; 6:24-27.
[29] Kartha R, Krishnamurthy S. I J Physiol Pharmacol 1978; 22(1):44-52.
[30] Hassard TH. Understanding biostatistics. St. Louis: Mosby- Year Book. 1991; 268-272.
[31] Kennet GA, Chaouloff F, Marcou M, Curzon G. Brain Res 1986; 382:416-421.

[32] Shimizu N, Oomura Y, Kai Y. Physiol Behav 1989; 46:835-841.
[33] Heinricks SC, Menzaghi F, Pich EM, Hauger RL, Koob GF. Brain Res 1993; 611:18-24.
[34] Nagaraja HS and Jeganathan PS. I J Physiol Pharmacol 2003; 47(1):94-100.
[35] Mc Laren GW, Mathews F, Fell R, Gelling M, Macdonals DW. Animal Welfare 2004; 13(3):337-341.
[36] Endo Y, Shiraki K. Physiol Behav 2000; 71:263-268.
[37] Szenasi G, Bencsath P, Takacs L. Acta Physiol Hung 1988; 72(1):93-98.
[38] Rowland NE and Antelman SM. Human obesity Science 1976; 191(4224):310-312.
[39] Bernatova I, Key MD, James BL, Morris M. Hypertension 2002; 40:768.
[40] Henry JP and Stephens PM. Clin Exp Pharmacol Physiol 1981; 8:483-487.
[41] Horie R, Yamori Y, Nara Y, Sawamura M, Mizushima S. Clin Exp Hypertens 1991; 13:859-864.
[42] Gelsema AJ, Schoemaker RG, Ruzika M, Copeland NE. J Hypertens 1994; 12(9):1019- 1028.
[43] Nagaraja HS and Jeganathan PS. I J Physiol Pharmacol 1999; 43: 53-59.
[44] Selye H. Br J Exper Patho 1936; 17:234-248.
[45] Marti O, Gavalda A, Jolin T, Armario A. Pyschoneuroendocrinol 1993; 18:67-77.
[46] Alario P, Gamallo A, Beato MJ, Trancho G. Physiol Behav 1987; 40:29-32.
[47] Wong Dona. J Cellular and Molecular neurobiology 2006; 26:889-898.
[48] Fontella FU, Siquiera IR, Vasconcellos AP, Tabajara AS, Netto CA, Dalmaz C. Neurochem Res 2005; 30:105.
[49] Liu J and Mori A. Int J Stress Mang 1994; 1:249- 263.
[50] Nayanatara AK, Nagaraja HS, Ramaswamy C, Bhagyalakshmi K, Ramesh bhat M, Damodara gowda KM, Venkappa S mantur. J Chinese clin med 2009; 4(21):92-97.
[51] Jain SK, Pandey SN, Srivasta RK, Ghosh SK. J Anatom Soc India 2000; 49:165-167.
[52] Champe PC, Harvey RA. Biochemistry, 2 ed. Lippincott-Raven Publishers 1994; 47-60.
[53] Lunderberg U, Fredrikson M, Wallin L, Melin B, Frankenhaeuser M. Pharmacol Biochem Behav 1989; 33:381-386.
[54] Muldoon MF, Herbert TB, Patterson SM, Kameneva M, Raible R , Manuck SB. Arch Intern Med 1995; 155:615-620.
[55] Haberland M, Fong D and Cheng L. Science 1988; 241:215-218.
[56] Obrosova IG. Int Rev Neurobiol 2002; 50:3 -35.
[57] Kyselova P, Zourek M, Rusavy Z, Trefil L, Racek J. Physiological Res 2002; 51:591- 595.