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EFFECTS OF ACUTE AND REPEATED IMMOBILIZATION STRESS ON OXYGEN CONSUMPTION OF THE ISOLATED INTERSTITIAL RATS' TESTES CELLS

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The aim of this study was to investigate the effects of acute and repeated immobilization stress on oxygen consumption of the isolated interstitial rats' testes cells (ISC). The ISC testes cells were isolated acording to Anakwe et al. The oxygen consumption by ISC testes was measured polarographically in vitro with a Clark-type oxygen electrode (YSI-5331, Yellow Springs Instrument), which was done in two phases of respiration: in phase V_4 (without ADP) and in phase V_3 (with ADP). Repeated immobilization stress (2 hours daily for 10 consecutive days) induced a fall in oxygen consumption in both phases of ISC rat's testes respiration (-10% V_4, -4% V_3), but this inhibition of respiration was not statistically significant ($p>0.05$). Acute immobilization stress (2 h) induced decrease in oxygen consumption in both phases of ISC rats' testes respiration (-49% V_4, -31% V_3) which was statistically significant ($p<0.01$). Our data suggest that acute and repeated immobilization stress reduce oxygen consumption of ISC testes cells. However, the mechanisams by which immobilization stress induces mitochondrial dysfunction, as well as mechanisms which develop an adaptive response to repeated immobilization remain unclear, so that further investigations of this mechanisms are required.

Key words: cell respiration, stress, testis, rat

INTRODUCTION

Stress is defined as disruption of homeostasis and stimuli that challenge homeostasis are designated as stressors (Rivier and Rivest, 1991).

Reproductive function in male primates or animals is suppressed by psychogenetic or somatic stress (Tache *et al*., 1976; Sapolsky, 1985). It is well established that acute immobilization stress lowers serum testosterone values primarily at the testicular level in the presence of unchanged serum luteinizing hormone (LH) levels. Meanwhile, chronic immobilization stress has inhibitory effects on the hypothalamic pituitary level and by lowering serum LH release, decreases serum testosterone concentrations (Maric *et al*., 1996; Dong *et al*.,

2004). Recent studies suggested that stress produces testicular germ cell apoptosis (Sasagawa *et al*., 2001).

Adenosine triphosphate (ATP), is used as a general energy source by all living cells. Approximately 95% of ATP formation in animal cells with aerobic type of metabolism occurs by oxidative phosphorylation (OXPHOS). OXPHOS consists of two functionally independent processes: oxidation of reduced substrates (expressed as respiration or oxygen consumption) and phosphorylation of ADP by inorganic phosphate. The latter, energy-consuming process occurs at the expense of energy released during the former.

Some of ATP-dependent energy-consuming processes in the testis are: chromatin remodelling, which disrupts histone-DNA contacts and induces nucleosome mobilization, regulation of sperm motility, maturation, fertilization as well as germ cell apoptosis (Lane, 2006). Recent studies have also shown that testes need ATP for the process for cholesterol transport to the outer mitochondrial membrane in the presence of StAR protein (Stocco *et al*., 2001; Hales *et al*., 2005).

Spermatogenesis in the seminiferous tubuli of the testis occurs under a high proliferation rate, suggesting considerable oxygen consumption. Because of the lack of blood vessels, the oxygen partial pressure in the lumen of the tubuli is very low (Wegner, 2005). However, the consequences of these environmental conditions on spermatogenesis, steroidogenesis and OXPHOS are unknown. The PAS domain and hypoxia-inducible factor-1alpha (HIF-1alpha) are found in environmental protein sensors involved in the perception of oxygen partial pressure, light intensity, redox potentials and voltage (Lysiak *et al*., 2009).

Although the testis has been described previously as functioning on the brink of hypoxia; the effects of stress on oxygen consumption of the testes cells have not been investigated yet.

The aim of this study was to investigate the effects of acute and repeated immobilization stress on oxygen consumption of the isolated interstitial rats' testes cells.

MATERIALS AND METHODS

Animals

Experiments were performed on adult male Wistar rats (n = 18, weight 250 ± 20 g) bred in the Institute of Physiology and raised under controlled environmental conditions (temperature 22 ± 2^{o}C; 14 h light/10 h dark) with food and water available *ad libitum.* All experiments were done with the approval of the institutional ethical committee and were conducted in accordance with the principles and procedures of the NIH Guide for Care and Use of Laboratory Animals. Animals were killed by decapitation immediately after the end of the stress session.

Immobilization stress protocol

The animals were divided into three groups: the repeated immobilization stress group, rats (n=6) were subjected to repeated immobilization stress for 2 h

for 10 consecutive days; the acute immobilization stress group, rats (n=6) were immobilized for 2 h, once; the control group, the rats (n=6) were left undisturbed.

Immobilization rats were bound to the supine position in a wooden board by fixing the rat limbs by thread to the wooden board according to Kvetnansky *et al*. (1970). Head motion was not limited. Rats were immobilized in the morning (from 8.00 to 10.00 h). At the end of the immobilization period rats' testes were quickly removed, decapsulated and used for further experiments.

Isolation and preparation of the interstitial rats' testes cells suspension

Interstitial rats' testes cells (ISC) were isolated by the method of Anakwe *et al*. (1985). Testes were dissociated by collagenase (1.2 mg/mL medium) at 34°C in a shaking water bath for 8 minutes, oscillating at 120 cycles/min. The resulting cell suspension was diluted with 6 vol of Medium 199 (M199) with 0.5% bovine serum albumin (M199-BSA 0.5 %) and incubated for 5 minutes at 4°C to allow the seminiferous tubule fraction to settle. The supernatant was decanted through nylon into another tube and centrifuged at 150 g for *5* min. The seminiferous tubule fraction was washed once more with 10 mL M199-BSA 0.5 %, and centrifuged at 150 g for 5 min. The resultant pellet of the interstitial rats' testes cells was resuspended in 5 mL of the medium. This interstitial rats' testes cells suspension were used immediately for further experiments.

Determining number and an assessment of cell viability of isolated interstitial rats' testes cells

In this study we determined the cell number and an assessment of cell viability at the suspension of ISC testes before oxygen consumption measurement. The number of live cells at the suspension was determined by "Trypan Blue" test at the hemocytometer. Trypan blue (0.2 %) is a vital stain used to selectively colour dead tissues or cells. Live cells with intact cell membranes are not coloured. Since cells are very selective for the compounds that pass through the membrane, Trypan blue is not absorbed in viable cells. However, it traverses the membrane in dead cells. Hence, dead cells were shown as distinctive blue colour under a microscope. The cell number in the suspension was calculated using the folowing formula:

$$\text{Cell number / mL suspension} = X \times 10 \times 2 \times 1000$$

X – mean cell number at 1 mm^2; 10 – depth of chamber, 2 – dilution; 1000 – cell number per mm^3.

Oxygen consumption measurements of the interstitial rats' testes cells suspension

Oxygen consumption of the interstitial rats' testes cells suspension was measured using Biological Oxygen Monitor (Model 5300, YSI, USA) with Clarck type electrode.

The electrode itself was located at the top of the chamber with oxygen-saturated medium (95% O_2 and 5% CO_2). Interstitial rats' testes cells suspension (1 mL) and supstrate (glutamate 0.5 M, 20 μL) were added to the medium (2 mL).

During these basal conditions, oxygen consumption was observed for 5 min (V_4 respiratory phase). Adenosine diphosphate (ADP, 0.1 mol/L, 10 μL) was added subsequently and cell respiration was observed for 3 min during the active maximum respiratory phase (V_3 respiratory phase). At the end of each V_3 respiratory phase, sodium dithionite was added in order to eliminate all oxygen from the medium. The control group was analysed in the same manner. Oxygen consumption of the interstitial rats' testes cells was expressed in $nlO_2/min/10^6$ cells.

Statistical analysis

All results were expressed as mean ± SD of eight repeated measurements. Statistical comparisons were made using a Student t test and $p<0.05$ was considered statistically significant.

RESULTS

An assessment of cell viability of isolated interstitial rats' testes cells

Calculated cell number in this study was 3.5 milion/mL. The cell viability at the suspension of ISC testes for each examined sample was higher than 80%.

The effect of ADP stimulation of respiration of the isolated interstitial rats' testes cells

ADP increased rate of oxygen consumption according V_4 respiratory phase in all examinated samples of ISC rat's testes was for the control group +261%, in the group of repeated immobilization stress +286% and in the group of acute immobilization stress +392% (Table 1).

Table 1. The effect of ADP on oxygen consumption of isolated ISC rats' testes

	V3/V4
Control group	+ 261%
Repeated immobilization stress group	+ 286%
Acute immobilization stress group	+392%

*pronounced by percentil change V_3 phase according to V_4 phase of respiration

The effect of repeated immobilization stress on oxygen consumption of the isolated interstitial rats' testes cells

The effects of repeated immobilization stress on oxygen consumption of the isolated interstitial rats' testes cells were shown in Figure 1 and Figure 2.

In the control group oxygen consumption was 63.63 ± 13.70 during V_4 respiratory phase and 229.67 ± 44.96 $nlO_2/min/10^6$ cells during V_3 respiratory phase, respectively.

In V_4 respiratory phase, the repeated immobilization stress induced a decrease of oxygen consumption relative to the control group (-10%) but that decrease was not statistically significant ($p>0.05$, $t=1.05$, $DF=14$).

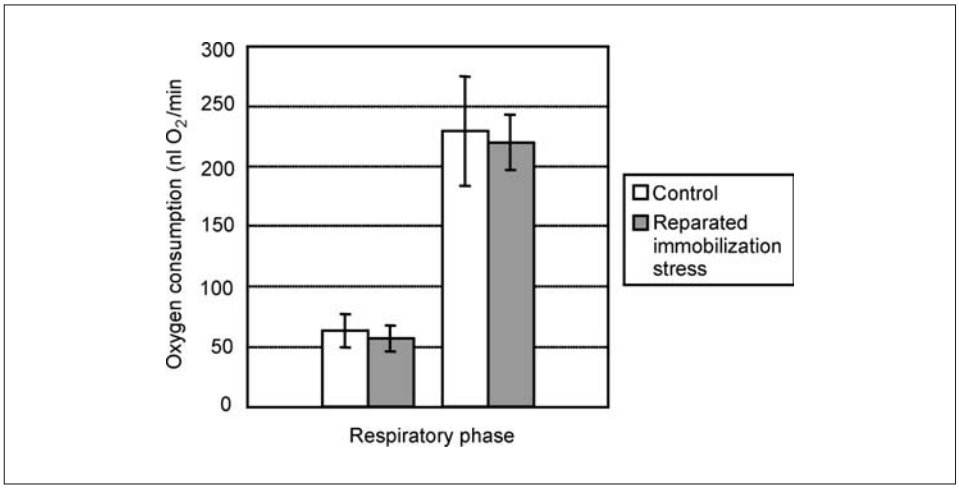

Figure 1. The effect of the repeated immobilization stress on oxygen consumption of isolated ISC rats' testes in V_4 and V_3 respiratory phases. The results are expressed as mean ± SD for eight repeated measurements (n=8) for each experimental group

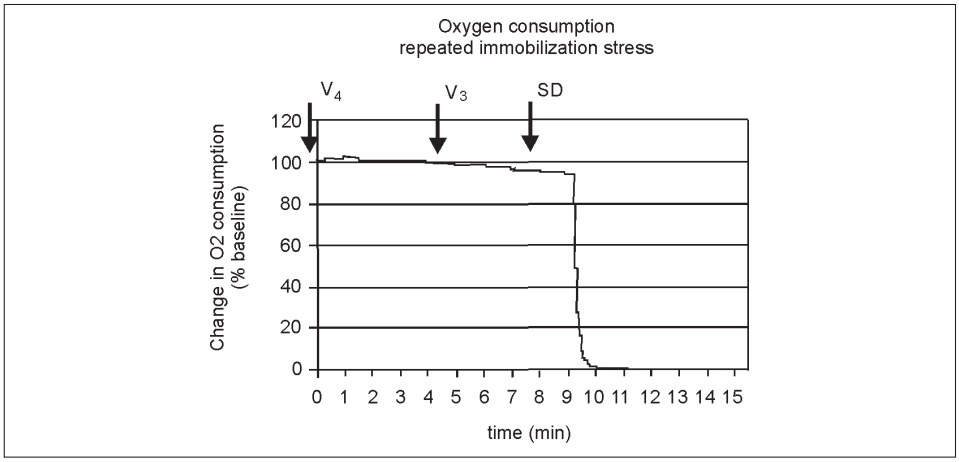

Figure 2. Original data recorded curves: the group of repeated immobilization stress. In V_4 respiratory phase interstitial rats' testis cells suspension and supstrate (glutamate 0.5 M, 20 μL) were added to the medium. After 5 minutes adenosine diphosphate (ADP, 0.1 mol/L, 10 μL) was added and cell respiration was monitored during V_3 respiratory phase. Sodium dithionite (SD) was added in order to eliminate all oxygen from the medium

In V_3 respiratory phase, the repeated immobilization stress induced decrease of oxygen consumption according the control group (-4%) but that decrease was not statistically significant ($p>0.05$, $t=0.49$, $DF=14$).

The effect of acute immobilization stress on oxygen consumption of isolated interstitial rats' testes cells

The effects of acute immobilization stress on oxygen consumption of the isolated interstitial rats' testes cells were shown in Figure 3 and Figure 4.

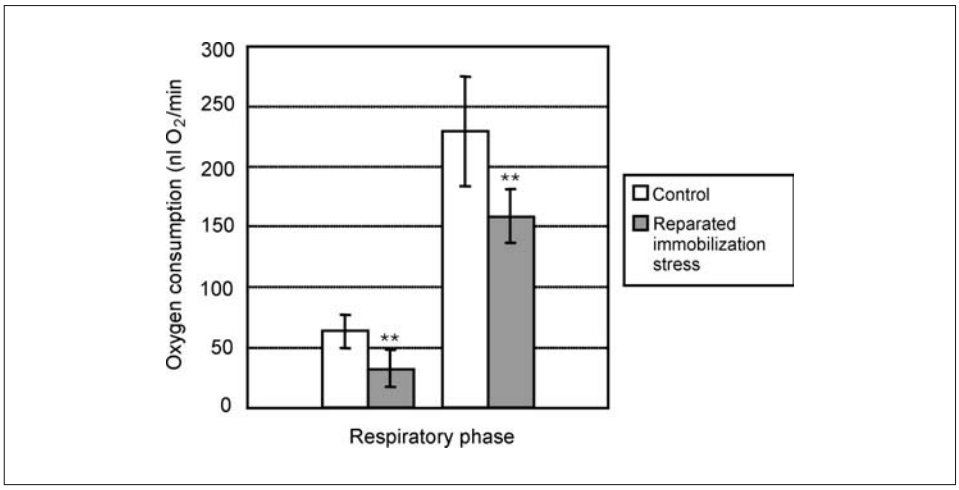

Figure 3. The effect of acute immobilization stress on oxygen consumption of isolated ISC rats' testes in V_4 and V_3 respiratory phases. The results are expressed as mean ± SD for eight repeated measurements (n=8) for each experimental group

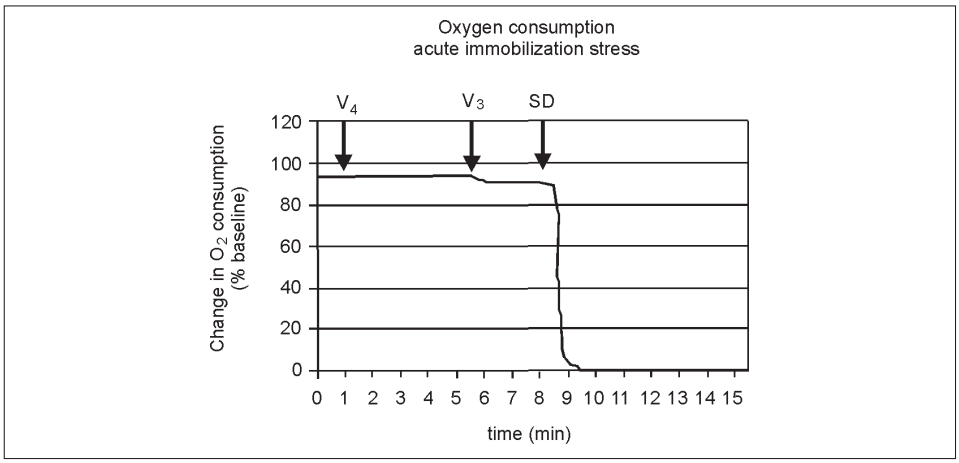

Figure 4. Original data recorded curves: the group of acute immobilization stress. In V_4 respiratory phase interstitial rats' testis cells suspension and supstrate (glutamate 0.5 M, 20 μL) were added to the medium. After 5 minutes adenosine diphosphate (ADP, 0.1 mol/L, 10 μL) was added and cell respiration was observeded during V_3 respiratory phase. Sodium dithionite (SD) was added in order to eliminate all oxygen from the medium

In the V_4 respiratory phase, the acute immobilization stress induced a statistically significant decrease of oxygen consumption relative to the control group (-49%) ($p<0.01$, $t=4.27$, $DF=14$).

In the V_3 respiratory phase, the acute immobilization stress induced statistically significant decrease of oxygen consumption according to the control group (-31%) ($p<0.01$, $t=3.98$, $DF=14$).

DISCUSSION

In the present study, the acute stress induced a significant inhibition of oxygen consumtion in comparison with the control group in both respiratory phases (V_4 : -49%, V_3 : -31%). Repeated immobilization stress also induced inhibition of oxygen consumtion in both respiratory phases (V_4 : -10%, V_3 : -4%), but this inhibition of respiration was not statistically significant.

Our results can not be compared with literature data because the effects of immobilization stress on oxygen consumption of the isolated interstitial rats' testes cells have not been investigated yet.

Meanwhile, many authors have shown that during stress Leydig cells produce nitric oxide (NO), a mediator of oxidative stress (Tatsumi *et al*., 1997; Kostic *et al*., 1998). Madrigal *et al*. have shown that chronic stress induced an increase of NO production via an expression of inducible NO synthase (iNOS) in the brain. These authors have also shown that chronic stress induced mitochondrial dysfunction, lipid peroxidation and glutathione depletion in the brain of adult male rats (Madrigal *et al*., 2001). A large number of other studies has shown that in different experimental models and in different tissues, NO induced inhibition of oxygen consumption and decreased tissue demand for oxygen (Denisenko and Kostenko, 2003; Kojic *et al*., 2006; Giulivi *et al*., 2006; Carreras and Poderosso, 2007). Our results of respiration inhibition of rat's testis ISC could be explained by the mechanism of increased nitric oxide production.

A possible mechanism by which acute immobilization stress significantly reduces respiration of rat's testes ISC besides an increased nitric oxide production may involve cellular damage by raised rate of lipid peroxidation (Kovács *et al*., 1996; Clarkson *et al*., 2007; Storey, 1996; Allen *et al*., 2006; Goyal and Anil, 2007). In the current study we did not assess the effect of immobilization stress on nitric and lipid reactive oxygen species (ROS) so it is not possible to discuss strictly the mechanisms which explain inhibition of respiration in the group exposed to acute immobilization stress.

Repeated immobilization stress induced reduction of oxygen consumption, but this inhibition was not statistically significant. It might be explained by an adaptive response to repeated immobilization. In our previous work we showed that basal and human chorionic gonadotropin-stimulated cAMP production by Leydig cells isolated from rats exposed to both acute and repeated immobilization was significantly reduced. Despite the reduced cAMP production, immunoblot analysis revealed increased immunoreactivity for both protein kinase A (PKA) and steroidogenic acute regulatory (StAR) protein in Leydig cells obtained from rats repeatedly exposed to immobilization. Also, the phosphorylation and production

of mature StAR protein was evident during exposure of rats to repeated immobilization treatment. Treatment with cholesterol, the steroid substrate transported into mitochondria by StAR, significantly increased androgen and progesterone production by Leydig cells isolated from rats exposed to repeated immobilization. In contrast, when other steroid substrates (22(R)-OH-cholesterol, pregnenolone, progesterone, Delta4-androstenedione) were present in the culture media, Leydig cell steroidogenesis was still reduced by immobilization. Thus, we concluded that PKA-mediated phosphorylation of StAR protein is an important mechanism in the adaptive response of Leydig cells to repeated immobilization (Kostic *et al*., 2008).

Losada (1988) and Sun (1991) showed that immobilization stress induced changes of central GABAergic function in rats. Other studies described involvement of nitic oxide in the regulation of stress susceptibility and adaptation in rats (Nadeem *et al*., 2006; Gulati *et al*., 2006). Smirin *et al*. (2000) showed that the adaptive response to stress possesses multiple NO-dependent protective effects and stimulates NO storage.

Teplyi and Gorden (2004) carried out the research on effects of immobilization stress on the relative mass of young and old rats reproductive organs. They found that vitamin E injection has protective effects against immobilization stress induced by reduction of the relative mass of reproductive organs.

Pogany (1983) reported that, in addition to weight loss, the irradiation of testicular tissue elicited distinct increases in oxygen consumption. The high metabolic activity was maintained for several weeks after the initial exposure and was most probably associated with the recovery phase of the testes when spermatogonia A are repopulating the germinal epithelium.

In order to evaluate the effects of exposition to continuous chronic hypobaric hypoxia (CCHH) and intermittent chronic hypobaric hypoxia (ICHH) on testis histology and on oxidative metabolism of spermatogenic cells (SC), male rats were exposed to a 4600-m simulated altitude (PO2: 89.6 mmHg). After 60 days, round spermatids from CCHH rats evidenced large oxygen consumption (QO_2) insensitive to inhibition by cyanide, a process that could be partly related to lipoperoxidation. Thus, exposure of male rats to CCHH and ICHH induced evident changes in testicular morphology and loss of spermatogenic cells, in all stages of the spermatogenic cycle. This post-meiotic spermatogenic cell loss in the testis correlated well with metabolic changes in round spermatids that evidenced a strong metabolic stress in these cells (Fabias *et al*., 2005).

A good index of functional integrity of the mitochondrial respiratory chain complexes during the experiments is the cell response to respiration stimulation by ADP (Nelson and Cox, 2005). In our experiments, comparing to the V_4 respiratory phase, the stimulation of respiration by ADP of isolated rat's testes ISC was present in the control group (+261%), in the repeated immobilization stress group (+265%), as well as in the acute immobilization stress group (+290%).

Immobilization model for stress research consits of complex stimuli, including pain, exercise, thermoregulation, circulatory and respiratory limitations. These stimuli induce maximal neuroendocrine activation with intense response of

catecholamines, corticosterone, prolactin and other hormones (cytokines) the role of which in oxygen consumption should be considered in our further work.

In conclusion, our data suggest that acute and repeated immobilizations stress reduce oxygen consumption of the isolated interstitial rats' testes cells. However, the mechanisms by which acute immobilization stress induce mitochondrial dysfunction, as well as mechanisms which develop an adaptive response to repeated immobilization remain unclear, thus further investigations of this mechanisms are required.

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EFEKTI AKUTNOG I PONOVLJENOG IMOBILIZACIONOG STRESA NA POTROŠNJU KISEONIKA IZOLOVANIH INTERSTICIJUMSKIH ĆELIJA TESTISA PACOVA

KOJIĆ ZVEZDANA, VIDENOVIĆ-IVANOV JELICA i KOSTIĆ TATJANA

SADRŽAJ

Cilj ovog rada je bio da se ispitaju efekti akutnog i ponovljenog imobilizacionog stresa na potrošnju kiseonika izolovanih intersticijumskih ćelija (ISĆ) testisa pacova. Intersticijumske ćelije testisa pacova izolovane su metodom Anakwe i sar (1985). Potrošnja kiseonika merena je pomoću kiseoničke elektrode Clark tipa, i to u toku V_3 (-ADP) i V_4 (+ADP) faze respiracije. Ponovljeni imobilizacioni stres (2 sata/dan, 10 dana) smanjio je respiratornu aktivnost izolovanih ISĆ testisa pacova u toku obe faze respiracije u odnosu na kontrolnu grupu, ali ova inhibicija nije bila stastistički značajna (-10% u V_4 i -4% u V_3 fazi, $p>0.05$). Akutni imobilizacioni stres (2 sata/dan, jednokratno) je, statistički značajno, ($p<0.05$) smanjio respiratornu aktivnost izolovanih ISĆ testisa pacova u toku obe faze respiracije u odnosu na kontrolnu grupu (-49% u V_4 i -31% u V_3 fazi). Dobijeni rezultati ukazuju da akutni i ponovljeni imobilizacioni stres smanjuje potrošnju kiseonika u izolovanim ISĆ testisa pacova. Mehanizmi ovog uticaja stresa na funkciju respiratornog lanca, kao i mehanizmi adaptivnog odgovora u toku ponovljenog imobilizacionog stresa, ostaju nejasni tako da su potrebna dalja ispitivanja.