# Effects of nutrition, social factors and chronic stress on the mouse Leydig cell testosterone production

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**ABSTRACT**: The testosterone production by Leydig cells of mice adversely affected with nutritional or social factors and exposed to chronic stress was studied *in vitro*. Both basal and gonadotrophin stimulated testosterone production were highly significantly decreased ($P < 0.01$) as in groups given the hypothyreotics or potassium nitrate alone as in combination with stress. Stimulated *in vitro* testosterone production was not significantly changed in groups fed with synthetic diets, however, in mice given modified diets and exposed to stress decreased responsivity of Leydig cells stimulated by gonadotrophin was found. Basal and stimulated testosterone production *in vitro* in most of gonadotrophin concentrations was non-significantly lower in mice affected only by stress when compared with undisturbed controls. In isolated male mice the basal testosterone production was significantly ($P < 0.05$) decreased and gonadotrophin stimulated production highly significantly ($P < 0.01$) decreased when compared with group caged males. The testosterone production was most severely suppressed in aggressive individuals. Serum testosterone levels were detected in all animals, corticosterone, $T_3$ and $T_4$ in selected groups of mice. We can conclude that the testosterone production was adversely affected by nutritional factors, and the impact was more profound when exerted together with chronic stress. The adverse effect of individual caging of male mice was also proved. ***For free full paper in pdf format see http://www.vri.cz/vetmed.asp***

**Keywords**: mice; chronic stress; hypothryeosis; potassium nitrate; fat-free diet; individual caging; aggressive and timid behaviour; Leydig cells *in vitro*; gonadotrophin; testosterone

## INTRODUCTION

Chronic exposure to stressful situations induces a highly complex set of neuroendocrine changes, dependent on the interaction between individual characteristics and situational factors (Mormede *et al*., 1990). During stress, the hypothalamic-pituitary-adrenal axis is activated, the glucocorticoid secretion increases (Barlow *et al.*, 1975; Pellegrini *et al.*, 1998; Věžník, 1998; Jeong *et al.*, 1999), and consequently, circulating testosterone levels are decreased via glucocorticoid receptors in Leydig cells (Lopez-Calderon *et al.*, 1991; Orr and Mann, 1992; Srivastava *et al.*, 1993; Pellegrini *et al.*, 1998; Věžník, 1998; Yazawa *et al.*, 1999) or a suppressed response to gonadoreline stimulation in the testosterone production assay (TPA) can be found (Věžník and Kozdera, 1990; Kozdera *et al.*, 1993). Low testosterone production adversely affects the quality of ejaculates and subsequent fertility of males. Therefore, one of the aims of our study was to assess the ability of the *in vitro* testosterone production by Leydig cells of mice adversely affected by nutritional or social factors combined with chronic stress. An excessive intake of nitrates found in drinking water (Písaříková *et al.*, 1996; Gelberg *et al.*, 1999) may induce intoxication (Bartík, 1985), and adversely affect the thyroid function and that causes reproductive disorders (Zralý *et al.*, 1997), as demonstrated when male mice were given either drugs inducing hypothyreodism, or potassium nitrate. An excessive intake of fat may increase the incidence of coronary artery diseases and arteriosclerosis and alter the male endogenous sexual hormone levels in men (Dorgan *et al.*, 1996) and the changes are more profound during stress (McCann *et al.*, 1999; Stoney *et al.*, 1999); the effect of a fat-free diet in comparison with a fat containing diet was also studied in our experiments. According to the indirect characteristics of the common syndrome of adaptation in male mice Šulcová (1981) proved that isolation had a stressful effect to the animals, and Nyska *et al.* (1998) found Leydig cell atrophy in individually caged males, and therefore another aim of this study was to examine the testosterone production in individually caged males with aggressive or timid behaviour.

## MATERIAL AND METHODS

### Experimental animals and experimental design

Experiment 1

The subject for this study were male Balb/c mice purchased from AnLab Brno, 3.5 months old, randomly allocated into 5 groups, 20 animals each. The mice were housed in cages in groups of ten, under identical conditions. All animals had *ad libitum* access to food and water. Groups 1 (control), 2 and 3 were fed a standard diet. Groups 4 and 5 were given special synthetic diets either containing 5% soybean oil (group 4) or without fat (group 5) which were prepared according to Kacerovský *et al.* (1990). Experimental groups were given drugs in drinking water for 5 weeks (group 2: 0.08% propylthiouracyl – Propycil 50, Solvay Pharmaceuticals GmbH, Germany; group 3: potassium nitrate – $KNO_3$ – 600 mg/l). Fourteen days after the beginning of the experiment, 10 mice were randomly selected from each experimental group and subjected to chronic stress according to a time schedule (groups 1b, 2b, 3b, 4b, 5b). All the stressors were exerted episodically so that no stereotype of the rhythm might occur. The remaining mice were given the same drugs without stress (groups 2a, 3a, 4a, 5a). Control group 1a was not disturbed. Body weights of all the mice were recorded at the beginning and in the end of the experiment. After five weeks, mice were decapitated in ether anaesthesia, their blood was harvested and blood serum obtained for consequent assessment of hormone levels. Testes were collected, Leydig cell suspensions were prepared and used for cultures.

The following stressors were used:

– sensory factors: noise produced by mutual action of two metal objects, irregularly exerted (5 min per session),
  20 times during experiment
– kinetic factors: swimming in water (20°C, 10 min per session),
  12 times during experiment
– psychical factors: movement restriction in plastic restrainers (3 h per session),
  8 times during experiment
  keeping in a dark place – a black box (20 min per session),
  9 times during experiment

Experiment 2

Forty male ICR mice, 3.5 months old, produced by VELAZ Prague obtained from the Pharmacological Institute (Faculty of Medicine, Masaryk University, Brno, Czech Republic) were used to assess the effect of social factors on the Leydig cell testosterone production. The mice were divided in the above mentioned Institute into 4 groups according to the classification by Šulcová *et al.* (1998), i.e. 1. isolated aggressive mice, 2. isolated timid mice, 3. group caged animals, counterparts of aggressive males, 4. group caged animals, counterparts of timid males. Blood was collected in the Pharmacological Institute from the intraorbital arc of mice under ether anaesthesia (routine of that laboratory) and serum hormone levels were determined. After sacrification, testes of the animals were used for preparation and culture of Leydig cell suspensions.

### Preparation and culture of testicular cells

Leydig cell suspensions were prepared by the method described by Mamode *et al.* (1983a). In brief, after collection, testes of mice were immediately placed into the tissue culture medium E 199 (USOL Prague, Czech Republic), supplemented with 2 % bovine serum (Bioveta, Ivanovice na Hané, Czech Republic), pH was adjusted to 7.2 with 7% sodium bicarbonate. Testes were decapsulated and digested with 0.062% collagenase (USOL Prague, Czech Republic) dissolved in the culture medium (5 ml per 10 testes) in a water-bath shaker at 34°C. After a 5 min incubation, the collagenase solution containing dispersed cells was transferred into test tubes and diluted with collagenase-free medium 1 : 1. Equal volume of collagenase solution in medium was added to the tissue. The digestion with collagenase was run three times and afterwards all suspensions were mixed. The rest of the tissue was washed with the collagenase-free fresh culture medium in petri dishes, agitated in a shaker for 5 min and filtered through a 4fold gauze net. In the filtrate additional cells were gained. Cell suspensions obtained during all the described steps of digestion and filtration were harvested by centrifugation at 140 G and 4°C for 10 min. The cell pellets were resuspended in culture medium and cell concentration was determined in a haemocytometer.

### Leydig cell stimulation with gonadotrophin *in vitro*

Aliquots of cell suspensions with the density $1 \times 10^6$/ml were incubated in a water-bath shaker for 3 h at 34°C in atmosphere of 95% $O_2$ and 5% $CO_2$ with or without gonadotrophin (Sergon, Bioveta, Ivanovice na Hané, Czech Republic). Concentrations of the stimulator were as follows: 7.8; 31; 62.5; 125; 250 I.U./l.

After incubation, the cell suspensions were centrifuged at 1 600 G and supernatants kept at –20°C until testosterone analysis.

### Analysis of testosterone, triodothyronine ($T_3$), thyroxine ($T_4$) and corticosterone

Testosterone levels in blood sera and supernatants were determined by radioimmunoassay (RIA) described

by Kozdera *et al.* (1993), $T_3$ and $T_4$ by a commercial radioimmunoassay kit (Humalab, Košice, Slovak Republic) and analysed by a RIA programme (Veterinary Research Institute, Brno, 1991). Corticosterone levels were determined by a RIA method (Hampl *et al.*, 1987) in the Institute for Endocrinology in Prague.

### Statistical analysis

The main statistical characteristics were evaluated by the programme STAT Plus v.1.10. (Matoušková *et al.*, 1993). In experiments 1 and 2, the Student's *t*-test and the Scheffe's test, respectively, were used to assess the significance of the differences between experimental and control groups of animals.

## RESULTS

### Experiment 1

As Figure 1 shows, serum testosterone and corticosterone levels in the stressed control group (1b) decreased by 13.00% and increased by 32.85%, respectively, in comparison with the non-stressed control group (1a). $T_3$ and $T_4$ levels in the stressed control group were reduced (Figure 1). In the stressed group, reduced basal and

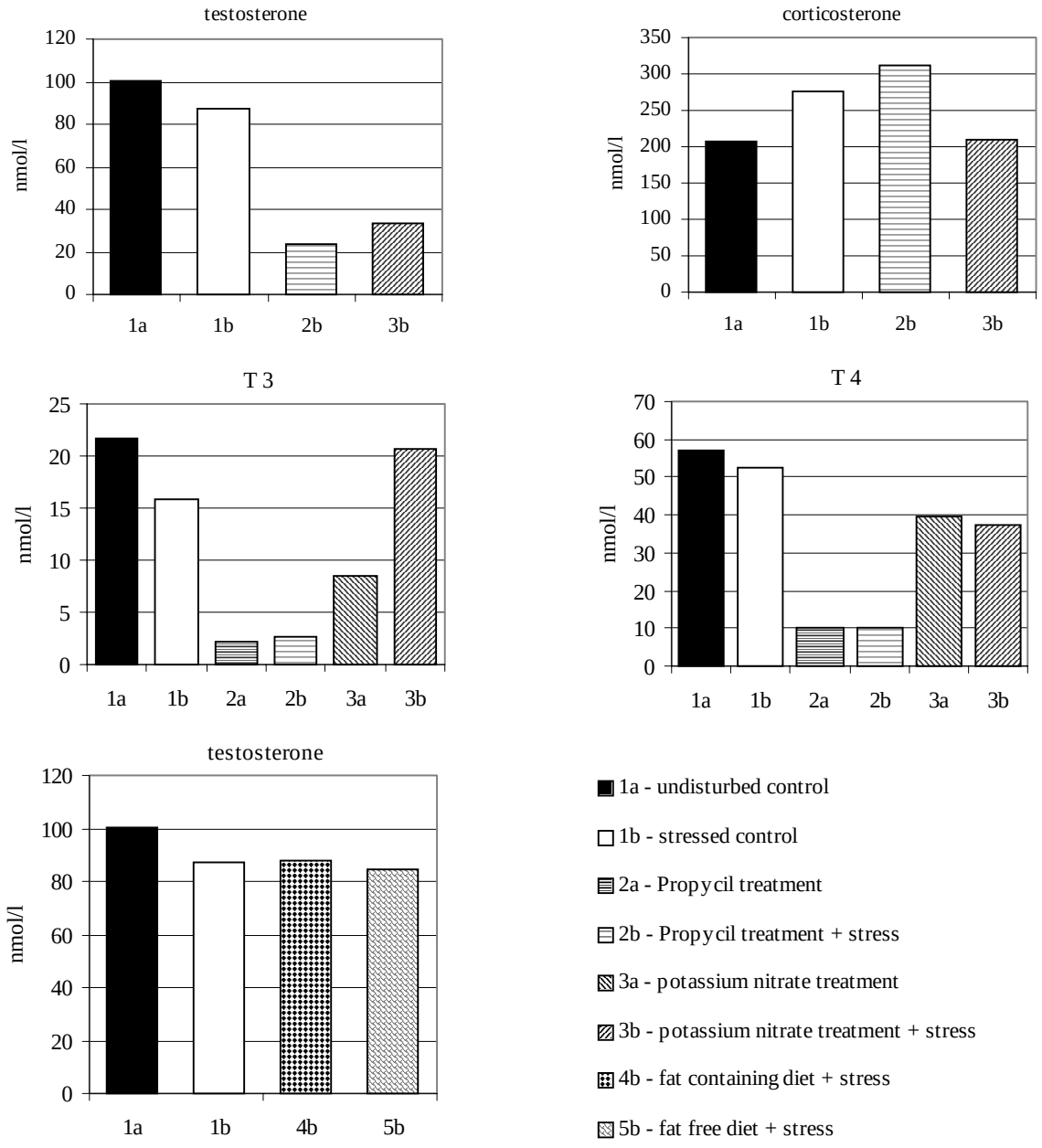



Figure 1. Serum testosterone, corticosterone, $T_3$ and $T_4$ levels in male mice

most of the Sergon stimulated *in vitro* Leydig cell testosterone production values were found in comparison with the non-stressed control (Figure 2). However, the differences were insignificant. The highest stimulation in the undisturbed control group did not increase the testosterone production in comparison with the stressed group.

$T_3$ and $T_4$ serum levels in groups given Propycil (2a, 2b) proved hypothyreosis. Reduced levels of $T_3$ and $T_4$ (Figure 1) were found in both these groups in comparison with the non-stressesed group (1a). In the Propycil-treated stressed group (2b), the decline in $T_4$ level was highly statistically significant ($P < 0.01$). Serum testosterone and corticosterone levels in mice treated with Propycil (2a) were not altered. However, in the group of stressed mice treated with Propycil (2b), the testosterone and corticosterone levels were decreased by 76.46% and increased by 49.76%, respectively, in comparison with the non-stressed group (Figure 1). The lowest Leydig cell responsivity to gonadotrophin stimulation was found in the stressed group treated with Propycil. This difference was highly statistically significant ($P < 0.01$) as in basal as in stimulated production with all the Sergon concentrations. Leydig cell testosterone production in the non-stressed mice treated with Propycil was also highly significantly decreased ($P < 0.01$) as in basal as in stimulated production by all the Sergon concentrations in comparison with the undisturbed control group (Figure 2).

In the stressed group of males treated with $KNO_3$ (3b), serum testosterone and corticosterone levels decreased by 66.59% and increased by 1.45%, respectively, in comparison with the non-stressed control group. Serum levels of the two hormones in the non-stressed $KNO_3$ treated group (3a) were not altered. Serum $T_3$ and $T_4$ levels in both the $KNO_3$ treated groups in comparison with the non-stressed control group were reduced (Figure 1). However, the differences were statistically insignificant. Basal and stimulated Leydig cell testosterone production *in vitro* in groups 3a and 3b were highly significantly decreased ($P < 0.01$) in comparison with the undisturbed control (Figure 3).

In groups of stressed mice fed with either of the synthetic diets (4b and 5b), the serum testosterone levels were reduced by 12.81% and 15.91%, respectively, in comparison with the non-stressed control group (Figure 1). A highly significant ($P < 0.01$) decline in basal Leydig cell testosterone production was found in the group of stressed mice fed with the fat-free diet in comparison with the non-stressed control group. In the other groups, no significant changes of basal values were found. Among the respective groups, significant differences were not revealed in most stimulation levels, although in the group fed with the fat-free diet (5a), *in vitro* testosterone production stimulated with the two highest Sergon concentrations used, reached higher levels than in the undisturbed control animals. In the group fed with the fat containing diet (4a), the testosterone production stimulated with the highest concentration of Sergon was higher than that in the non-stressed control group (Figure 4).

Body weight changes during the experiment are documented in Figure 5. In most groups exposed to stress, the body weight insignificantly decreased during the experimental period. However, undisturbed control mice, the group of non-stressed mice given nitrate and both the groups fed the synthetic fat-containing diet gained the weight during the experimental period.

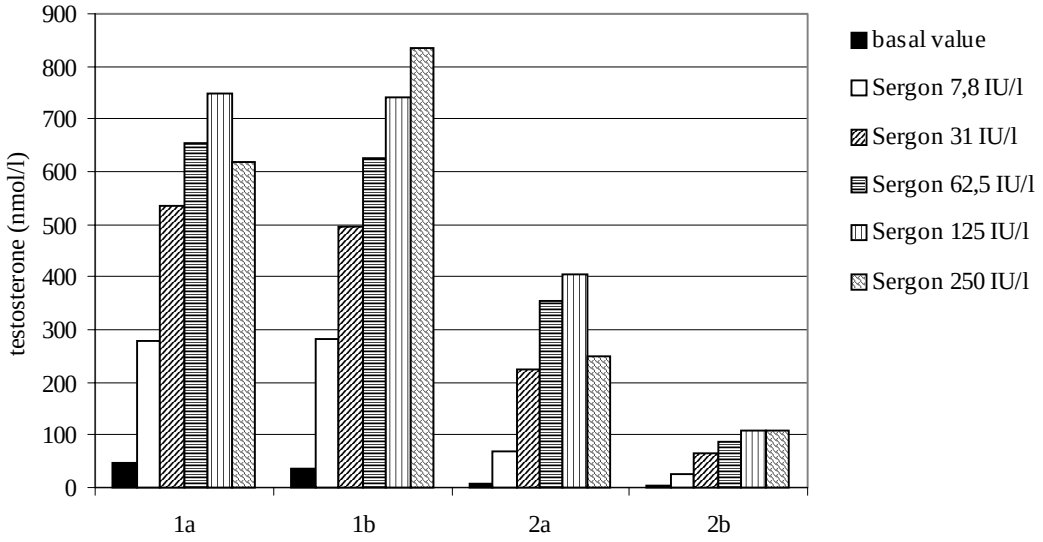



Figure 2. Effect of Propycil treatment on mouse Leydig cell testosterone production *in vitro*

1a – undisturbed control, 1b – stressed control, 2a – Propycil treatment, 2b – Propycil treatment + stress

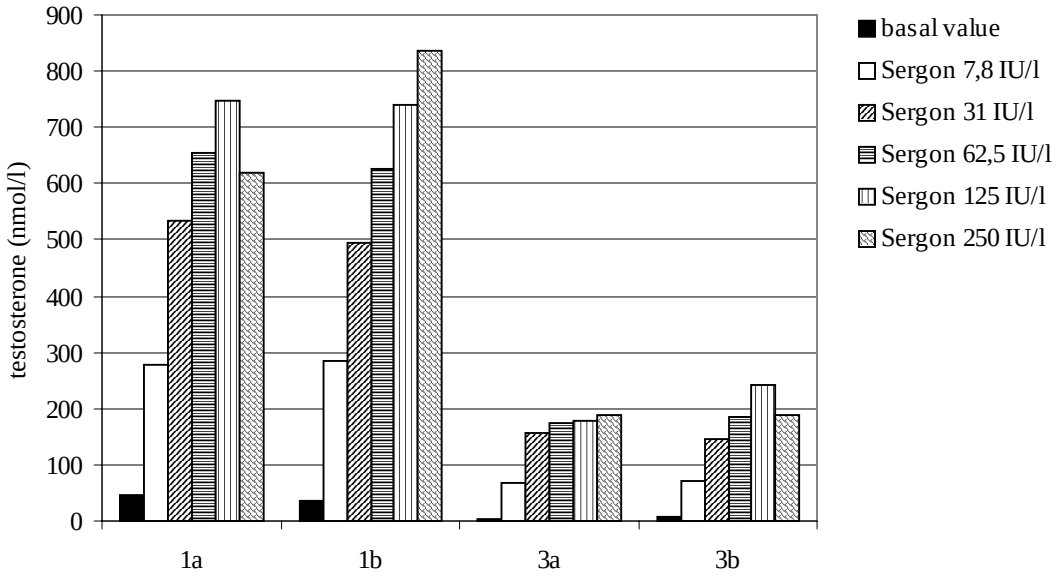



Figure 3. Effect of potassium nitrate treatment on mouse Leydig cell testosterone production *in vitro*

1a – undisturbed control, 1b – stressed control, 3a – potassium nitrate treatment, 3b – potassium nitrate treatment + stress

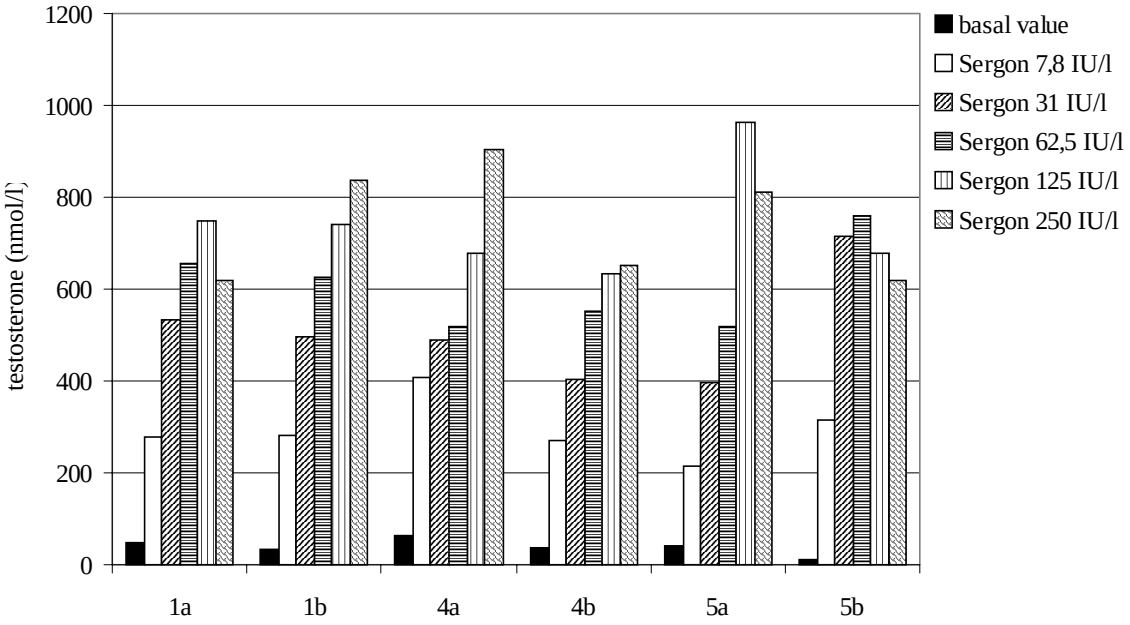



Figure 4. Testosterone production by Leydig cells of mice fed synthetic diets (*in vitro* assay)

1a – undisturbed control, 1b – stressed control, 4a – synthetic diet with fat, 4b – synthetic diet with fat + stress, 5a – fat-free synthetic diet, 5b – fat-free synthetic diet + stress

**Experiment 2**

Statistical analysis revealed insignificant differences of serum testosterone levels among the four groups (Figure 6). However, differences of serum corticosterone levels were highly significant ($P < 0.01$). The Scheffe's test revealed significant ($P < 0.05$) differences between respective groups in basal Leydig cell testosterone production *in vitro,* and highly significant ($P < 0.01$) differences in testosterone production stimulated with all the concentrations of Sergon used. The capability of Leydig cells of singly-caged males to produce testosterone was lower than that of mice caged in groups and the *in vitro* testosterone production was lower in isolated aggressive than timid animals (Figure 7).

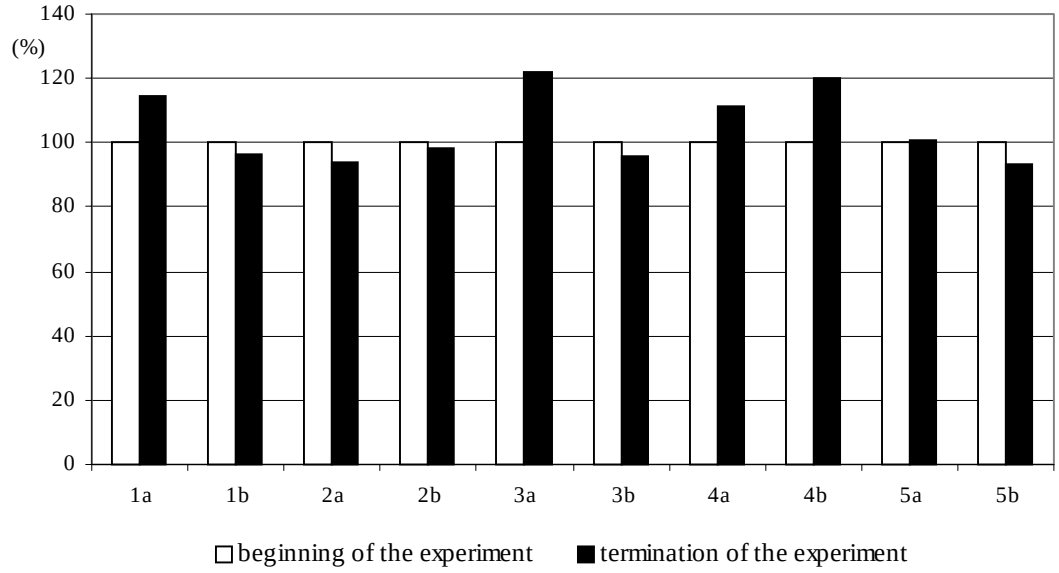



Figure 5. Body weight changes in mice during the experiment

1a – undisturbed control, 1b – stressed control, 2a – Propycil treatment, 2b – Propycil treatment + stress, 3a – potassium nitrate treatment, 3b – potassium nitrate treatment + stress, 4a – fat containing diet, 4b – fat containing diet + stress, 5a – fat-free diet, 5b – fat-free diet + stress

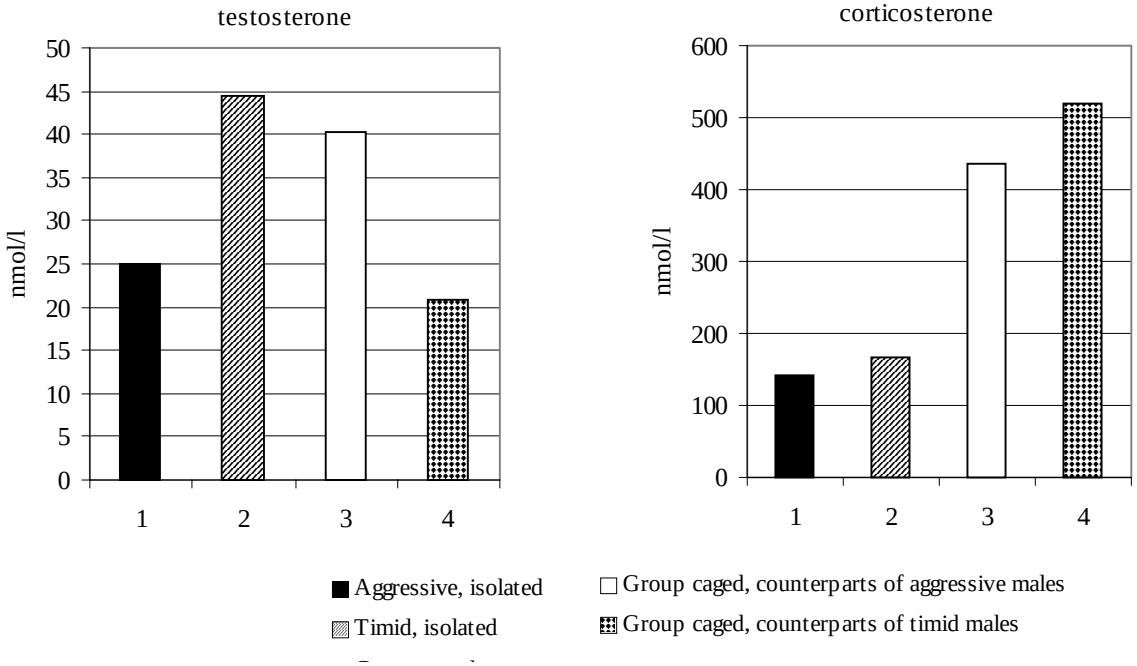
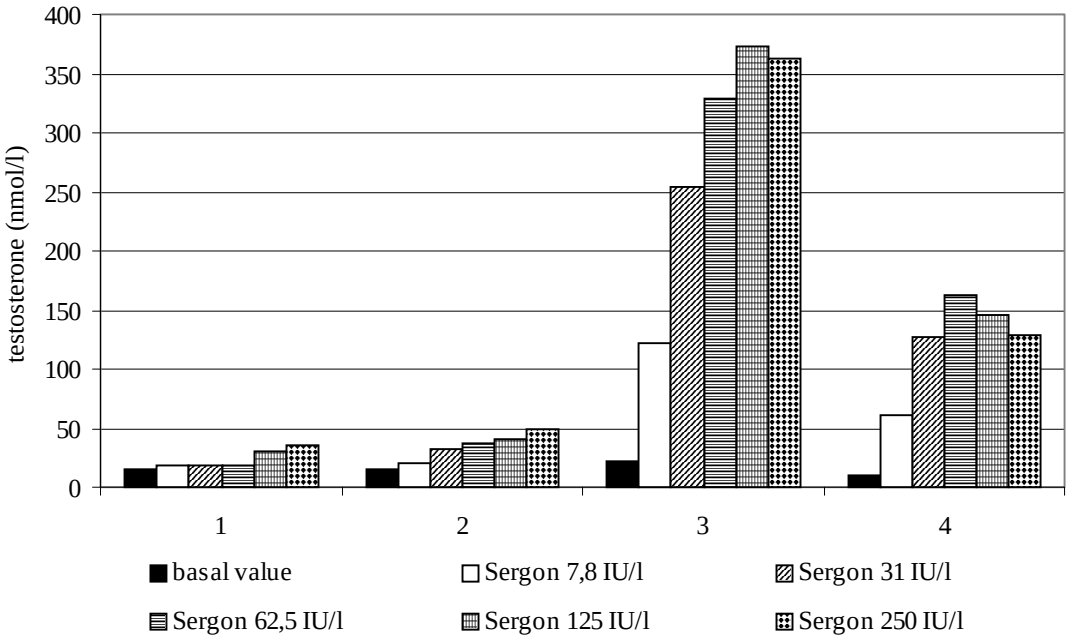



Figure 6. Serum testosterone and corticosterone levels in male mice (Experiment 2)

## DISCUSSION

It is likely that hyperactivity of the hypothalamic-adrenal axis is involved in mediating the effect of stress on the testes (Srivastava *et al.*, 1993) and increased glucocorticoid levels are associated with reduction of testosterone biosynthesis by Leydig cells (Orr *et al.*, 1994) which are the primary site of glucocorticoid binding in the testis (Hardy and Ganjam, 1997). In our study, the serum testosterone levels were decreased by 76.46%; 66.59% and 13.00% in stressed mice treated with Propycil, potassium nitrate and control group exposed to stress, respectively, in comparison with non-disturbed controls. These findings are in accordance with the re-




7. Effect of a long-time isolation on mice Leydig cell testosterone production *in vitro*

1 – aggressive, isolated, 2 – timid, isolated, 3 – group caged, counterparts of aggressive males, 4 – group caged, counterparts of timid males

sults obtained by Orr and Mann (1992) and Srivastava *et al.* (1993) who proved reduced plasma testosterone levels after immobilisation stress by 24 and 55%, respectively. In the study by Věžník (1998) serum basal testosterone levels in stressed bulls were decreased by 62% in comparison with controls. Twenty-four hours after the last immobilisation, testosterone levels were the same as in control animals, although the percentages of apoptotic cells in the stressed group were significantly higher than in controls (Yazawa *et al.*, 1999). Therefore it can be assumed that the levels of these hormones may be affected by the interval between the last stressor exerted and blood collection. Serum corticosterone levels in stressed mice in our experiments were increased by 49.76; 1.45 and 32.85% in animals treated with Propycil, potassium nitrate and controls exposed to stress, respectively, in comparison with non-disturbed controls which is consistent with the findings by Srivastava *et. al*. (1993) and Ottenweller *et al.* (1992) who proved increased serum corticosterone levels after stress exerted. The impact of stress and Propycil or potassium nitrate on hormone levels was higher than the effect of the stress alone. Saxena *et al*. (1990) observed marked pathological changes in the rat testis after combined treatment with lead and stress in comparison with either alone. We can conclude that stress exerted to an adversely affected organism is more profound, followed by a reduction in testosterone production, in comparison with the impact of stress exerted to organisms which are expected to be in a good physiological state.

The *in vitro* stimulation of testicular cells with gonadotrophin in this study resulted in an increased testosterone production as in mice in the study by Mamode *et al.* (1983b) as in bulls in the study by Faldíková *et al.* (1990). Leydig cell sensitivity to gonadotrophins decreases in stressed organisms which is in accordance with Charpenet *et al.* (1981) who found stress-induced decreased basal testosterone production and Orr and Mann (1992) who proved reduced testosterone production by hCG stimulated Leydig cell suspensions. In our study, a decreased basal *in vitro* Leydig cell testosterone production in mice exposed to stress alone was found in comparison with the undisturbed control; in most gonadotrophin concentrations, the testosterone production was non-significantly reduced, which is consistent with the findings by Maric *et al.* (1996). Leydig cells obtained from the undisturbed control were not able to produce more testosterone when stimulated by the highest Sergon concentration used, and moreover, the production was slightly decreased. The decline might have been caused by the depletion of Leydig cell receptors in this group of mice. Similar results were described by Faldíková *et al.* (1990). When the results obtained from all the groups studied are considered, it can be concluded that the ability of Leydig cells to produce testosterone after stimulation *in vitro* was significantly reduced as in mice treated by Propycil or potassium nitrate alone, as in combination with stress. The most marked effect was observed in the group of mice exposed to Propycil and stress. Serum

corticosterone levels were highest in this group and we can assume that the Leydig cell function was inhibited by that hormone. The testosterone production *in vitro* was highly significantly decreased in groups given potassium nitrate which is consistent with the results in the study by Zralý *et al.* (1997) who found suppressed testicular responsivity to gonadotrophin-releasing hormone in bulls given potassium nitrate. As treatment with Propycil as with potassium nitrate resulted in decreased $T_3$ and $T_4$ serum levels, which is also consistent with the results obtained by Zralý *et al.* (1997) who proved a marked decline in tyroxine levels in bulls after feeding potassium nitrate. It demonstrates impaired thyroid function during the treatment.

Leydig cells obtained from stressed male mice fed different synthetic diets showed decreased *in vitro* responsivity to gonadotrophin in comparison with unstressed animals given different diets. These results show a more profound effect of stress combined with the need of adaptation of the animals to changed diets.

Chronic stress caused the body weight decrease in the stressed control group of mice, in groups of animals treated with Propycil or nitrate, and mice given the fat-free diet. However, in stressed mice fed the fat-containing synthetic diet, that effect was not observed. It might be explained by the fact that the synthetic diet contained soybean oil which can promote a transient hyperphagia (Kamara *et al.*, 1998) and consequently, the increased fat intake may reduce the stress or increase the resistance to stress (Mlekusch *et al.*, 1998).

In the study of the effect of male mice behaviour on testosterone production *in vitro*, the lowest responsivity to gonadotrophin was found in isolated aggressive males. *In vitro* testosterone production by Leydig cells of singly housed timid mice was lower in comparison with males housed in groups, although it was higher than in singly housed aggressive mice. Decreased testosterone production in singly housed mice might have been induced by isolated housing as it can be a stressful factor which can produce Leydig cell atrophy (Nyska *et al.*, 1998). In the study by Šulcová (1981) the isolated aggressive mice had heavier adrenals and significantly more marked involutions of thymi in comparison with timid animals. It was expressed more in the aggressive than the timid mice which is consistent with our findings of the lowest Sergon stimulated *in vitro* responsivity of Leydig cells obtained from singly-caged aggressive mice. However, the serum corticosterone levels in isolated male mice in our study were not increased. The adrenals might have likely been exhausted by stress of isolation exerted for 6 weeks and they could not produce increased amounts of corticosterone any more. We can also assume that the stress induced by isolation of that duration might have been the reason of lower testosterone levels which is consistent with the *in vitro* production. As Šulcová (1981) suggests a correlation between the body-built and the agonistic behaviour, it might be interesting to study the relationships between these characteristics.

We can conclude that the effects of nutritional or social factors and chronic stress on Leydig cell testosterone production *in vitro* were proved. These results contributed to the knowledge of multifunctional adverse effects of combined stress on male reproduction.

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