UC 577,1; 61 ISSN 0354-3447

Jugoslov. Med. Biohem. 22: 11–17, 2003

Originalni naučni rad
Original paper

EFFECT OF ACUTE STRESS ON CIRCULATORY LEVELS OF ADRENOCORTICOTROPIC HORMONE IN RATS LOCALLY HEAD-IRRADIATED WITH X-RAYS

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Summary: In order to study the effects of ionizing radiation on the pituitary gland before and after an acute stress attack, rats aged 8 days were locally head-irradiated with a single dose of 9.6 Gy of X-rays. One group of animals was irradiated under hypothermia (rectal temp. 5.96 °C). At the age of 46 days, the animals were sacrificed and circulatory adrenocorticotropic hormone levels were measured by the RIA method. Basal ACTH levels were lower in the irradiated animals when compared to the non-irradiated controls ($p<0.05$). After an acute stress attack, the irradiated animals showed significantly lower ACTH levels compared to the non-irradiated controls ($p<0.001$). Animals subjected to hypothermia during irradiation, after an acute stress attack, showed significantly higher ACTH levels in comparison to animals irradiated under normal body temperature ($p<0.001$). It may be concluded that a dose of 9.6 Gy of X-rays reduces the post-stress response of the pituitary gland and that hypothermia effectively protects the pituitary gland when ionizing radiation is concerned.

Key words: X-rays, hypothermia, ACTH, rats

Introduction

When the head region of humans and mammals is subjected to ionizing irradiation, unwanted side-effects may occur such as damage to salivary glands (1, 2), adverse effects on the developing dentition, especially in young children (3) as well as in mammals (4). The pituitary gland is often in the field of radiation exposure which may as a consequence be also damaged (5). Mosier and Jansons (6) showed that in the rats whose heads were exposed to X-rays, had significantly lower pituitary masses than the non-irradiated controls. A similar loss of pituitary mass was achieved by exposing the head region of 8-days-old rats to doses between 3.84 Gy and 7.68 Gy (7). Local irradiation of the head region of 8-days-old female rats with doses of 7.68 Gy and 6.72 Gy resulted in a significant delay in the opening of the vaginal membrane which precedes puberty in the female rat (8).

In order to minimize the unwanted effects of ionizing radiation, numerous substances have been tested as radioprotectors. Beside various chemicals and hormones, hypothermia has been shown to be a very efficient radioprotector. Smith and Grenan (9) were among the first to demonstrate the effectiveness of hypothermia as a radioprotector in mammals. They showed that marmots irradiated with a dose of 6.50 Gy of X-rays during the state of hibernation (rectal temperature 4.40 °C) lived significantly longer than animals irradiated under normal body temperature. Exposure of pregnant rats at day 13 of gestation to X-rays while in a state of hypothermia (doses of 0.025, 0.04 and 0.05 C/Kg, rectal temperature 16 18 °C), resulted in a high survival rate of their offspring with a significant reduction of teratogenic malformations and all adult females remained fertile (10). Hypothermia has shown to be an efficient radioprotector not only in the case of tooth development after the exposure of rats to X-rays (11,12), but also in preserving the trace element homeostasis in bone tissue (13).

Considering the fact that the pituitary gland is sensitive to ionizing radiation, the objective of this study was to investigate effects of ionizing radiation on the pituitary gland by monitoring the basal and stress levels of adrenocorticotropic hormone (ACTH) in rats

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exposed to X-rays. In addition, as hypothermia is an efficient radioprotector for a variety of mammalian tissues and organs, the aim of this report was also to analyze the efficiency of hypothermia as a radioprotector in the case of the pituitary gland after its exposure to ionizing radiation.

Materials and Methods

Experimental animals

In our experiments, we used male rats of a Wistar strain bred at the »Vinča« Institute. The animals were kept under standard conditions: room temperature 22.0 ± 2.0 °C, lights on from 7:00 to 19:00 hours. The animals were fed standard rat pellets and had access to drinking water *ad libitum* after having been weaned by the mother at the age of 30 days.

At the age of 8 days (the day of birth taken as day 0), male pups were divided into the following four groups: i) normal controls, ii) normal controls to be subjected to hypothermia, iii) animals to be irradiated with a single dose of X-rays and iv) animals to be irradiated with a single dose of of X-rays under hypothermia. Prior to commencement of the experiments and after, the pups were kept 5 7 per litter. After having been weaned at the age of 30 days, the young rats were separated from their nursing mothers and kept 5 to 7 animals per cage, until sacrificed at the age of 46 days.

Irradiation procedures

The heads of 8-day-old male rats were exposed to a single dose of X-rays from a »Philips« set under the following conditions: 200 Kv, 16 mA, filter-Cu 0.5 mm, FSD-34 cm, dose rate 0.7135 Gy/min and room temp. 18.0 °C. The bodies of the animals were protected by a »U« shaped lead shield 5 mm thick, anteriorly reaching just behind the ears. The second group of animals was irradiated under the same conditions as above, with the difference that, prior to the start of the radiation procedure, their rectal temperature was reduced to an average of 5.96 ± 0.46 °C (n= 10) as measured by a medical »Ellab« (Coppenhagen) electronic thermometer with a medical probe made of Cu/CuNi alloy inserted into the rectum. The state of hypothermia in these animals was achieved by confining the animals in glass jars (250 mL) with a glass stopper and placing them in a refrigerator at 4 °C until the desired rectal temperature was achieved, within 25 30 min. This procedure is similar to that described by Andjus (14). As it takes 13.52 minutes of radiation exposure to achieve the dose of 9.6 Gy with our machine, the pups spontaneously warm their bodies from the surrounding, so that after 10 minutes the rectal temperature is 10.15 ± 0.47 °C (n= 10) and after 15 minutes, the rectal temperature is 12.90 ± 0.45^{3}°C (n=10).

The third group of animals was subjected only to hypothermia and the fourth group served as normal intact controls. As the animals that were irradiated were immobilized during the irradiation procedure with medical plaster tape, so were the two non-irradiated groups immobilized for the time the irradiation procedure lasted.

Adrenocorticotropic hormone assay

At the age of 46 days, all animals were sacrificed between 9:00 and 10:00 hrs by decapitation with a small animal guillotine. From each of the four animal groups, part of the animals were sacrificed without inflicting an acute stress, while the remaining animals from each group were 5 minutes prior to sacrifice subjected to an acute stress. Stress was executed by a subcutaneous injection of 1mL/100g saline into the hind leg. The whole manipulative procedure lasted 15 20 s (15). In order to avoid stress, all animals had their body masses measured the previous day.

After sacrifice, the pituitary gland was removed from each animal and weighed on an analytical measuring scale. Trunk blood was collected in cold polystyrene tubes using a teflon funnel, the tubes containing 1 mg EDTA per 1 mL blood. The samples were placed on ice until centrifuged at 4 °C for 20 min at 3000 g. Plasma was separated into cold polystyrene tubes and 250 IU/mL of trasylol was added into each tube. Plasma was stored at 18 °C until assayed for ACTH. A RIA kit (CIS-France) was used for estimating plasma ACTH in 100 μL of unextracted plasma as previously described (16). Briefly, this radioimmunoassay system uses ^{125}I as tracer, hormone used for labeling being a synthetic human ACTH 1-39. The ACTH antiserum was raised in rabbit by injection of ACTH 1-24 coupled to bovine albumin. The minimal detectable level of hormone for this assay system is 20 pg/mL. The non-specific binding was 2.3%, and specific binding 24.1%. The intra-assay and inter-assay coefficient of variation as determined by the analysis of replicate plasma pool samples were 5.9% (n=8) and 8.9% (n=8), respectively.

Statistical analysis

The data are expressed as the mean ± S.E.M. (standard error of the mean) and were analyzed for statistical significance by the Student's t-test. One-way ANOVA was also used for statistical analysis of multiple means for hormone concentrations. The level of statistical significance was set at $p < 0.05$.

Results

Body mass

The normal control animals showed significantly higher body mass (138.19 ± 4.43 g, n=18) when compared to the animals irradiated with 9.6 Gy (99.29

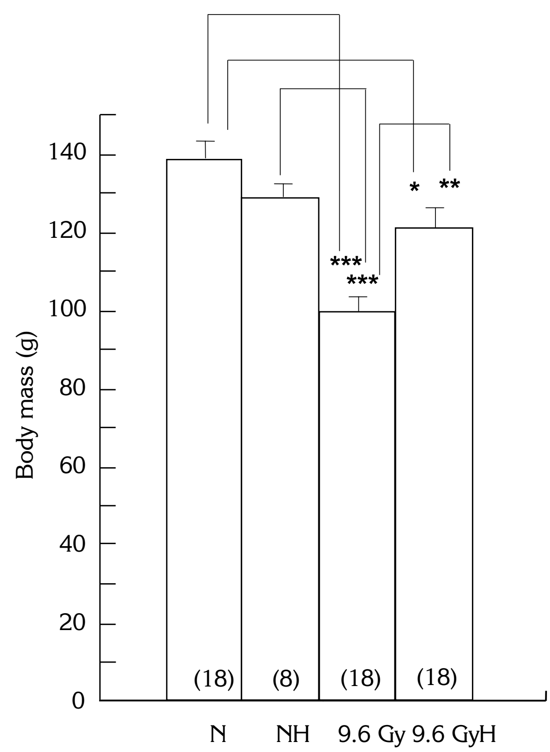

Figure 1. Body mass (g) of rats exposed to X-rays and non-irradiated rats.
Values expressed as mean ± s.e.m; N-normal controls; NH-normal controls subjected to hypothermia 9.6 Gy-rats with head region irradiated with a dose of 9.6 Gy of X-rays. 9.6 GyH-rats with head region irradiated with a dose of 9.6 Gy of X-rays and subjected to hypothermia No of animals ( ); Level of statistical significance: * p<0.02, ** p<0.01, *** p< 0.001.

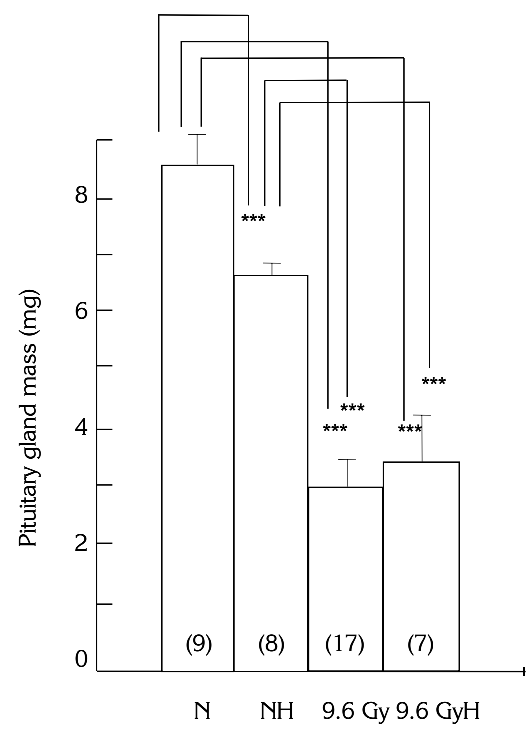

Figure 2. Pituitary gland mass (mg) of rats exposed to X-rays and non-irradiated rats.
Values expressed as mean ± s.e.m; N-normal controls; NH-normal controls subjected to hypothermia; 9.6Gy-rats with head region irradiated with a dose of 9.6 Gy of X-rays; 9.6 GyH-rats with head region irradiated with a dose of 9.6Gy of X-rays and subjected to hypothermia; No of animals ( ); Level of statistical significance: *** p<0.001.

± 3.64 g, n=18, p<0.001) and the animals irradiated with 9.6 Gy under hypothermia (120.47 ± 5.12 g, n=18, p<0.02). Normal control rats subjected only to hypothermia had a significantly higher body mass (128.44 ± 3.28 g, n=8) when compared to the 9.6 Gy irradiated animals (p<0.001). Animals irradiated with 9.6 Gy under hypothermia, had a significantly higher body mass when compared to the irradiated animals not subjected to hypothermia (p<0.01) (*Figure 1).*

Pituitary gland mass

As demonstrated in *Figure 2*, it may be seen that the normal control rats had a significantly higher pituitary mass (8.47 ± 0.50 mg, n=9) when compared to the pituitary mass of the controls subjected only to hypothermia (6.63 ± 0.19 mg, n=8, p<0.001), irradiated animals (3.09 ± 0.45 mg, n=17, p<0.001) and irradiated animals subjected to hypothermia (3.51 ± 0.77 mg, n=7, p<0.001). Rats subjected only to hypothermia showed a significantly higher pituitary mass when compared to both irradiated groups (p<0.001). Comparing the two irradiated groups of which one was subjected to hypothermia during X-ray irradiation, no significant difference in the masses of the pituitary were detected (p>0.1).

Concentrations of circulatory plasma ACTH

Basal concentrations of plasma ACTH showed minor differences between the groups (ANOVA, $F_{3,51}$= 2.003417, p>0.05). The basal level of ACTH in normal controls (50.02 ± 3.45 pg/mL, n=21) was significantly higher when compared to the ACTH basal levels of rats irradiated with 9.6 Gy of X-rays (37.93 ± 5.71 pg/mL, n= 13, p<0.05), see *Figure 3.* No significant differences in basal ACTH levels were detected between the normal controls subjected to hypother-

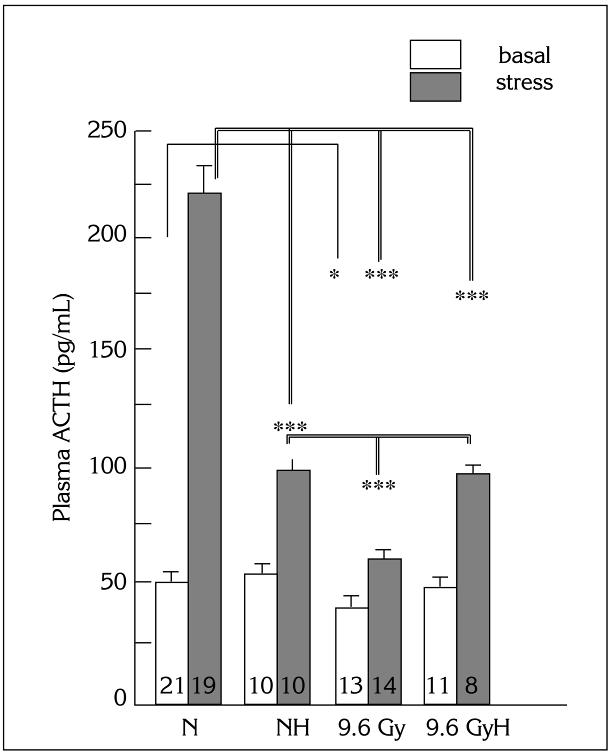

Figure 3. Basal and stress values of circulatory plasma ACTH concentrations (pg/mL) of rats exposed to X-rays and non-irradiated rats.
Values expressed as mean ± s.e.m; N-normal controls NH-normal controls subjected to hypothermia. 9.6 Gy-rats with head region irradiated with a dose of 9.6 Gy of X-rays 9.6 GyH-rats with head region irradiated with a dose of 9.6 Gy of X-rays and subjected to hypothermia; No of animals ( ); Level of statistical significance:* $p<0.05$, ***$p<0.001$.

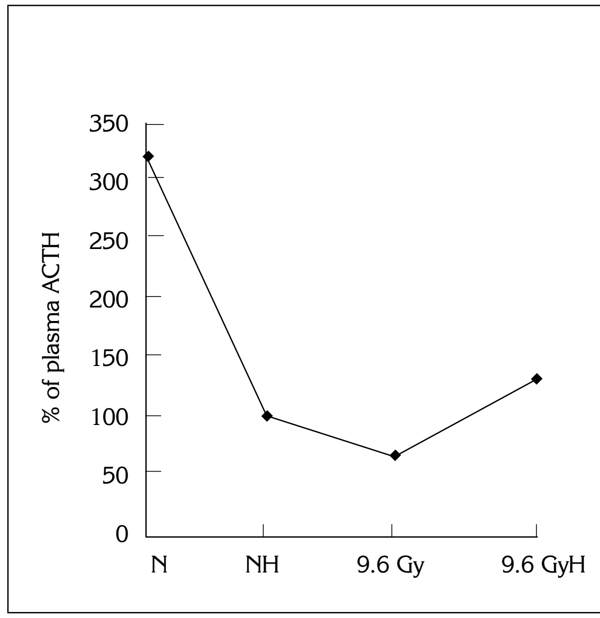

Figure 4. Percentage of plasma ACTH increase after an acute stress compared to basal levels.
N-normal controls; NH-normal controls subjected to hypothermia. 9.6 Gy-rats with head region irradiated with a dose of 9.6Gy of X-rays; 9.6 GyH-rats with head region irradiated with a dose of 9.6 Gy of X-rays and subjected to hypothermia.

mia (54.02 ± 6.45 pg/mL, n = 10) and the animals irradiated with 9.6 Gy of X-rays while under hypothermia (44.34 ± 4.81 pg/mL, n= 11).

In contrast to the basal ACTH levels, the patterns of ACTH plasma levels show significant differences between the animal groups as detected in the plasma 5 minutes after initiation of an acute stress (ANOVA, $F_{3,47}= 21.750351$, $p<0.0001$ (*Figure 3*). The stress response of ACTH as measured in the plasma was highest in normal controls (211.98 ± 20.27 pg/mL, n=19) when compared to the hormone levels in: normal controls subjected to hypothermia (102.23 ± 4.34 pg/mL, n=10, $p<0.001$), rats irradiated with 9.6 Gy of X-rays (63.53 ± 3.76 pg/mL, n= 14, $p<0.001$) and animals irradiated under conditions of hypothermia (97.03 ± 8.48 pg/mL, n= 8, $p<0.001$). In addition, levels of plasma ACTH after stress were significantly higher in normal control animals subjected only to hypothermia and those irradiated with 9.6 Gy of X-rays while under hypothermia when compared to the stress ACTH plasma levels from animals irradiated with 9.6 Gy ($p<0.001$).

It is of interest to indicate to the fact that when compared to the basal ACTH levels, the highest stress response of ACTH was in the case of normal controls (an increase of ACTH level by 323.92%, *Figure 4*) while the lowest increase was detected in the case of animals irradiated with 9.6 Gy of X-rays, 67.41%. Control animals subjected to hypothermia had a 89.25% post-stress rise of ACTH and the irradiated animals subjected to hypothermia a 118.83% increase of ACTH level after stress.

Discussion

In the present report, we confirmed the previous findings that in our strain of Wistar rats, the acute stress response of ACTH in normal control rats is highest 5 min after an initial acute stress attack induced by a single saline injection (15). The basal levels of circulatory ACTH did not differ significantly between all the groups of animals in our experiment, a slight difference being detected only between the normal control rats and those animals irradiated with 9.6 Gy of X-rays ($p<0.05$). This may be explained by the fact that in rats under other altered physiological conditions; basal levels of circulatory ACTH may not always show significant differences when compared to corresponding control animals, as for instance in pinealectomized rats (17). On the other hand, basal values of ACTH may differ to a certain extent in rats whose bodies are exposed to 4.2 Gy of gamma rays from a ^{60}Co source when compared to corresponding non-irradiated controls (18).

The significantly lower stress levels of circulatory ACTH between the normal controls and rats irradiated with X-rays as in our case, may be explained by the direct effect of ionizing radiation on the pituitary gland. Pantić and Hristić (19) demonstrated that a single dose of 11.52 Gy of X-rays applied to the head region of male adult rats resulted in decreased amounts of granular endoplasmatic reticulum and regression of Golgi bodies in the adrenocorticotropic cells, implying decreased protein synthesis. This may also explain the highest percentage of post-stress response of ACTH in the normal controls as compared to their basal ACTH levels (323.92%). The reduced circulatory ACTH post-stress levels in our irradiated animals may be attributed also to the effect of ionizing radiation on lipopolysaccharides (LPS) for which it is known to raise the concentrations of ACTH after stress. Ionizing radiation disintegrates that part of the LPS molecule complex, which is responsible for toxicity including an enhanced production of cytokines, which trigger the hypothalamo-pituitary-adrenal axis (20).

The significantly higher post-stress level of ACTH and relatively high increase compared to basal levels (118.83%) in animals irradiated with 9.6 Gy of X-rays under hypothermia as compared to the post-stress circulatory levels of animals irradiated with 9.6 Gy, may be attributed to the protective effect of hypothermia, which has been shown to be a protective agent for a wide variety of tissues when ionizing radiation is considered (9 13). Hypothermia exerts its protective effect by reducing the O2 tension in the body tissues as a result of a reduction of the breathing rate of animals subjected to deep hypothermia (10, 21). Cooling tissues to between 5 °C and 15 °C, oxygen consumption is reduced to 1 and 15% respectively when compared to oxygen consumption in normal tissue (22). The lowered amount of O2 in the tissues has as a consequence a lower rate of free radical and hydrogen peroxide formation which are known to be the key mechanism responsible for producing oxidative damage by ionizing radiation resulting in pathophysiological event (23).

It is more complex to explain the fact that our control animals subjected only to hypothermia showed a significantly lower post-stress circulatory ACTH level when compared to the normal controls. The rectal temperature of 5.96 °C should not have harmed the pituitary gland, as it has been reported that tissues in the rat are damaged below 2.2[3]°C (24). It should be also noted that the mass of the pituitary gland relative to body mass in the cooled animals is 0.0052%, while in the normal controls this value is 0.0061%. An explanation to these differences between the two control groups in circulatory ACTH concentrations and pituitary gland mass may be the stress of hypothermia itself to which one group of the control animals was subjected at the age of 8 days. There are reports that an early environment can contribute substantially to the development of stable individual differences in the hypothalamic-pituitary-adrenal axis (25). It is suggested that rudimentary, adaptive responses to stress could be modified by environmental events, in our case, hypothermia. The environmental effects influence the differentiation of neurons, which are ultimately involved in the negative feedback regulation of the hypothalamic-pituitary-adrenal axis, most notably, changes in glucocorticoid receptor gene expression in the hippocampus and frontal cortex.

One should bear in mind that in our irradiation procedures, the pineal gland was also in the field of radiation and that the pineal gland is also sensitive to ionizing radiation, which reduces basal melatonin synthesis in the rat pineal glands (26). As animals which have been pinealectomized, after an acute stress attack show a far greater post stress rise of circulatory ACTH levels than control animals (15, 17), one would expect a far greater ACTH stress response in our irradiated animals. In our case, it may be postulated that the pineal glands remained active and were not affected by irradiation or, this gland recovered by the time the rats were subjected to acute stress (38 days after irradiation exposure) and succeeded in exerting its usual inhibitory modulatory effect on ACTH release after an acute stress attack.

From our experimental results, it may be concluded that a single dose of 9.6 Gy of X-rays applied to the head region of 8-days-old rats, has a negative effect on the pituitary gland as detected by measuring the basal and stress levels of circulatory ACTH at the age of 46 days. It may also be concluded that subjecting rats to hypothermia during irradiation, the pituitary gland is significantly protected from the negative effects of ionizing radiation.

Acknowledgement. This research was supported by the Serbian Ministry for Science, Technology and Development, project No 1956. The authors thank the colleagues from the Laboratory for Radiation Protection and Environment of the »Vinča« Institute for help with dosimetry and irradiation procedures.

EFEKAT AKUTNOG STRESA NA CIRKULATORNI ADRENOKORTIKOTROPNI HORMON U PACOVA ČIJA JE GLAVA IZLOŽENA LOKALNOM OZRAČIVANJU X-ZRACIMA

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Kratak sadržaj: U cilju ispitivanja delovanja jonizujućeg zračenja na hipofiznu žlezdu pacova pre i posle akutnog stresa, 8 dana stari pacovi su podvrgnuti jednokratnom ozračivanju sa 9.6 Gy X-zraka. Jedna grupa životinja je ozračena u stanju hipotermije (rektalna temp. 5.96^{3}°C). Na 46. dan starosti, životinje su žrtvovane i nivoi adrenokortikotropnog hormona u cirkulaciji su mereni RIA metodom. Bazalni nivoi ACTH su bili niži u ozračenim životinjama u odnosu na neozračene kontrole ($p<0.05$). Nakon akutnog stresa, ozračene životinje su pokazale značajno niže vrednosti ACTH u odnosu na neozračene kontrole ($p<0.001$). Životinje podvrgnute hipotermiji tokom ozračivanja, nakon akutnog stresa su imale veći nivo ACTH u odnosu na životinje ozračene pod normalnom telesnom temperaturom ($p<0.001$). Može se zaključiti da doza od 9.6 Gy X-zraka umanjuje stresni odgovor hipofizne žlezde a hipotermija efikasno štiti hipofiznu žlezdu kada je u pitanju jonizujuće zračenje.

Ključne reči: X-zraci, hipotermija, ACTH, pacovi

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Received: January 10, 2003

Accepted: February 11, 2003