Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v34i4.10568

Effect of pain related of sex and estrous cycle on blood plasma glucose, free fatty acids and corticosterone in rats

Elzira Diniz de Moraes[1*], Cássia Thaís Bussamara Vieira Zaia[2], Dimas Augusto Morozin Zaia[3], Angelo Alexander Torres dos Santos[2], Rachel Cezar de Andrade Ribeiro[2] and Andreia Lopes da Silva[2]

*[1]Coordenadoria de Pesquisa e Pós-graduação da Faculdade Integrado, Instituto de Ensino Superior de Londrina, Av. Duque de Caxias, 1290, 86015-000, Londrina, Paraná, Brazil. [2]Laboratório de Neurociências, Fisiologia e Metabolismo, Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, Londrina, Paraná, Brazil. [3]Departamento de Química, Universidade Estadual de Londrina, Londrina, Paraná, Brazil. *Author for correspondence. E-mail: elziramor@gmail.com*

ABSTRACT. Sex differences related with pain have been studied and evidences suggesting influence of sex steroid hormones on the thresholds of pain. Experimental nociception has been test using formalin as a model of nociceptive stimulus. Association of stress, pain and metabolic and hormonal changes has not been explored. The aim of this study was to compare metabolic and hormonal changes between male rats and female rats in proestrus and estrus cycle after painful stimulus by formalin into the masseter muscle. Male and female Wistar rats (200-250 g b.w.) were submitted to an injection of formalin (F, 1.5%) or saline (S, 9.9%) into the masseter muscle and after 0 (N, control group without injection), 5, 15, 30 or 60 minutes they were decapitate and blood was collected to measure biochemical parameters. Plasma estradiol concentration (pg dL^{-1}) was significantly higher in proestrus (106.3 ± 4.3, $n = 45$, $p < 0.05$) group compared to the estrous group (89.4 ± 3.5, $n = 43$). Blood plasma concentration of glucose (mg dL^{-1}) was increased after 5 and 15 minutes of injection of formalin or saline in the animals, but in the estrus group the increase was bigger than in the others. Free fatty acids levels increased in the estrous group after 5, 15 and 30 minutes and also the corticosterone levels and these concentrations were significantly different ($p < 0.05$) from either male or female animals in proestrus state. The results obtained suggesting that estradiol is related to a sensibility to pain and the estrus stage is related to stress and the estrous cycle has a modulator effect on pain and nociceptive sensibility.

Keywords: sex differences, nociception, stress, hormones, rats.

Resposta de dor relacionada ao sexo e ciclo estral em concentrações plasmáticas de glicose, ácidos graxos livres e corticosterona em ratos

RESUMO. Estudos experimentais têm demonstrado a existência de diferenças sexuais na resposta de dor, e as evidências sugerem a influência de hormônios sexuais na experiência dolorosa. O objetivo deste estudo foi o de comparar as alterações metabólicas e hormonais entre machos e fêmeas em proestro e estro após o estímulo doloroso por formalina no músculo masseter. Ratos machos e fêmeas Wistar (peso: 200-250 g) foram submetidos a uma injeção de formalina (grupo F, 1,5%) ou salina (grupo S, 9,9%) no músculo masseter e depois de 0 (grupo N, controle sem injeção), 5, 15, 30 ou 60 minutos foram decapitados e retirou–se o sangue para dosagens bioquímicas. A concentração plasmática de estradiol (pg dL^{-1}) foi significativamente maior no proestro ($106,3 \pm 4,3$, $n = 45$, $p < 0,05$) em comparação com o grupo em estro ($89,4 \pm 3,5$, $n = 43$). A concentração sanguínea de glicose plasmática (mg dL^{-1}) aumentou após 5 e 15 minutos da injeção de formalina ou salina nos animais, mas no grupo estro o esse aumento foi maior. A concentração plasmática de ácidos graxos livres e de corticosterona demonstrou níveis elevados no grupo estro após 5, 15 e 30 minutos apresentando uma diferença significante ($p < 0,05$) em relação aos animais machos ou fêmeas em proestro. Os valores de glicose, ácidos graxos livres e corticosterona mais elevados nas fêmeas em estro sugerem que a fase do ciclo estral pode estar interferindo na resposta de estresse, podendo estar relacionada com a diminuição na concentração de estradiol.

Palavras-chave: diferenças sexuais, nocicepção, estresse, hormônios, ratos.

Introduction

Converging lines of evidence suggest that there are important sex-related influences on the experience of pain. Women report more pain than men and they are at great risk for developing many forms of chronic pain (LE RESCHE; DAO, 2000; UNRUH, 1996). Laboratory studies consistently report lower pain threshold and tolerance among women and abundant nonhuman animal research indicates sex differences in nociceptive responses

and endogenous pain modulation (BERKLEY, 1997; FILLINGIM; GEAR, 2004). This sex differences may be related to the effect of steroid hormones on the developing and adult nervous systems response to pain (CRAFT et al., 2004; KELLY et al., 2003), and also neuromodulatory roles of sex steroid hormones on the opioid system (DAWSON-BASOA; GINTZLER, 1998) can be involved.

The cutaneous formalin test is a valid and reliable model of nociception (ABBOTT et al., 1995), and nociceptive formalin stimulus has been associated to activate the pituitary-adrenocortical system in the awake rats (TAYLOR et al., 1998).

Elevation of circulating glucose levels, at a time to a marked degree, can occur during a variety of stressful situations (ZAIA et al., 1997) including nociception, and short-term ether exposure provokes stress associated with rapid and marked increase in the plasma levels of corticosterone (NASCIMENTO CURI et al., 1990).

Thus, considering sex-related influences in pain response and the effects elicited by the injection of formalin into deep tissues not yet been explored by metabolic parameters, the aim of this study is to compare metabolic and hormonal changes between males rats and females rats in proestrus and estrus cycle after painful stimulus by formalin into the masseter muscle.

Material and methods

Animals

This study was carried out using male (230-250 g) and female (200-225 g) Wistar rats, housed in standard clear plastic cages with soft bedding (five/cage), which were given free access to standard laboratory chow and tap water were available *ad libitum*. They were maintained in a temperature-controlled room (23 ± 1°C) with a 12/12h light-dark cycle (with lights on at 6:00h) for at least 1 week prior the experiments. Estrous cycle stage was determined using vaginal smears and rats were followed for at least two full cycles. Male and regularly cycling females in proestrus and estrus were decapitated after 0 minutes (control group, the N group, without any injection) 5, 15, 30 and 60 min. of injection into the masseter muscle and blood was collected to determinate blood plasma concentrations metabolites and hormones. Those animals were also subjected to formalin (F group) or saline (S Group, injection control group) pain stress and sacrificed by 5, 15, 30 and 60 min. of injection into the masseter muscle and blood was collected to determinate blood plasma concentrations metabolites and hormones. All the experiments were performed between 10:00 to 12:00h a.m. The study was conducted in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (ZIMMERMANN, 1983).

Vaginal smear

Every morning between 8:00 and 9:00 a.m. vaginal secretion was collected with a plastic pipette filled with 10 μL of normal saline (NaCl 0.9%) and one drop was collected with a clean tip from each rat. Vaginal fluid was placed on glass slides. The material was observed under a light microscope, without the use of the condenser lens, with 10 and 40 x objective lenses using the methods of Long and Evans (1922). Cellular types and the proportion among them allowed the observer to define the estrous cycle phase of the rat. A proestrus smear primarily consisted of a predominance of nucleated epithelial cells; an estrus smear primarily consisted of anucleated cornified cells; a metestrus smear consisted of the same proportion among leukocytes, cornified, and nucleated epithelial cells; and a diestrus smear primarily consisted of a predominance of leukocytes (MARCONDES et al., 2002).

Procedure for pain or stress procedure

The rats received a 50 μL injection of either saline or formalin (1.5% from 3 wt wt^{-1}, formaldehyde, diluted in 0.9% saline). Diluted formalin solutions were prepared from commercially (Sigma) available stock formalin further diluted in isotonic saline to 1.5%. The stock formalin is an aqueous solution of 37% of formaldehyde. The injection was performed through a 30-gauge needle connected to a cannula consisting of a polyethylene tube that it was also connected to a Hamilton syringe (50 μL) previously filled with formalin or saline.

Analytical determinations

Trunk blood was collected just after decapitation in heparinized test tubes and centrifuged at 3,000 rpm for 15 min. Aliquots of plasma were removed and stored at -20°C for further use. Plasma glucose was assayed by spectrophotometric method using commercially available KIT (Glicose-Enz-Color, BioDiagnóstica, PR, Brazil) based on the oxidase peroxidase method (TRINDER, 1969). Plasma corticosterone was measured by fluorimetric analysis (GUILLEMIN et al., 1958), free fatty acids (FFA) were also measured by spectrophotometric method (FALHOLT et al., 1973); and estradiol was measured by chemiluminescence's method using

commercially available KIT (Estradiol-6II, Bayer Corporation) as a gift from Análise® Produtos e Serviços para Laboratórios Ltda. (São Paulo State, Brazil).

Statistical analysis

Statistical comparisons were performed using *t*-Student test or one-way analysis of variance (ANOVA). Multiple post-hoc comparisons were performed using Student-Newman-Keuls test was used to determine statistical significance among the groups as well as among time points in the same group. Statistical significance was set at the $p < 0.05$ level. Data are presented ion the figures and text as means ± SEM.

Results

Blood plasma glucose response to stress

Five and fifteen minutes after injection, a significant increase [$F(9, 65) = 12.10$; $p < 0.001$] in blood plasma glucose was observed to male rats (group S and group F) and after 60 min. it reached normal values compared with the normal group (N). It differs from the estrous female group which a significant increase was observed in the blood plasma glucose concentration only after 5 minutes of formalin injection compared with saline group. The proestrus group was different from the estrus group 5 and 15 minutes after injection of formalin (Figure 1).

Free fat acid response to stress

The blood plasma concentration of free fat acids was increased [$F(9, 50) = 6.55$; $p < 0.001$] in the estrus group after 5, 15, and 30 minutes of formalin injection compared to the male and female proestrus groups (Figure 1). The S group did not differ from the N group during the studied period.

Corticosterone response to stress

The corticosterone concentration in blood plasma of male group increased [$F(9, 56) = 5.69$; $p < 0.001$] after 5 and 15 minutes of formalin or salin injection. In the female proestrus group a significative increase was also observed in 5 and 15 minutes after the saline or formalin injection [$F(9, 48) = 3{,}86$; $p < 0{,}001$] compared with the N group; On the other hand, the increase of this hormone was more significant [$F(9, 52) = 15.78$; $p < 0.0001$] in the estrus group than when compared to female proestrus and male rats, and this increase was still shown after 30 minutes (Figure 1).

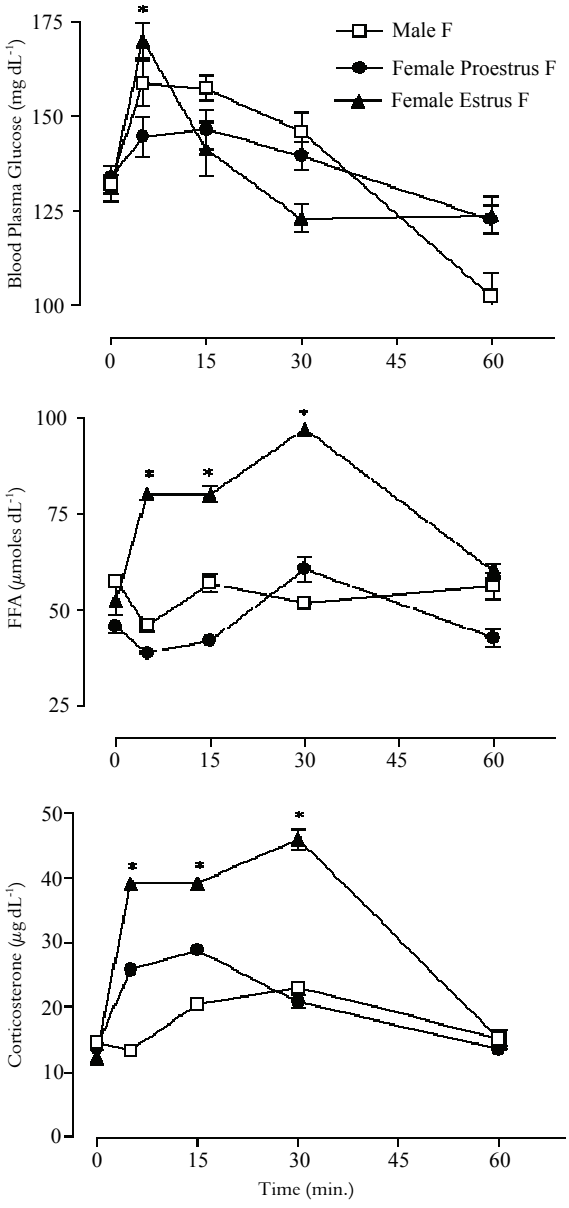

Figure 1. Temporal evolution of plasma glucose (mg dL^{-1}), free fatty acids (μg dL^{-1}) and corticosterone (μg dL^{-1}) of male and female rats in proestrus and estrus submitted to injection of formalin (F, 1.5%) in muscle. Values expressed as mean ± E.P.M. *Difference ($p < 0.05$) between estrus female group and the groups male and female proestrus, in its time.

Discussion

The prevalence of pain conditions that includes symptoms of masseter muscle pain, such as temporomandibular disorders and fibromyalgia syndrome, are significantly greater in women than they are in men (CAIRNS et al., 2001). Similar to the emerging literature documenting sex differences in pain, studies in animals also demonstrate sex differences by environmental stimulus such as pain stress (CRAFT et al., 2004). The response to stress involves the activation of both catecholaminergic

neurons of the locus ceruleus, and corticotropin releasing hormone and arginine-vassopressin neurons of the paraventricular nuclei of the hypothalamus (SÁNCHEZ et al., 2002). These general responses will lead to the activation of the efferent sympathetic-adrenomedullary system and the hypothalamic-pituitary-adrenal axis (ZAIA et al., 1997). In the present study this activation was evidenced by the analysis of plasma corticosterone and blood plasma glucose and free fatty acids (FFA).

The increase in glycemia five minutes after formalin injection for the three groups, demonstrated normal pain stress response which agreement finds to Nascimento Curi et al. (1990), Zaia et al. (1997) and Sánchez et al. (2002). The observation of increase in corticosterone and free fat acids levels after stress has been previously reported also (KOVACS et al., 1996; NASCIMENTO CURI et al., 1990). Time course of blood plasma glucose undemonstrated significant sex differences in formalin pain response among male and female groups. However the increase in free fat acids and corticosterone profile in estrous female suggest that there is a influence of sex hormones on pain perception.

Determination of estradiol in the proestrus and estrus groups showed that the concentration of this hormone is higher in all the estrous animals during the time of the experiments than in all the animals. The precise influence of gonadal steroids hormones in pain is not well understood, therefore there are possible neuromodulatory roles of sex hormone on the opioid system. Clinical studies suggest that women obtain greater pain relief from kappa-opioid analgesics than men (GEAR et al., 1996). Dynorphin is an endogenous opioid peptide with a high affinity for the kappa-opioid receptor and its up-regulation in the spinal cord coincide whit an elevation in pain thresholds in rats during pregnancy and parturition (BRADSHAW et al., 2000). High levels of ovarian steroids occurring in pregnancy have previously been show to increase both the amount of spinal dynorphin and spinal kappa-opioid receptors mediated antinociception (CHANG et al., 2000). This effect is thought to be mediated through the interactions of elevated levels of estrogen and progesterone with the kappa-opioid systems (DAWSON-BASOA; GINTZLER, 1998). Many studies report interactions between estrogens and the opioid system. Estrogens may modulate brain opioid peptide mRNA levels, opioid peptide levels, opioid receptor density, and opioid receptor-mediated signal transduction (CRAFT et al., 2004). Estrogens were found to induce μ-opioid receptor internalization in the medial preoptic nucleus and the posterodorsal medial amygdala nucleus (CECCARELLI et al., 2003). μ-Opioid receptor internalization also can be reversed by progesterone, and estrogens act on neurons that are presynaptic to the μ-opioid receptors to augment the release of endogenous opioid peptides (SINCHAK; MICEVYCH, 2001). A review by Fillingim and Gear (2004) concluded that high levels of estrogens were associated whit decrease opioid analgesia among females, based on studies of rodents at different phases of the estrous cycle. Results of this find suggest that during the estrus stage, when circulating sex steroids are their lowest levels (MARCONDES et al., 2002) sensitivity to pain likely greater to those of the males and female proestrus. It is agreement with the sequence of changes in endogenous gonadal steroids leading to the proestrus LH surge find to Berglund et al. (1988) which shown to desensitize opiate receptors following exogenous hormone administration. In cycling female rodents, opioids often are at least potent or effective in estrus female compared to females tested in others stages, and this suggest that estradiol is responsible for cycle-related changes in opioid analgesia (CRAFT et al., 2004).

The injection of formalin but not saline into the masseter muscle produces quantitative and stereotyped nociceptive behaviors at about 10 minutes after the injection characterized by flinching the head quickly, by tumbling the head to the inject side and by rubbing the orofacial region. A similar inefficiency of saline in inducing nociceptive behaviors has already been noted in temporomandibular joint pain (ROVERONI et al., 2001). A experiment whit with glutamate (nociceptive agent) into the rat masseter muscle demonstrated evoked activity in Aδ mechanoreceptive afferents that were shown to project to the caudal brain stem, a region documented to be a critical relay of nociceptive input form jaw muscles as well as other craniofacial tissues (CAIRNS et al., 2001). One of the characteristics of the formalin responses is its biphasic pattern. The initial responses (5 minutes) is generally attributed to a direct effect of formalin on the sensory receptors (DUBUISSON; DENNIS, 1977) and the later response (after 5 minutes) is related to the subsequent development of inflammation and spinal cord sensitization (SHIBATA et al., 2001). The first phase of formalin pain response was observed for all groups in blood plasma glucose concentration and the second phase was evidently clear in estrus female group to free fatty acids and corticosterone concentrations.

Conclusion

In conclusion, the increase in blood plasma glucose, free fatty acids and corticosterone levels suggest a pain stress response influenced by sex hormones and the increase observed in the female estrus group permit suppose that there is a greater pain response in this group.

Acknowledgements

We thank Dr. Laurita C. A. Ribeiro for the estradiol analysis and the Análise® Produtos e Serviços para Laboratórios Ltda., São Paulo State, Brazil, for gift of the KIT for estradiol determination. This study was supported by CNPq grant; Elzira Diniz de Moraes thanks for CAPES fellowship.

References

ABBOTT, F. V.; FRANKLIN, K. B. J.; WESTBROOK, R. F. The formalin test: scoring properties of the first and second phases of the pain response in the rats. **Pain**, v. 60, n.1, p. 91-102, 1995.

BERKLEY, K. J. Sex difference in pain. **Behavioral and Brain Sciences**, v. 20, n. 3, p. 371-380, 1997.

BERGLUND, L. A.; DERENDORF, H.; SIMPKINS, J. W. Desensitization of brain opiate receptor mechanisms by gonadal steroid treatment that stimulate luteinizing hormone secretion. **Endocrinology**, v. 122, n. 6, p. 2718-2726, 1988.

BRADSHAW, H.; MILLER, J.; LING, Q.; MALSNEE, K.; RUDA, M. A. Sex difference and phases of estrous cycle alter the response of spinal cord dynorphin neurons to peripheral inflammation and hyperalgesia. **Pain**, v. 85, n. 1-2, p. 93-99, 2000.

CAIRNS, B. E.; HU, J. W.; ARENDT-NIELSEN, L.; SESSLE, B. J.; SVENSSON, P. Sex-related differences in human pain and rat afferent discharge evoked by injection of glutamate into the masseter muscle. **Journal of Neurophysiology**, v. 86, n. 2, p. 782-791, 2001.

CECCARELLI, I.; FIORENZANI, P.; MASSAFRA, C.; ALOISI, A. M. Long-term ovariectomy changes formalin-induced lincking in female rats: the role of estrogens. **Reproductive Biology and Endocrinology**, v. 1, n. 24, p. 559-66, 2003.

CHANG, C. P.; AICHER, A. S.; DRAKE, C. T. Kappa opioid receptors in rat spinal cord vary across the estrous cycle. **Brain Research**, v. 861, n. 5, p. 168-72, 2000.

CRAFT, R. M.; MOGIL, J. S.; ALOISI, A. M. Sex differences in pain and analgesia: the role of gonadal hormones. **European Journal of Pain**, v. 8, n. 5, p. 397-411, 2004.

DAWSON-BASOA, M.; GINTZLER, A. R. Gestational and ovarian sex steroid antinociception: synergy between spinal kappa and delta opioid systems. **Brain Research**, v. 794, n. 1, p. 61-67, 1998.

DUBUISSON, D.; DENNIS, S. G. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. **Pain**, v. 4, n. 2, p. 161-74, 1997.

FALHOLT, K.; LUND, B.; FALHOLT, W. An easy colorimetric micromethod for routine determination of free fatty acids in plasma. **Clinica Chimica Acta**, v. 46, n. 2, p. 105-111, 1973.

FILLINGIM, R. B.; GEAR, R. W. Sex differences in opioid analgesia: clinical and experimental findings. **European Journal of Pain**, v. 8, n. 5, p. 413-425, 2004.

GEAR, R. W.; MIASKOWSKI, C.; GORDON, N. C.; PAUL, S.; HELLER, P. H.; LEVINE, J. D. Kappa-opióids produce significantly greater analgesia in women than in men. **Nature Medicine**, v. 2, n. 11, p. 1248-1250, 1996.

GUILLEMIN, R.; CLAYTTON, G. W.; SMITH, J. D.; LIPSCOMB, H. S. Measurement of free corticosteroids in rat plasma: physiological validation of method. **Endocrinology**, v. 63, n. 3, p. 349-357, 1958.

KELLY, M. J.; OIU, J.; WAGNER, E. G.; RONNEKLEIV, O. K. Rapid effects of estrogen on G protein-coupled receptor activation of potassium channels in the central nervous system (CNS). **Journal of Steroid Biochemistry and Molecular Biology**, v. 83, n. 1-5, p. 187-193, 2003.

KOVACS, P.; JURANEK, I.; STANKOVICOVA, T.; SVEC, P. Lipid peroxidation during acute stress. **Pharmazie**, v. 51, n. 1, p. 51-53, 1996.

LE RESCHE, L.; DAO, T. T. Gender differences in pain. **Journal of Orofacial Pain**, v. 14, n. 13, p. 169-84, 2000.

LONG, J. A.; EVANS, H. M. The estrous cycle in the rat and its associated phenomena. In: **Memoirs University of California**. Berkeley: University of California Press, 1922. p. 1-148, v. 6.

MARCONDES, F. K.; BIANHI, F. J.; TANNO, A. P. Determination of the estrous cycle phases of rats: some helpful considerations. **Brazilian Journal of Biology**, v. 62, n. 4A, p. 609-614, 2002.

NASCIMENTO CURI, C. M. P. O.; ZAIA, C. T. B. V.; RIBEIRO, E. B.; DOLNIKOFF, M. S. Glycemic response to stress stimulaton by ether exposure in adrenalectomyzed rats. **Pharmacology Biochemistry and Behavior**, v. 37, n. 3, p. 339-403, 1990.

ROVERONI, C. R.; PARADA, C. A.; VEIGA, M. C. F. A.; TAMBELINI, C. H. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. **Pain**, v. 94, n. 2, p. 185-91, 2001.

SÁNCHEZ, O.; AMAU, A.; PAREJA, M.; POCH, E.; RAMÍREZ, I.; SOLEY, M. Acute stress-induce tissue injury in mice: differences between emotional and social stress. **Cell Stress**, v. 7, n. 1, p. 36-46, 2002.

SHIBATA, M. O.; TAKAHASHI, H.; INOKI, R. Modified formalin test: characteristic biphasic pain response. **Pain**, v. 38, n. 3, p. 347-352, 2001.

SINCHAK, K.; MICEVYCH, P. E. Progesterone blockade of estrogen activation of μ-opioid receptors regulates reproductive behavior. **Journal of Neuroscience**, v. 21, n. 15, p. 5723-5729, 2001.

TAYLOR, B. K.; AKANA, S. F.; PETERSON, M. A.; DALLMAN, M. F.; BASBAUM, A. I. Pituitary-adrenocortical responses to persistent noxious stimuli in

the awake rat: endogenous corticosterone does not reduce nociception in the formalin test. **Endocrinology**, v. 139, n. 5, p. 2407-2413, 1998.

TRINDER, P. Determination of blood-glucose using an oxidase peroxidase system with a non-carcinogenic chromogen. **Annals of Clinical Biochemistry**, v. 6, n. 5, p. 24-29, 1969.

UNRUH, A. M. Gender variations in clinical pain experience. **Pain**, v. 65, n. 2-3, p. 123-167, 1996.

ZAIA, C. T. B. V.; ZAIA, D. A. M.; DELATTRE, E.; GAZIRI, L. C. J.; DOLNIKOFF, M. S.; TIMO-IARIA, C. Effect of chemical stimulation of the dorsomedial hypothalamic nucleus on blood plasma glucose, triglycerides, and free fatty acids in rats. **Brain Research Bulletin**, v. 42, n. 3, p. 195-198, 1997.

ZIMMERMANN, M. Ethical guidelines for investigations of experimental pain in conscious animals. **Pain**, v. 16, n. 2, p. 109-110, 1983.

Received on July 1, 2010.
Accepted on February 10, 2011.