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Fisioterapeuta

INFLUÊNCIA DO ESTRESSE SOBRE A ANSIEDADE E NOCICEPÇÃO INDUZIDA NA ATM DE RATAS NAS FASES DE ESTRO E PROESTRO

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Doutora em Odontologia, Área de Concentração em Fisiologia Oral.

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RESUMO

As disfunções têmporo-mandibulares são mais prevalentes em mulheres e a influência dos hormônios gonadais sobre estas disfunções ainda não estão totalmente esclarecidas. Uma vez que a exposição tanto ao estresse agudo quanto ao crônico podem apresentar diferentes respostas comportamentais; o objetivo deste estudo foi avaliar os efeitos do estresse agudo, sub-crônico e crônico sobre a ansiedade e nocicepção induzida pela injeção de formalina na articulação temporomandibular (ATM) de ratas nas fases de estro e proestro. Foi avaliada também a relação entre os níveis sanguíneos de corticosterona após os diversos protocolos de estresse. Ratas Wistar nas fases de estro ou proestro foram submetidas a uma sessão de estresse agudo por imobilização (15 min, 30 min ou 1 h), ou expostas ao estresse sub-crônico (3 dias – 1 h/dia), ou crônico (40 dias – 1 h/dia). Logo depois, foram (1) submetidas ao teste da formalina na ATM para avaliação da nocicepção; ou (2) submetidas ao teste do labirinto em cruz elevado para a avaliação da ansiedade; ou (3) mortas imediatamente para coleta de sangue e mensuração hormonal da corticosterona plasmática por radioimunoensaio. Finalmente, foi avaliado o papel do receptor kapa-opióide nas alterações nociceptivas induzidas pelo estresse. Para isso, o antagonista seletivo κ-opióide, nor-BNI (200 μg/ 25 μl) ou salina foi administrado 24 h antes da avaliação da nocicepção. Os resultados mostraram que todos os protocolos de estresse aumentaram significativamente os níveis de corticosterona, não apresentando diferença em relação às fases do ciclo estral. Em relação à nocicepção, os animais submetidos ao estresse agudo (1 h), tanto na fase de estro quanto na fase de proestro, apresentaram diminuição nas respostas comportamentais nociceptivas (analgesia) quanto comparados ao controle. A injeção local de nor-BNI reverteu parcialmente a analgesia induzida pelo estresse na fase proestro, mas não em estro. Quanto à ansiedade, houve um aumento significativo da ansiedade nas ratas submetidas ao estresse agudo (1 h) e um aumento da atividade locomotora nas ratas estressadas cronicamente quando comparadas ao controle. Concluiu-se que: 1) o estresse agudo (1 h) causa analgesia em ratas nas fases de estro e proestro, sendo este efeito maior em proesto; 2) o receptor κ-opióide reverte parcialmente a analgesia induzida pelo estresse em fêmeas na fase de proestro; e 3) o estresse agudo (1 h) aumenta o nível de ansiedade em fêmeas nas fases de estro e de proestro.

Palavras-chave: Dor facial, Teste da formalina, Articulação têmporo-mandibular, Ciclo estral.

ABSTRACT

Temporo-mandibular disorders are more prevalent in women and the influences of gonadal hormones on these disorders are not fully clarified. Since the exposure to both acute and chronic stress may have different behavioral responses; the purpose of this study was to evaluate the effects of acute, sub-chronic and chronic stress on behavioral nociceptive responses induced by the injection of formalin in the temporomandibular joint (TMJ) of rats in the phases of estrus and proestrus. It was also assessed the relationship between blood levels of corticosterone after the various protocols of stress. Wistar female rats in estrus or proestrus were submitted to a session of acute stress by immobilization (15 min, 30 min or 1 h), or exposed to sub-chronic stress (3 days - 1 hr / day) or chronic (40 day - 1 hr / day). Soon after, they were (1) submitted to the formalin test to evaluate the nociception, or (2) submitted to the elevated plus-maze test for the evaluation of anxiety, or (3) killed immediately to collect blood and measure hormonal plasma corticosterone by radioimmunoassay. Finally, was assessed the role of kappa-opioid receptor on nociceptive alterations induced by stress. For this, the antagonist selective κ-opioid nor-BNI (200μg/ 25 μl) or saline was administered 24 hours before the evaluation of nociception. The results showed that all protocols of stress significantly increased levels of corticosterone, but there were no significant difference in relation to the phases of the estrous cycle. In relation to the nociception, animals submitted to acute stress (1 h), both in estrus and in proestrus phases, showed a decrease in the nociceptive behavioral responses (analgesia) when compared to control. The local injection of nor-BNI partially reverted the analgesia induced by stress in proestrus phase, but not in estrus. As for anxiety, there was a significant increase in anxiety levels in rats submitted to acute stress (1 h) and an increase in locomotor activity in chronically stressed rats when compared to control. It was concluded that: 1) the acute stress (1 h) induce analgesia in rats in the estrus and proestrus phases, being this effect higher in proestrus, 2) the receptor κ-opioid partially reverses the analgesia in females in proestrus phase, and 3) acute stress (1 h) increase the anxiety levels in females in estrus and proestrus phases.

Key Words: Facial pain, Formalin test, Temporomandibular joint, Estrous Cycle.

SUMÁRIO

INTRODUÇÃO

Dentre as estruturas craniomandibulares, a articulação temporomandibular (ATM) é considerada uma das principais fontes desencadeantes de dor da região orofacial. A dor é considerada uma das formas de sensibilidade somestésica, onde os receptores sensoriais específicos responsáveis pela captação de estímulos nocivos, os nociceptores, que são terminações nervosas também presentes na ATM (Aghabeigi, 1992), são sensíveis às diversas formas de estímulos: mecânicos, térmicos ou químicos. Além dos estímulos nocivos, alterações nos estados emocionais tanto de humanos (Barlow et al., 1996), como de animais (King et al., 1996) podem alterar fortemente a reatividade à sensação dolorosa.

A diferença entre os resultados encontrados na literatura em relação à dor (Fillingim and Ness, 2000) e as divergências em relação aos efeitos do estresse sobre a nocicepção (Terman et al., 1986) não surpreendem já que diferentes metodologias têm sido empregadas e a resposta de estresse depende de fatores como a natureza, a intensidade e a duração do estímulo estressor. A influência do estresse na nocicepção é largamente baseado em modelos experimentais de nocicepção em animais (Clemente et al., 2004; Le Bars et al., 2001), e apresentam respostas distintas. Diversos trabalhos têm demonstrado analgesia induzida por estresse tanto em humanos (Bandura et al., 1987; Droste et al., 1991) como em animais (Lapo et al., 2003; Mogil et al., 1996; Wiedenmayer and Barr, 2000).

Os clássicos efeitos analgésicos do estresse representam uma vantagem sob o ponto de vista evolutivo, pois permitem ao organismo responder rapidamente frente a uma situação de perigo em presença de injúria, ao mesmo tempo em que conserva energia mantendo o animal livre de dor. Os efeitos analgésicos induzidos por estressores são comparados àqueles causados pela morfina em doses de 5-10 mg/kg, porém a duração dos efeitos dos estressores são relativamente menores, desaparecendo aproximadamente dentro de 30 minutos (Girardot and Holloway, 1984; Snow and Dewey, 1983).

Muitos modelos experimentais medem a resposta nociceptiva de tecidos superficiais, como o "tail-flick" (Gamaro et al., 1998), "hot-plate" (King et al., 2003) e injeção de formalina na pata (Aloisi et al., 1998). É importante salientar que condições de dor profunda diferem de tecidos dolorosos superficiais. O teste da formalina na ATM é um teste sensível a várias classes de drogas analgésicas (Hunskaar and Hole, 1987;

Tjolsen et al., 1992), causa dor inflamatória tônica (Teng and Abbott, 1998), e é um válido modelo de dor orofacial profunda (Roveroni et al., 2001).

Outro fator que deve ser considerado em relação à dor é a influência dos hormônios sexuais, pois devem ser os responsáveis pela maior prevalência de disfunções temporomandibulares (DTMs) em mulheres, uma vez que estudos epidemiológicos demonstram que a dor associada às DTMs é 2-2,5 vezes mais comum no sexo feminino, em idade reprodutiva (Locker and Slade, 1988), do que no sexo masculino (LeResche, 1997).

Para se testar os efeitos do estresse em um modelo de dor profunda, utilizando-se ratas em diferentes fases hormonais, se faz necessário compreender as seguintes fases do ciclo sexual dos roedores, chamado de ciclo estral, que é constituído por quatro fases: (1) metaestro, período no qual a progesterona está elevada e o estrógeno e LH estão baixos; (2) diestro, caracterizado pelo aumento do estrógeno no final do dia e queda da progesterona; (3) proestro, onde o estrógeno, a progesterona, o LH e o FSH atingem os níveis máximos e; (4) estro, período no qual as fêmeas estão sexualmente receptivas e o estrógeno, a progesterona, o LH e o FSH atingem os menores níveis (Fillingim and Ness, 2000).

Estudo realizado com animais demonstrou que a magnitude da resposta nociceptiva induzida pela administração de formalina na região da ATM é significativamente maior em ratas na fase diestro, que equivale à fase de baixo nível circulante de estrógeno, quando comparadas com as fêmeas na fase proestro e machos (Clemente et al., 2004). Similarmente, estudo realizado em humanos demonstrou que a dor na ATM em mulheres é mais elevada durante períodos de baixo nível circulante de estrógeno (LeResche et al., 2003).

Associando-se o teste da formalina na ATM com a imobilização prévia para causar estresse, foi demonstrado que em ratos machos, o estresse agudo (1h) e crônico (40 dias - 1h) aumenta os níveis plasmáticos da corticosterona, confirmando que a imobilização provoca estresse, e os animais cronicamente estressados apresentaram quadro de hiperalgesia quando comparados com o controle (Gameiro et al., 2005). Além desses resultados, também foi demonstrado que os animais submetidos a estresse agudo (15 min, 30 min, 1h), sub-crônico (3dias -1h) e crônico (40 dias - 1h) apresentaram maior nível de ansiedade, confirmado pelo tempo de menor permanência nos braços abertos no labirinto de cruz elevado, quando comparados ao grupo controle (Gameiro et al., 2006).

Devido a diversas variáveis envolvidas em um modelo experimental destinado ao estudo da relação entre dor e estresse, devem ser consideradas as variáveis fisiológicas e comportamentais envolvidas em uma situação de estresse; e considerando a relação existente entre estresse e crises de dor facial (Suvinen et al., 1997) e também a capacidade do estresse em conjunto com diferentes fases do ciclo estral alterar a nocicepção, estudos sobre o mecanismo das alterações nociceptivas induzidas pelo estresse nas dores profundas são relevantes para a pesquisa.

PROPOSIÇÃO

Os objetivos do presente trabalho foram:

- Verificar o efeito do estresse agudo, sub-crônico e crônico sobre as respostas comportamentais nociceptivas induzidas pelo teste da formalina na ATM de ratas na fase de estro e proestro.

- Avaliar a participação do sistema κ-opióide nas possíveis alterações nociceptivas induzidas por situações estressantes.

- Avaliar a relação entre os diversos protocolos de estresse e: 1) os níveis sanguíneos de corticosterona plasmática e 2) os níveis de ansiedade nas fases de estro e proestro do ciclo estral.

CAPÍTULOS

O presente estudo foi realizado em formato alternativo, conforme deliberação da Comissão Central de Pós-Graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) n° 001/98.

CAPÍTULO 1

Influence of sex on temporomandibular disorder pain: a review of occurrence and development

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Article Review

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Abstract

Aim: The aim of this study was to develop a narrative literature review using international research to present the influence of sex on occurrence and development of temporomandibular disorder (TMD) pain. **Methods**: The data sources were computer-based searches in PubMed between 1987 and Feb 2008 using appropriate keywords. For inclusion in this review, articles had to meet the following criteria: be written in English; include human and nonhuman subjects; be published a full-text paper in a peer-reviewed medical journal. **Results**: The studies considered eligible for this narrative review presented results in agreement with the difference in sex and orofacial pain. Patients were almost always adults, with particular focus on patients' sex. Clinical conditions were predominantly TDM pain. Since sexual dimorphism was detected in TMD pain, the results are focused on women. **Conclusion**: The findings of this review suggest that there is difference in the occurrence and development of pain according to the individual's sex, women being more susceptible to TMD pain.

Keywords:
Temporomandibular disorder; pain; sex difference; review.

Introduction

Being male or female is one of the most important predictors of an individual's health. Compared to women of similar age, women outnumber men for stress-related bodily complaints such as chronic pain[1]. The most common cause of chronic facial pain conditions involves temporomandibular disorder (TMD)[2]. TMD pain is the most common symptom that compels patients to seek therapy; its management, however, mostly involves a multidisciplinary approach[2]. Dentists, orthodontists, psychologists, physical therapists, and physicians work together to address the condition of the patient with TMD[3]. For a very long period, the sex of subjects used to study pain was rarely taken into account in either basic or clinical studies[4]. Epidemiological studies on nonpatient populations in the early 1970s reported that the prevalence of TMD signs and symptoms was similar for men and women. Studies of TMD signs and symptoms in nonpatients revealed either no gender difference or a somewhat greater prevalence among women. In the 1990s, a longitudinal study[5], however, showed that the course of TMD symptoms differed significantly with respect to gender: women who had reported symptoms during adolescence consistently reported symptoms 1 decade later, whereas only 60% of men reported symptoms later.

In view of the need for dealing with pain during TMD treatment, the objective of this review was to present the influence of sex on the occurrence and development of TMD pain.

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Identification and Review of Studies

Computer-based searches in PubMed full-text paper electronic database were conducted using combinations of the following keywords: pain, temporomandibular disorder, orofacial pain, sex-related difference, sexual dimorphism and gender difference pain. Reference sections from published articles in the field were also used as sources. No attempt was made to contact study authors. To be included in this review, articles had to meet the following criteria: (1) be written in English; (2) include human and nonhuman subjects; (3) be published as a full-text paper in a peer-reviewed medical journal between 1987 and Feb 2008.

Occurrence of Temporomandibular Disorder Pain

Chronic orofacial pain affects approximately 10% of adults

and up to 50% of the elderly. There is evidence that sex differences in masticatory muscle pain and tenderness emerge as early as 19 years of age[1]. Childbearing-age women, mainly those in their 40s, seek treatment for orofacial pain more frequently in comparison with men by a 2:1 ratio. Although women are more likely to seek medical care for pain, they also report more pain for which they do not seek treatment[6]. Moreover, the difference between the two sexes is multifaceted, involving the occurrence of chronic pain, the type of pain syndromes experienced, the characteristics of the complications that develop, etc. There could be several reasons for the higher reactivity of women compared to men to a similar painful stimulation, ranging from genes to hormonal and cultural influences[4].

Converging lines of evidence suggest that there are important sex-related influences on the experience of pain. Women report more pain than men and are at greater risk for developing many forms of chronic pain[7]. Laboratory studies consistently report lower pain threshold and tolerance among women, and these effects are moderate in magnitude. In addition to these human data, abundant nonhuman animal research indicates sex differences in nociceptive responses. While the clinical implications of these sex differences in pain responses are not yet precisely defined, it is becoming increasingly clear that future improvements in the effectiveness of pain management will require taking the patient's sex into account[8].

TMD refers to a group of conditions, whose principal symptom is pain in the masticatory muscles and/or temporomandibular joints on palpation and during function (e.g., chewing, mouth opening, speech)[9-10]. The classification and epidemiology of orofacial pain presents challenges because of the many anatomic structures involved, diverse causes, unpredictable pain referral patterns and symptoms, and a lack of consensus with regard to differential diagnostic criteria[6,11].

A number of aspects of the prevalence pattern of TMD suggest that reproductive hormones may play a role in these pain conditions[12]: the prevalence of TMD pain prior to adolescence is low (2-4%), and does not seem to differ for boys and girls. However, prevalence rates are higher in adult women than in adult men, and the prevalence is lower for women in the postmenopausal years than for those of reproductive age[13]. The existence of sex differences in pain and analgesia, and the fact that the developmental profile of some types of pain clearly parallels reproductive function strongly suggest that gonadal steroid hormones significantly influence pain[14].

TMD are 1.5-2 times more prevalent in women than in men in the community, and 80% of treated cases of TMD are women[15-16]. Moreover, women are at significantly greater risk than men of experiencing TMD-related disability, which is associated with significant use of health services and increased use of opioid and sedative hypnotic medications. In addition, treatment of TMD can be associated with severe iatrogenic consequences[17]. Furthermore, chronic TMD has been found to interfere with normal social activity and interpersonal relationships and to negatively affect the ability to maintain employment[3]. Individuals react to stressful events in different ways, and differences in the physiological stress response are important determinants of health. A stressful stimulus results in the activation of several physiological pathways including the hypothalamic-pituitary-adrenal axis (HPAA) and the autonomic nervous system. A considerable body of research during recent years has linked the function of both of these systems with the pathogenesis of several common disorders, including coronary arterial disease, type 2 diabetes, metabolic syndrome, depression and stress-related bodily complaints. Importantly, both systems show a clear sex-specific pattern of response. Therefore, stress reactivity is a major candidate for a mechanism explaining why some diseases are more common in men and others in women[1].

TMD is usually manifested by one or more of the following signs or symptoms: pain, joint sounds, limitation in jaw movement, muscle tenderness, and joint tenderness. It also is commonly associated with other symptoms affecting the head and neck region such as headache, ear-related symptoms, and cervical spine disorders. Patients with chronic TMD frequently report symptoms of depression, stress, anxiety, poor sleep quality, and low energy[3,18]. Knowing what biological mechanisms underlie such profound differences may be extremely helpful in elucidating the pathogenesis of various common disorders, a crucial step in developing their prevention and treatment[1]. The prevalence of several pain conditions located in the craniofacial region and the mechanisms that underlie sex-related differences remain obscure and probably involve both physiological and psychosocial factors[9,19].

Development of Orofacial Pain

Nociception results from the activation of primary afferent nociceptors and the transmission of the nociceptive information to the spinal cord from where it is relayed to supra spinal levels. Following tissue injury and inflammation, primary afferent nociceptors are sensitized by mediators released from diseased or damaged tissue or from the immune system in such a way that previously slight or ineffective stimulation becomes effective in inducing nociception. This primary sensory nociceptor sensitization is referred to as hyperalgesia[20].

Inflammatory pain is a pervasive problem and usually results in both spontaneous pain and hyperalgesia. Although the hyperalgesic state does not necessarily involve ongoing pain, the nociceptive threshold is lowered in this state, and the application of a nonnoxious mechanical, thermal, or chemical stimulus induces a nociceptive behavior response. However, spontaneous

inflammatory pain is characterized by a continuous endogenous stimulation of nociceptors caused by the release of inflammatory mediators that directly stimulate them. Postsurgical or traumatic pain is usually referred to as spontaneous pain in a hyperalgesic state[21].

Inflammatory temporomandibular joint (TMJ) conditions can result in TMJ hyperalgesia produced by peripheral sensitization of TMJ nociceptors and by central sensitization of the nociceptive neurons of the trigeminal brainstem sensory nuclear complex. Peripheral sensitization, as well as central sensitization is characterized by an increase in the neuronal membrane excitability by inflammatory mediators released at the site of injury and by neuropeptide and excitatory amino acid released at the trigeminal brainstem sensory nuclear complex, respectively. Some of the inflammatory mediators released at the site of injury including PGE2 are present at high levels in the synovial fluid of patients with TMD. During hyperalgesic states, the nociceptive threshold is lowered and a nonnoxious stimulus such as jaw movement can induce pain, and noxious stimulus can also induce increased pain. The inflammatory mediators released at the site of tissue injury, such as prostaglandins, sensitize nociceptors. Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used to manage inflammatory pain. The analgesic action of these drugs results from the blockade of prostaglandins synthesis, thus preventing the peripheral sensitization of nociceptors[21-22].

The spinal cord has been shown to be a CNS region in which components of opioid analgesic pathways and their regulation manifest sexual dimorphism. For example, the density of the kappa-opioid receptor (KOR) and its distribution within axon terminals differs between the spinal cord of male and female rodents[23]. Functional KOR are located within the TMJ of rats; peripherally acting KOR agonists could be of benefit in the treatment of TMJ pain, especially in women[24], because the analgesic effect of a class of drugs, nalbuphine, pentazocine and butorphanol, which are thought to induce analgesia predominantly by action on KOR, produce greater analgesia in women[25].

The receptors of gonadal steroids are referred to as the hormones produced by the ovaries and testes (gonads). They are present in many brain areas including some involved in pain transmission and modulation[4]. The man products of the tests are the androgens: testosterone and dihydrotestosterone. The ovaries primarily produce two types of steroid hormones: estrogens (e.g., estradiol, estriol, estrone) and progestins (e.g., progesterone; so-called because it promotes gestation and pregnancy). Testosterone is a precursor to estradiol, so the ovaries also make testosterone. Conversely, estradiol is a metabolite of testosterone, so the testes also produce some estrogens. The aromatization of testosterone to estradiol is greatly facilitated by the enzyme aromatase. This means that tissues containing aromatase can convert testosterone to estrogen and thereby make use of estrogen through estrogen receptors. In women, testosterone is produced in the adrenal cortex (25%) and ovaries (25%) and by transformation (50%) in the liver, kidneys, bowel, lungs, adipose tissue, and CNS[26]. Furthermore, since TMJ pain in women is highest at times of lowest estrogen, the effects of peripherally acting kappa opioid receptor agonists on the treatment of TMJ pain in women across the menstrual cycle should be better evaluated[24].

The physiological basis for the sex-related difference in analgesic response to a KOR agonist is not completely known. It is possible that a male related hormone, such as testosterone, interacts negatively with KOR agonists; or that female-related hormones, such as progesterone or estrogen, potentiate the action of KOR[27]. Thus, the treatment of choice for TMD is conservative because the symptomatology of the condition is often improved by the use of medication, occlusal splints, physical therapy, and orthodontic treatment[3].

Influence of female gonadal hormones on orofacial pain

During the menstrual cycle, serum levels of estrogen and progesterone fluctuate. In women, estrogen and progesterone levels are both relatively low at the beginning of the cycle. During the follicular phase, estrogen levels gradually increase, peaking prior to ovulation, and then moderately decrease during the luteal phase. Progesterone levels rapidly increase after ovulation, peaking during the middle of the luteal phase. At the end of the luteal phase, both estrogen and progesterone levels drastically decrease[28]. However, menopause induces changes in the endogenous hormone balance: ovarian production of estrogens dramatically decreases. Thereafter, the adrenal cortex is responsible for estrogen production via aromatization of androgens to estradiol in peripheral tissue (e.g., fat), which is significant in obese postmenopausal women. Nevertheless, few researchers determine testosterone and estradiol blood concentrations in their experimental subjects at the time of testing[14].

Several mechanisms by which hormones could influence TMD pain can be postulate[29]. Peripherally, hormones could act directly on the temporomandibular joint and associated soft tissues. For example, estrogen is known to increase joint laxity, at least during pregnancy, and laxity of the temporomandibular joint is thought to play a role in the development of some of these disorders[30]. Another possibility is that estrogen enhances a number of specific inflammatory responses in the TMJ, and estrogen receptors have been found only in the TMJ tissues of female primates, but not in males[31].

The classic animal experiment testing the relationship between hormones and nociception involves ovarectomizing female animals to examine the effects of

hormone deficit, and then replacing hormones exogenously and observing the effects of hormone replacement. Such studies are obviously not feasible in humans. However, the natural experiment of postmenopausal hormone replacement therapy presents an interesting parallel to the animal model: hormones are depleted (either slowly with the natural aging process, or more abruptly by surgery) and then replaced from exogenous sources. If female reproductive hormones increase the risk of a particular pain condition, those post-menopausal women who replace their depleted endogenous hormones from exogenous sources would be hormonally more similar to younger women than those postmenopausal women, who chose not to use hormone replacement therapy, and the users of hormone replacement therapy would be expected to be at higher risk of the specific pain condition[31].

Few studies have investigated the role of hormonal fluctuations in the frequency or intensity of musculoskeletal pains, such as TMD, where episodes tend to be longer than for headache. There was a report on the variability of myofascial pain of TMD over three menstrual cycles in 12 female subjects. Users of oral contraceptives tended to show less variable pain intensity levels, and fewer pain-free days than women experiencing hormonal fluctuations related to their naturally occurring menstrual cycles. However, the differences were not statistically significant and a predominant temporal pattern could not be discerned in this small sample[13].

TMD pain, abdominal pain, migraine and tension-type headache are more prevalent in adult women than in men. Epidemiological studies have also found higher prevalence of these conditions, and sometimes back pain, among adolescent girls when compared to boys. Recently, use of hormone replacement therapy in postmenopausal women has been identified as a risk factor for back pain and TMD pain[32].

Concluding, the findings of this narrative literature review suggest that there is difference in the occurrence and development of pain according to sex, women being more susceptibly to orofacial pain. However, the conclusions drawn from these studies have considerable methodological limitations and this area requires further assessment using stricter randomized controlled trials to assess the difference between sexes. It is hoped that this review will highlight the fact that the patient's sex should be taken into account during TMD therapy.

References

1. Kajantie E, Philips DIW. The effects of sex and hormonal status on the physiological response to acute phychosocial stress. Psychoneuroendocrinology. 2006; 31: 151-78.
2. Gameiro GH, Andrade AS, Nouer DF, Veiga MCFA. How many stressful experiences contribute to the development of têmporo-mandibular disorders? Clin Oral Invest. 2006; 10: 261-8.
3. McNeely ML, Armijo Olivo S, Magee DJ. A systematic review of the effectiveness of physical therapy interventions for temporomandibular disorders. Phys Ther. 2006; 86: 710-25.
4. Aloisi AM, Bonifazi M. Sex hormones, central nervous system and pain. Horm Behav. 2006; 50: 1-7.
5. Isberg A, Hagglund M, Paesini D. The effect of age and gender on the onset of symptomatic temporomandibular joint disk displacement. Oral Surg Oral Med Pathol Oral Radiol Endod. 1998; 85: 252-7.
6. Shinal RM, Fillingim RB.Overview of orofacial pain: epidemiology and gender differences in orofacial pain. Den Clin N Am. 2007; 51: 1-18.
7. Sherman JJ, LeResche L. Does experimental pain response vary across the menstrual cycle? A methodological review. Am J Physiol Regul Integr Comp Physiol. 2006; 291: R245-6.
8. Fillingim RB, Gear RW. Sex differences in opioid analgesia: clinical and experimental findings. Eur J Pain. 2004; 8: 413-25.
9. Cairns BE. The influence of gender and sex steroids on craniofacial nociception. Headache. 2007; 47: 319-24.
10. Fischer L, Clemente JT, Tambeli CH. The protective role of testosterone in the development of temporomandibular joint pain. J Pain. 2007; 8: 437-42.
11. Song PC, Schwartz J, Blitzer A. The emerging role of botulinum toxin in the treatment of temporomandibular disorders. Oral Dis. 2007; 13: 253-60.
12. Butkevich IP, Barr GA, Vershinina EA. Sex differences in formalin-induced pain in prenatally stressed infant rats. Eur J Pain. 2007; 11: 888-94.
13. LeResche L, Mancl L, Sherman JJ, Gandara B, Dworkin SF. Changes temporomandibular pain and other symptoms across the menstrual cycle. Pain. 2003; 106: 253-61.
14. Craft RM, Mogil JS, Aloisi AM. Sex differences in pain and analgesia: the role of gonadal hormones. Eur J Pain. 2004; 8: 397-411.
15. Von Korff M, Dworkin SF, LeResche L, Kruger A. An epidemiologic comparison of pain complaints. Pain. 1988; 32: 173-83.
16. Dworkin SF, Huggins KH, LeResche L, Von Korff M, Howard J,Truelove E, Sommers E. Epidemiology of signs and symptoms in temporomandibular disorders: clinical signs in cases and controls. J Am Dent Assoc. 1990: 120: 273-81.
17. Von Korff M, Dworkin SF, LeResche L.Graded chronic pain status: an epidemiologic evaluation. Pain. 1990; 40: 279-91.
18. Gameiro GH, Gameiro PH, Andrade AS, Pereira LF, Arthuri MT, Marcondes FK et al. Nociception and anxiety-like behavior in rats submitted to different periods of restraint stress. Physiol Behav. 2006; 87: 643-9.
19. Wiesenfeld-Hallin Z. Sex differences in pain perception. Gend Med. 2005; 2: 137-45.
20. Oliveira MCG, Pelegrini-da-Silva A, Parada CA, tambeli CH. 5-HT acts on nociceptive primary afferents through an indirect mechanism to induce hyperalgesia in the subcutaneous tissue. Neuroscience. 2007; 145: 708-14.
21. Jorge S, Parada CA, Ferreira SH, Tambeli CH. Interferential therapy produces antinociception during application in various models of inflammatory pain. Phys Ther. 2006; 86: 800-8.
22. Rodrigues LLFR, Oliveira MCG, Pelegrini-da-Silva A, Veiga MCFA, Parada CA, Tambeli CH. Peripheral sympathetic component of the temporomandibular joint inflammatory pain in rats. J Pain. 2006; 7: 929-36.
23. Harris JA, Chang PC, Drake CT. Kappa opioid receptors in rat spinal cord: sex-linked distribution differences. Neuroscience. 2004; 124: 879-90.
24. Clemente JT, Parada CA, Veiga MCFA, Gear RW, Tambeli CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neuroscience Lett. 2004; 372: 250-5.
25. Gear RW, Gordona NC, Miaskowski C, Paul SM, Heller PH, Levine JD. Sexual dimorphism in very low dose nalbuphine postoperative analgesia. Neuroscience Lett. 2003; 339: 1–4.

26. Brody DJ, Bracken MB. Short interpregnancy interval: a risk factor for low birth weight. Am J Perinatol. 1987; 4: 50-4.
27. Liu NJ, von Gizycki H, Gintzler AR. Sexually dimorphic recruitment of spinal opioid analgesic pathways by the spinal application of morphine. J Pharmacol Exp Ther. 2007; 322: 654-60.
28. Kuba T, Quinones-Jenab V. The role of female gonadal hormones in behavioral sex differences in persistent and chronic pain: Clinical versus preclinical studies. Brain Res Bull. 2005; 66: 179-88.
29. Arthuri MT, Gameiro GH, Tambeli CH, Veiga MCFA. Peripheral effect of a kappa opioid receptor antagonist on nociception evoked by formalin injected in TMJ of pregnant rats. Life Sci. 2005; 76: 1177-88.
30. Westling L. Temporomandibular joint dysfunction and systemic joint laxity. Swed Dent J Suppl. 1992; 81: 1-79.
31. LeResche L, Saunders K, Von Korff MR, Barlow W, Dworkin SF. Use of exogenous hormones and risk of temporomandibular disorder pain. Pain. 1997; 69: 153-60.
32. Le Resche L, Mancl LA, Drangsholt MT, Saunders K, Von Korff M. Relationship of pain and symptoms to pubertal development in adolescents. Pain. 2005; 118: 201-9.

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CAPÍTULO 2

Article

The effects of restraint stress on nociceptive responses evoked by formalin injected in TMJ of female rats

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Abstract

In this study the temporomandibular joint (TMJ) formalin test was used to evaluate the effects of acute, sub-chronic and chronic restraint stress on nociceptive behavioral responses in female rats stratified into proestrus and estrus phases of the estrous cycle. Animals were submitted to one session of acute restraint stress (15 min, 30 min or 1 h), three sessions of sub-chronic stress (3 days-1h/ day) or exposed to chronic stress (40 days-1 h/ day). Then, animals were immediately killed to collect blood for hormonal radioimmunoassay determinations; or submitted to the TMJ formalin test to evaluate nociception. All stress protocols significantly raised the levels of corticosterone. Rats submitted to acute (15 min and 30 min), sub-chronic and chronic restraint presented similar performance to unstressed controls in the TMJ formalin test, whereas those rats stressed for 1 h showed a decrease in nociceptive responses, both in proestrus and estrus phases. The stress-induced analgesia was higher in the proestrus than in the estrus females. To evaluate the role of kappa-opiate receptors in this process, the selective receptor κ-opioid antagonist nor-BNI (200 μg or saline) was injected into the TMJ twenty-four hours prior to acute restraint stress (1 h) and TMJ formalin test. The local nor-BNI injection partially reverted the stress-induced analgesia in the proestrus phase, but not in the estrus phase. These findings suggest that 1) acute stress (1 h) can produce analgesia both in proestrus and estrus female rats, being this effect higher in the proestrus phase; 2) the κ-opioid receptor activation is involved in the stress-induced analgesia observed being this effect higher in the proestrus phase.

Keywords: Stress; Analgesia; Formalin test; Temporomandibular joint; Estrous cycle.

Introduction

Temporomandibular dysfunctions are pain conditions of the masticatory muscles and temporomandibular joint (TMJ) (Dworkin and LeResche 1992; Denucci, Dionne et al. 1996) with frequent spreading to adjacent regions of the head and neck (Bereiter 2001) and with greater prevalence, severity, and duration in women than in men (LeResche 1997; Fischer, Clemente et al. 2007). These findings might be attributed to a pronociceptive effect of ovarian hormones on TMJ pain modulation (LeResche 1997; Cairns, Sessle et al. 2001; Craft, Mogil et al. 2004). It was previously demonstrated that the injection of formalin into the rat's TMJ induces a behavioral nociceptive response significantly lower in male than in female rats (Clemente, Parada et al. 2004).

One possible explanation for the sexual dimorphism in the TMJ nociceptive responses can be related to specific opioid mechanisms. As seen before, functional kappa opioid receptors are located within the TMJ of rats. Activation of these receptors suppresses formalin-induced TMJ nociceptive behavior in both males and females, but significantly more in females (Clemente, Parada et al. 2004). The analgesic system in women is more sensitive than in men when κ-opioid receptor agonists are used (Arthuri, Gameiro et al. 2005). It is possible that a male related hormone, such as testosterone, interacts negatively with κ-opioid agonists (testosterone is present in both sexes, since the circulating testosterone levels in female subjects are typically about 10% of those observed in male subjects (Fischer, Clemente et al. 2007); or that female-related hormones, such as progesterone or estrogen, potentiate the action of κ-opioid (Gear, Gordon et al. 1996).

Recently, it has been demonstrated that female sex hormones can modulate the adrenal medullary function to produce sexual dimorphism in baseline mechanical nociceptive

threshold, as well as in epinephrine-induced hyperalgesia in rats (Khasar, Dina et al. 2005).

Considering that mechanisms regulating stress-induced changes in nociception can produce alterations in endogenous opioid system (Przewlocki, Lason et al. 1987; Amit and Galina 1988; Yamada and Nabeshima 1995; Gear, Gordon et al. 1996), together with the fact that repeated stress (chronic) can produce hyperalgesia in male rats due to alterations in the activity of opioid systems (Gameiro, Andrade Ada et al. 2005), we hypothesized that the different stress system activation in male and female rats can be involved in the sexual dimorphism in the TMJ nociception modulation.

Chronic generalized pain, characterized by diffuse lowered pain threshold, such as fibromyalgia and irritable bowel syndromes, disproportionately affect women (Buskila 2001; Yunus 2002) and in many cases stress may precede or is comorbid with symptoms of generalized pain syndromes (Davis, Zautra et al. 2001; Raphael, Janal et al. 2004). However, as far as the orofacial region is concerned, particularly as regards pain in TMJ, which is more prevalent in women (LeResche 1997), little is known about the effect of female sex hormones and the possible involvement of the stress activation on TMJ nociception.

Considering that the nociceptive behavioral responses elicited by the injection of formalin into the TMJ represent a valid and reliable model of deep orofacial pain (Roveroni, Parada et al. 2001), this study aimed to evaluate the effects of acute, sub-chronic and chronic restraint stress on the nociceptive behavioral responses induced by TMJ formalin test in female rats in estrus and proestrus phases.

Methods

1. Animals

This study was carried out in 3-month-old female rats obtained from Centro Multidisciplinar de Investigação Biológica (CEMIB), UNICAMP, Brazil. The rats were housed in groups of five and maintained in a temperature-controlled room (23±1°C) with a 12/12 h light–dark cycle (lights on at 6:00 a.m.) and food and water were available ad libitum. Animals were handled for at least 1 week prior to the experiments, which were approved by the Committee on Animal Research of the University of Campinas (protocol 938-1) and according to IASP (International Association for the Study of Pain) guidelines for the study of pain in animals (Zimmermann 1983). Procedures were performed between 08:00 a.m. and 1:00 p.m.

2. Estrous phase determination

Proestrus and estrus phases were determined by daily microscope examination of vaginal smears taken by gentle lavage, between 8:00 and 10:00 a.m. Proestrus was identified by the predominance (>70%) of nucleated epithelial cells and estrus by anucleated cornified cells, in rats with at least two consecutive regular 4-day cycles (Smith, Freeman et al. 1975; Marcondes, Bianchi et al. 2002). These phases were chosen because they represent phases of high and low ovarian hormonal levels, respectively (Butcher, Collins et al. 1974).

3. Stress exposure

All animals were stressed by restraint. In the acute model, the animals were stressed during 15 min, 30 min or 1 h for a single exposure (Gamaro, Xavier et al. 1998). In the sub-chronic model the animals were stressed 1 h daily for 3 days (Quintero, Moreno et al. 2000), and females started the procedures of sub-chronic restraint stress two phases of estrous cycle (two days) before the pre-determined phases. In the chronic model, 1 h daily, 5 days per week for 40 days (Ely, Dapper et al. 1997; da Silva Torres, Cucco et al. 2003), females were submitted to estrous phase determination in the thirtieth second day of restraint stress until the fortieth second day. The experiments were carried out between the thirty-eighth and fortieth second day, because it is not possible to predict the exactly estrous phase in the fortieth day.

Restraint was carried out by placing the animal in a plastic restraint device (adjustable in size depending on the animal's weight). The area of the tube could be adjusted individually and the tube was held firmly in place with adhesive tape. There was a 1-cm hole in the far end for breathing. The control group was not submitted to restraint. The restraint procedure was carried out in a separate quiet room between 9:00 and 11:00 a.m.

4. Hormonal assay

Blood of proestrus and estrus females were collected by decapitation under basal conditions (within 30 s of removal from the home cage; also referred to as the 0 min time point) or collected at the end of each restraint (within 30 s of removal from the restrainer). Rats were decapitated, and the blood was collected in tubes containing heparin. The blood was centrifuged at 2500 rpm, 4ºC for 10 min. The serum was collected and frozen at -20°C until use. Plasma corticosterone levels were determined

by radioimmunoassay after plasma extraction using ethanol as previously described (Castro, Figueiredo et al. 1995).

5. Testing procedure for TMJ pain

The design of this study follows that used by Roveroni et al, 2001. Testing sessions took place between 10:00 and 12:00 a.m. in a quiet room maintained at (23±1°C). Immediately after the period of stress procedures, each animal was briefly anesthetized by inhalation of halothane to allow the TMJ injection. Rats received a 50 μl injection of formalin solution prepared from commercially (SIGMA) available stock formalin (an aqueous solution of 37% of formaldehyde) further diluted in 0.9% NaCl (saline) to concentration of 1.5% into the left TMJ region. The injections were performed via a 30-gauge needle introduced into the left TMJ capsule. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 μl) previously filled with formalin 1.5%. Following the TMJ injection, the rat was placed in the test chamber (30 x 30 x 30 cm mirrored-wood chamber with glass at the front side) and nociceptive behavioral responses characterized by rubbing the orofacial region (amount of time—seconds) and flinching the head (number of head flinches) were quantified for 30 min (10 blocks of 3 min). Considering that the flinching of the head behavior followed a uniform pattern of 1 s in duration, each flinching was expressed as 1 s. The combination (sum) of both behaviors provides a better measure of pain intensity than any single behavior (Roveroni, Parada et al. 2001; Gameiro, Arthuri et al. 2003). At the end of each experiment, Evans blue dye (0.1%, 5 mg/ kg – SIGMA) was injected systemically (via cardiac) in order to confirm the TMJ injection site at postmortem, as previously described (Haas, Nakanishi et al. 1992) by the visual

examination of formalin-induced plasma extravasation of Evans blue dye bond to plasma protein.

6. Assessing opioid system on nociception induced by stress

In case of stressed animals (acute, sub-chronic or chronic stress) presenting a significant reduction on nociception indicating analgesia, the sensitivity to a specific opioid antagonist to kappa receptor was tested. The kappa opioid antagonist nor-binaltorphimine (nor-BNI) (Binder, Machelska et al. 2001) (200 μg/ 25 μl – SIGMA) was dissolved in saline. Because it has been reported that nor-BNI may not be selective for kappa opioid receptors until several hours after its administration (Schmidt, Tambeli et al. 2002), nor-BNI was administered into the left TMJ region one day (24 hours) prior to the experiment.

7. Statistical analysis

Statistical analysis of plasmatic corticosterone data was made using Two-way ANOVA on Ranks. The sum of rubbing and flinching responses exhibited by each animal was computed. The data were analyzed by Two-way ANOVA on Ranks followed by Tukey or Student–Newman–Keuls post-hoc tests, as appropriate. All values are given as mean ± standard deviation (SD). A level of 5% was taken as evidence of statistical significance. All statistical analyses were performed using SIGMA STAT version 3.0 for Windows – licensed to University of Campinas.

Results

1. Effects of stress procedures on plasmatic corticosterone

This experiment was carried out to define the efficacy of different various restraint protocols in inducing stress-like hormonal modifications. The corticosterone levels in female rats during proestrus and estrus phases of the estrous cycle are shown in Fig 1. There was a significant increase in plasmatic corticosterone levels after the various stress protocols when compared to controls ($p < 0.001$). The differences between the phases of the estrous cycle were not significant ($p = 0.689$).

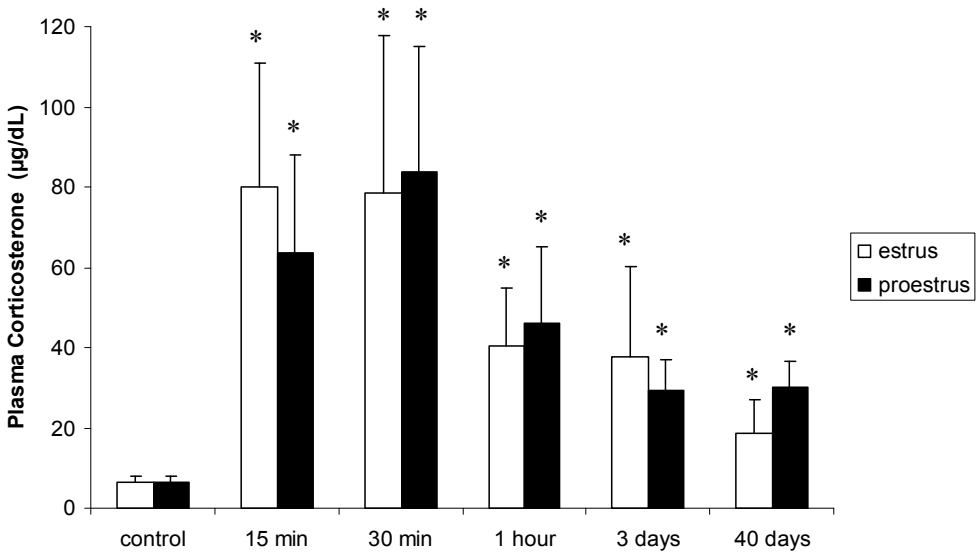

Fig 1. Basal and stress-induced plasmatic corticosterone level after the different stress procedures in proestrus and estrus phases of estrous cycle. Each column represents the mean and error bars indicate the standard deviation (S.D). Number of subjects was set as n= 8/group. (*) Indicates significant difference when compared to controls ($p<0.001$, Two-way ANOVA).

2. Effect of acute stress on nociceptive behavioral responses

The exposure to a single restraint session for 15 min or 30 min did not affect the nociceptive responses evoked by formalin 1.5% injected in TMJ of rats (Fig. 2). There were no statistical differences ($p = 0.146$) between the controls (non-stressed) and the stressed groups in the estrus and proestrus phase, but there was a significant difference between estrus vs proestrus in control rats ($p < 0.001$).

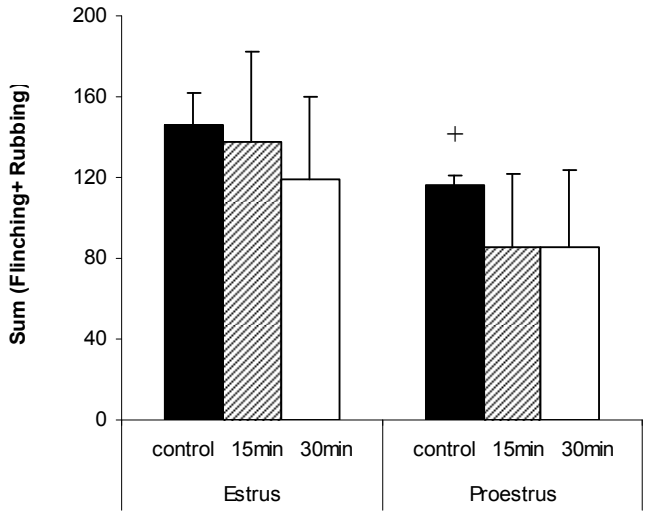

Fig. 2. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 µl, 1.5%) previously submitted to 15 min or 30 min of restraint (n= 6/group) or left undisturbed in their home cage (n= 6/group) in the estrus and proestrus phase. Each column represents the mean. Error bars indicate the SD. No significant differences were found in nociceptive responses for controls vs. stressed groups ($p = 0.146$, Two-way ANOVA). (+) Significant difference between estrus vs proestrus in control rats ($p < 0.001$, Two-way ANOVA).

The exposure to a single restraint session for 1 h affected the nociceptive responses evoked by formalin 1.5% injected in TMJ of rats (Fig. 3). The 1 h stressed animals presented analgesia (a significant decrease in the nociceptive behavioral responses) when compared to controls. A statistically significant decrease on nociceptive responses in the stressed (1 h) proestrus group was observed when compared to the estrus group ($p < 0.001$).

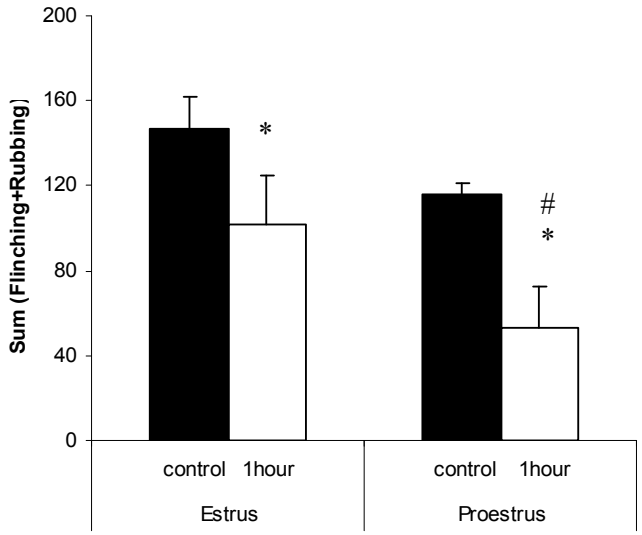

Fig. 3. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 µl, 1.5%) previously submitted to 1 h of restraint (n= 6/group) or left undisturbed in their home cage (n= 6/group) in estrus and proestrus phases. Each column represents the mean. Error bars indicate the SD. (*) Significant difference between the controls and stressed groups, ($^{\#}$) significant difference between the stressed group of different phases (estrus vs proestrus) – Two-way ANOVA.

3. Effect of sub-chronic stress on nociceptive behavioral responses

Three restraint sessions (1 h daily for 3 days) did not affect the nociceptive responses evoked by formalin 1.5% injected in TMJ of rats in estrus and proestrus phase (Fig. 4). There was no statistical difference ($p = 0.146$) between the controls (non-stressed) and the stressed groups.

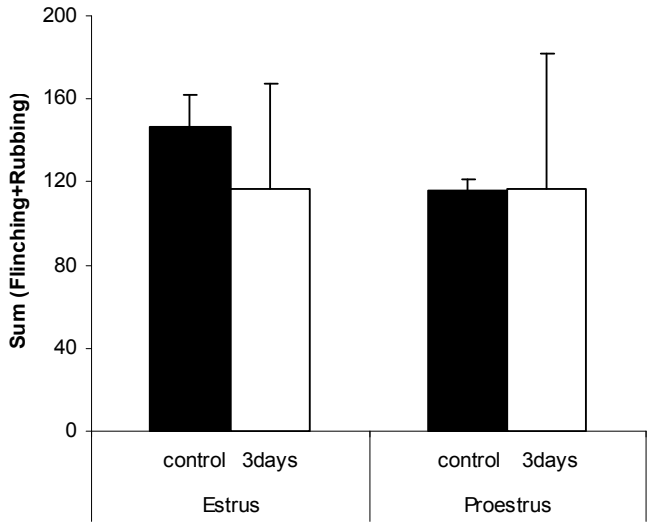

Fig. 4. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 µl, 1.5%) previously submitted to 1h daily for 3 days of restraint (n= 6/group) or left undisturbed in their home cage (n= 6/group) in estrus and proestrus phases. Each column represents the mean. Error bars indicate the SD. No significant differences were found in nociceptive responses for controls vs. stressed groups ($p = 0.146$, Two-way ANOVA).

4. Effect of chronic stress on nociceptive behavioral responses

The exposure to chronic restraint sessions for 1 h daily for 40 days did not affect the nociceptive responses evoked by formalin 1.5% injected in TMJ of rats in estrus and proestrus phase (Fig. 5). There were no statistical differences ($p > 0.05$) between the controls (non-stressed) and the stressed groups.

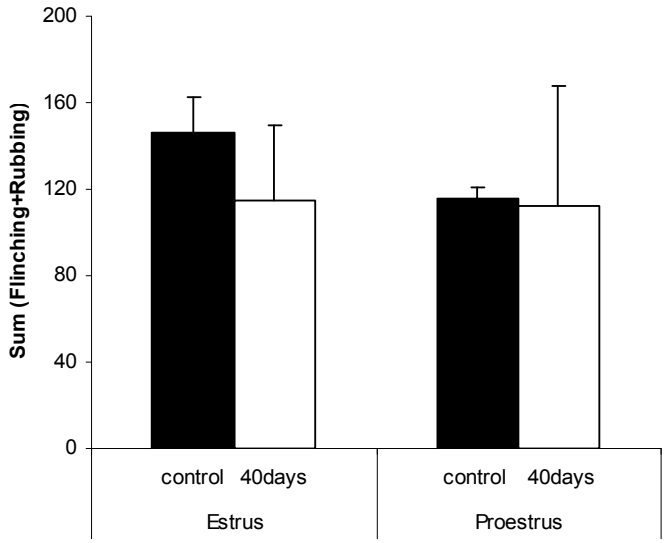

Fig. 5. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 μl, 1.5%) previously submitted to 1 h/40 days of restraint (n= 6/group) or left undisturbed in their home cage (n= 6/group) in estrus and proestrus phases. Each column represents the mean. Error bars indicate the SD. No significant differences were found in nociceptive responses for controls vs. stressed groups ($p > 0.05$, Two-way ANOVA).

5. Effect of nor-BNI on nociception in rats submitted to acute restraint stress

The local pre-administration (24 h before the stress and TMJ formalin test) of nor-BNI partially revert the stress-induced analgesia in the proestrus females ($p < 0.009$; Fig. 6). This effect was not observed in the estrus females (Fig. 6).

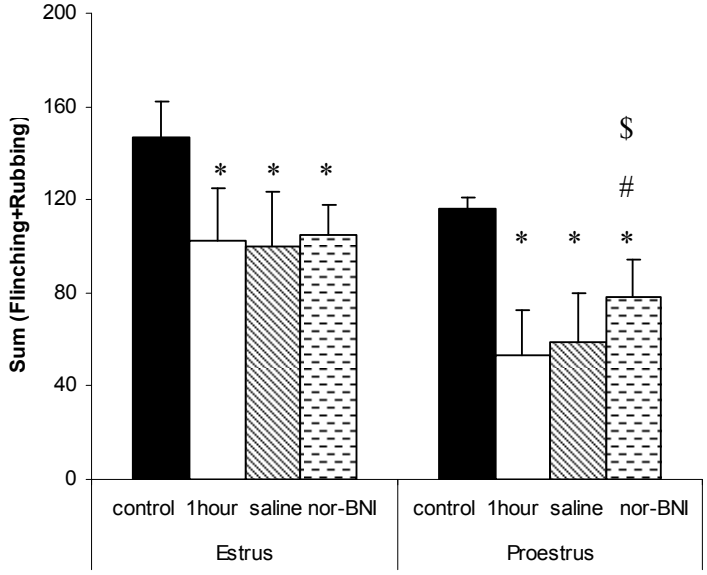

Fig. 6. Effects of control, stressed (1 h), saline (+ 1 h) and nor-BNI (+1 h) on formalin-treated animals (50 µl, 1.5%) in estrus and proestrus phases (n= 6/group). Each column represents the mean. Error bars indicate the SD. (*) Indicates significant difference when compared to controls ($p < 0.001$). ($^{\#}$) indicates significant difference when compared to different phases (estrous and proestrus) ($p < 0.001$). ($^{\$}$) Indicates significant difference when compared to stressed groups (nor-BNI vs 1 h) ($p < 0.009$) – Two-way ANOVA.

6. Effect of nor-BNI on nociception in rats not submitted to stress

The proestrus phase affected the nociceptive responses evoked by formalin 1.5% injected in TMJ of rats not submitted to restraint stress. The decrease in the sum of nociceptive behaviors (flinching+rubbing) was statistically significant ($p < 0.001$, Fig. 7) when the proestrus female rats were compared to the estrus. However, the local pre-administration of nor-BNI did not affect the nociceptive responses in both estrus and proestrus females (Fig.7).

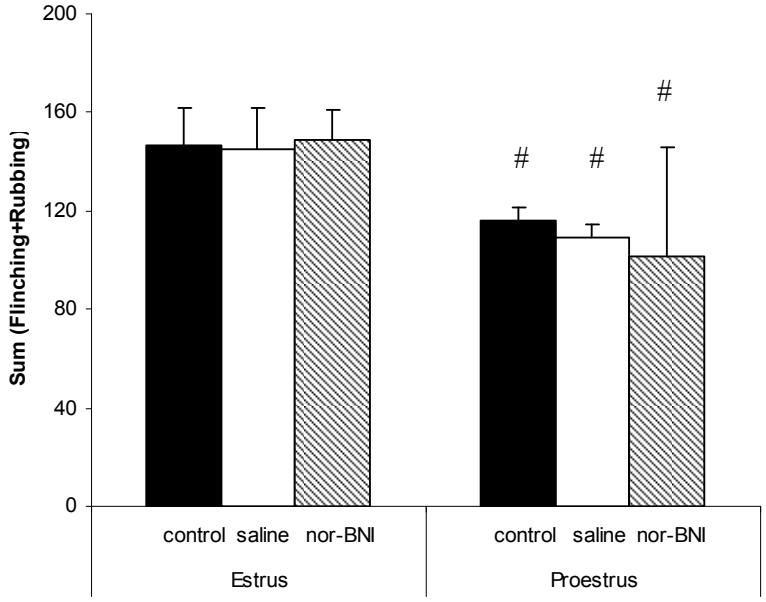

Fig. 7. Effects of nor-BNI and control saline group on unstressed formalin-treated animals (50 µl, 1.5%) in estrus and proestrus phases (n= 6/group). Each column represents the mean. Error bars indicate the SD. ($^{\#}$) Indicates significant difference when compared to estrus group ($p < 0.001$, Two-way ANOVA).

Discussion

Numerous animal studies have demonstrated that pain-like behavior evoked by cutaneous (Martinez-Gomez, Cruz et al. 1994), deep, or visceral tissue stimulation (Kayser, Berkley et al. 1996; Giamberardino, Berkley et al. 1997; Ness, Lewis-Sides et al. 2001) varies throughout the estrous cycle. LeResche et al. (2003), using a human clinical assay, suggested that temporomandibular pain in women is highest at times of lowest estrogen. In animals, during the estrous cycle, prolactin, LH and FSH remain low and increase in the afternoon of the proestrus phase. Estradiol levels begin to increase at metestrus, reaching peak levels during proestrus and returning to baseline at estrus. Progesterone secretion also increases during metestrus and diestrus with a decrease afterwards. Then the progesterone value rises to reach its second peak towards the end of proestrus (Spornitz, Socin et al. 1999; Marcondes, Bianchi et al. 2002). Although studies in non–human beings generally support the view that gonadal hormones influence nociception in females, the factors involved in mediating this effect are not well understood (Terner, Lomas et al. 2005).

Moreover, it seems that there are no experimental studies regarding the effects of stress on modulation of nociceptive input from articular tissue in female rats in estrus and proestrus phases

A variety of environmental and/or stressful stimuli have been shown to elicit analgesia, a phenomenon often referred to as stress-induced analgesia (SIA) (Watkins, Cobelli et al. 1982; Furuta, Onodera et al. 2003; Gameiro, Andrade Ada et al. 2005; King, Devine et al. 2007). For example, some evidence suggests that female rats are most sensitive to thermal nociceptive stimuli in proestrus (Kayser, Berkley et al. 1996; Vincler, Maixner et al. 2001) when estrogen and progesterone levels peak, whereas others suggest peak

sensitivity during estrus (Martinez-Gomez, Cruz et al. 1994; Kayser, Berkley et al. 1996; Stoffel, Ulibarri et al. 2003) when estrogen and progesterone levels are relatively low. These discrepancies might, in part, be related to the type of nociceptive stimulus used in the experiments. For example, lower nociceptive thresholds are typically observed during estrus with mechanical and electrical nociceptive stimuli (Kayser, Berkley et al. 1996), while no differences in thresholds are observed across phases with chemical stimuli (Vincler, Maixner et al. 2001) and higher thresholds are observed during estrus and proestrus with visceral stimuli (Bradshaw, Temple et al. 1999; Terner, Lomas et al. 2005). In the present study, a single exposure (1 h) to restraint stress reduced the nociceptive behavioral responses evoked by nociceptive chemical stimulation (formalin 1.5%) of the female rat's TMJ in estrus and proestrus phases, but the stress-induced analgesia was higher in the proestrus phase. This finding is in accordance with those obtained in another study (Ryan and Maier 1988), in which female rats with high hormonal levels exhibited higher stress-induced analgesia in the tailshocks test.

The evidence indicating that gonadal hormones influence this pain sensitivity comes from studies demonstrating a trend for higher pain thresholds and tolerance levels during the follicular phase when estrogen levels peak (Riley, Robinson et al. 1999; Terner, Lomas et al. 2005). These results support the hypothesis suggested by other authors that elevated hormone levels are responsible for reduced pain sensitivity (Medina, Dawson-Basoa et al. 1993; Liu and Gintzler 2000; Gupta, Kelson et al. 2001; Tall and Crisp 2004; Arthuri, Gameiro et al. 2005). The higher susceptibility to pain behavior in the present study was also observed in female rats with lower hormonal levels.

In the current study, the relationship between hormonal variations and nociceptive responses induced by stress was evaluated. A significant increase in plasma corticosterone level was observed after acute (15 min, 30 min, 1 h) restraint stress sessions, although only the 1 h stress was able to alter the nociception evoked by the TMJ formalin test. It is well established that the types of stressor, its intensity, duration, as well as the type of the nociceptive model used, affect the stress-induced changes on pain modulation (Gameiro, Gameiro et al. 2006). On the other hand, when animals are repeatedly submitted to the same stressor, some behavioral and physiological consequences of stress exposure are reduced (habituation). For example, corticosterone levels are reduced after repeated exposure to the same stressor (Marti and Armario 1998; Torres, Gamaro et al. 2001). In this study, sub-chronic and chronic restraint stress didn't present any difference on corticosterone levels and nociceptive behavioral responses when compared to controls.

We have already showed that restraint stress can release endogenous opioids (Gameiro, Andrade Ada et al. 2005), but this effect was not able to induce analgesia in male rats submitted to acute restraint stress before the TMJ formalin test. Therefore, the present results suggest that female sex hormones can modulate the stress-induce analgesia, since female rats (in both estrus and proestrus phases) exhibited a decreased nociceptive responses in the TMJ formalin test, and this analgesic effect was higher in the proestrus females. We suggest that female rats are more prone to exhibit stress-induced changes, regarding both the hormonal and behavioral ones. The higher corticosterone levels after the stress procedures, when compared with those that we previously found in males, also support this hypothesis.

Stress and opioid agents act throughout the neuraxis to modulate sensory, motor, autonomic, motivational and emotional responses to nociceptive stimulation (Houshyar, Cooper et al. 2001; Hebb, Poulin et al. 2005; King, Devine et al. 2007). Studies examining the involvement of exogenous opioids in the peripheral modulation of TMJ pain (Bakke, Hu et al. 1998; Cai, Cairns et al. 2001) support the presence of peripheral opioid receptors in TMJ that may have a role in modulating nociceptive responses (Clemente, Parada et al. 2004; Arthuri, Gameiro et al. 2005). In normal cycling females, morphine and buprenorphine (opioid agonist) were generally most potent in metestrus and proestrus and least potent in estrus (Terner, Lomas et al. 2005). In the last experiment, we tested the nociceptive responses in control and single restrained (1 h) rats previously injected with nor-BNI (200 μg/ 25 μl) (opioid antagonist) in the TMJ formalin test. Our results demonstrated that the local pre-administration of nor-BNI reduced the stress-induced analgesia in proestrus females. The effect of nor-BNI in the estrus females was not significant, indicating that higher hormonal levels can increase the stress-induced analgesia via opioid mechanism. Although it has been described that nor-BNI induces hyperalgesia after several hours from its administration (Schmidt, Tambeli et al. 2002), this finding was not observed in our study, since the nor-BNI applied to unstressed rats did not evoke a hyperalgesic effect. This result is also supported by those found in the study of Arthuri, Gameiro et al. (2005).

The present data indicate that κ-receptors are in part involved in mediating the stress-induced analgesia in female rats. The administration of nor-BNI before formalin in unstressed rats did not alter the nociceptive responses in the TMJ formalin test, suggesting that the κ-opioid antagonist selectivity of nor-BNI observed in the present results was related to the gonadal hormones.

To conclude, acute restraint stress (1 h) can produce analgesia in proestrus and estrus female rats, but this effect is higher in the proestrus phase. The sub-chronic and chronic restraint stress didn't affect the nociceptive responses in female rats. Moreover, this study demonstrates that κ-opioid receptor activation is involved in the stress-induced analgesia observed in the proestrus phase. The data observed in this study could be of clinical value and very important for understanding the neurobiological mechanisms concerning temporomandibular disorders and the relationship among sex hormones, nociception, and stress.

Acknowledgments

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References

Amit, Z. and Z. H. Galina (1988). "Stress induced analgesia plays an adaptive role in the organization of behavioral responding." Brain Res Bull **21**(6): 955-8.

Arthuri, M. T., G. H. Gameiro, et al. (2005). "Peripheral effect of a kappa opioid receptor antagonist on nociception evoked by formalin injected in TMJ of pregnant rats." Life Sci **76**(10): 1177-88.

Bakke, M., J. W. Hu, et al. (1998). "Morphine application to peripheral tissues modulates nociceptive jaw reflex." Neuroreport **9**(14): 3315-9.

Bereiter, D. A. (2001). "Sex differences in brainstem neural activation after injury to the TMJ region." Cells Tissues Organs **169**(3): 226-37.

Binder, W., H. Machelska, et al. (2001). "Analgesic and antiinflammatory effects of two novel kappa-opioid peptides." Anesthesiology **94**(6): 1034-44.

Bradshaw, H. B., J. L. Temple, et al. (1999). "Estrous variations in behavioral responses to vaginal and uterine distention in the rat." Pain **82**(2): 187-97.

Buskila, D. (2001). "Fibromyalgia, chronic fatigue syndrome, and myofascial pain syndrome." Curr Opin Rheumatol **13**(2): 117-27.

Butcher, R. L., W. E. Collins, et al. (1974). "Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat." Endocrinology **94**(6): 1704-8.

Cai, B. B., B. E. Cairns, et al. (2001). "Sex-related suppression of reflex jaw muscle activity by peripheral morphine but not GABA." Neuroreport **12**(16): 3457-60.

Cairns, B. E., B. J. Sessle, et al. (2001). "Temporomandibular-evoked jaw muscle reflex: role of brain stem NMDA and non-NMDA receptors." Neuroreport **12**(9): 1875-8.

Castro, M., F. Figueiredo, et al. (1995). "Time-course of hypothalamic CRH and pituitary ACTH contents, and pituitary responsiveness to CRH stimulation after bilateral adrenalectomy." Horm Metab Res **27**(1): 10-5.

Clemente, J. T., C. A. Parada, et al. (2004). "Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint." Neurosci Lett **372**(3): 250-5.

Craft, R. M., J. S. Mogil, et al. (2004). "Sex differences in pain and analgesia: the role of gonadal hormones." Eur J Pain **8**(5): 397-411.

da Silva Torres, I. L., S. N. Cucco, et al. (2003). "Long-lasting delayed hyperalgesia after chronic restraint stress in rats-effect of morphine administration." Neurosci Res **45**(3): 277-83.

Davis, M. C., A. J. Zautra, et al. (2001). "Vulnerability to stress among women in chronic pain from fibromyalgia and osteoarthritis." Ann Behav Med **23**(3): 215-26.

Denucci, D. J., R. A. Dionne, et al. (1996). "Identifying a neurobiologic basis for drug therapy in TMDs." J Am Dent Assoc **127**(5): 581-93.

Dworkin, S. F. and L. LeResche (1992). "Research diagnostic criteria for temporomandibular disorders: review, criteria, examinations and specifications, critique." J Craniomandib Disord **6**(4): 301-55.

Ely, D. R., V. Dapper, et al. (1997). "Effect of restraint stress on feeding behavior of rats." Physiol Behav **61**(3): 395-8.

Fischer, L., J. T. Clemente, et al. (2007). "The protective role of testosterone in the development of temporomandibular joint pain." J Pain **8**(5): 437-42.

Furuta, S., K. Onodera, et al. (2003). "Involvement of adenosine A1 receptors in forced walking stress-induced analgesia in mice." Methods Find Exp Clin Pharmacol **25**(10): 793-6.

Gamaro, G. D., M. H. Xavier, et al. (1998). "The effects of acute and repeated restraint stress on the nociceptive response in rats." Physiol Behav **63**(4): 693-7.

Gameiro, G. H., S. Andrade Ada, et al. (2005). "The effects of restraint stress on nociceptive responses induced by formalin injected in rat's TMJ." Pharmacol Biochem Behav **82**(2): 338-44.

Gameiro, G. H., M. T. Arthuri, et al. (2003). "Effects of ethanol on deep pain evoked by formalin injected in TMJ of rat." Life Sci **73**(26): 3351-61.

Gameiro, G. H., P. H. Gameiro, et al. (2006). "Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress." Physiol Behav **87**(4): 643-9.

Gear, R. W., N. C. Gordon, et al. (1996). "Gender difference in analgesic response to the kappa-opioid pentazocine." Neurosci Lett **205**(3): 207-9.

Giamberardino, M. A., K. J. Berkley, et al. (1997). "Pain threshold variations in somatic wall tissues as a function of menstrual cycle, segmental site and tissue depth in non-dysmenorrheic women, dysmenorrheic women and men." Pain **71**(2): 187-97.

Gupta, D. S., A. B. Kelson, et al. (2001). "Ovarian sex steroid-dependent plasticity of nociceptin/orphanin FQ and opioid modulation of spinal dynorphin release." J Pharmacol Exp Ther **298**(3): 1213-20.

Haas, D. A., O. Nakanishi, et al. (1992). "Development of an orofacial model of acute inflammation in the rat." Arch Oral Biol **37**(5): 417-22.

Hebb, A. L., J. F. Poulin, et al. (2005). "Cholecystokinin and endogenous opioid peptides: interactive influence on pain, cognition, and emotion." Prog Neuropsychopharmacol Biol Psychiatry **29**(8): 1225-38.

Houshyar, H., Z. D. Cooper, et al. (2001). "Paradoxical effects of chronic morphine treatment on the temperature and pituitary-adrenal responses to acute restraint stress: a chronic stress paradigm." J Neuroendocrinol **13**(10): 862-74.

Kayser, V., K. J. Berkley, et al. (1996). "Estrous and sex variations in vocalization thresholds to hindpaw and tail pressure stimulation in the rat." Brain Res **742**(1-2): 352-4.

Khasar, S. G., O. A. Dina, et al. (2005). "Estrogen regulates adrenal medullary function producing sexual dimorphism in nociceptive threshold and beta-adrenergic receptor-mediated hyperalgesia in the rat." Eur J Neurosci **21**(12): 3379-86.

King, C. D., D. P. Devine, et al. (2007). "Opioid modulation of reflex versus operant responses following stress in the rat." Neuroscience **147**(1): 174-82.

LeResche, L. (1997). "Epidemiology of temporomandibular disorders: implications for the investigation of etiologic factors." Crit Rev Oral Biol Med **8**(3): 291-305.

LeResche, L., L. Mancl, et al. (2003). "Changes in temporomandibular pain and other symptoms across the menstrual cycle." Pain **106**(3): 253-61.

Liu, N. J. and A. R. Gintzler (2000). "Prolonged ovarian sex steroid treatment of male rats produces antinociception: identification of sex-based divergent analgesic mechanisms." Pain **85**(1-2): 273-81.

Marcondes, F. K., F. J. Bianchi, et al. (2002). "Determination of the estrous cycle phases of rats: some helpful considerations." Braz J Biol **62**(4A): 609-14.

Marti, O. and A. Armario (1998). "Anterior pituitary response to stress: time-related changes and adaptation." Int J Dev Neurosci **16**(3-4): 241-60.

Martinez-Gomez, M., Y. Cruz, et al. (1994). "Assessing pain threshold in the rat: changes with estrus and time of day." Physiol Behav **55**(4): 651-7.

Medina, V. M., M. E. Dawson-Basoa, et al. (1993). "17 beta-estradiol and progesterone positively modulate spinal cord dynorphin: relevance to the analgesia of pregnancy." Neuroendocrinology **58**(3): 310-5.

Ness, T. J., A. Lewis-Sides, et al. (2001). "Characterization of pressor and visceromotor reflex responses to bladder distention in rats: sources of variability and effect of analgesics." J Urol **165**(3): 968-74.

Przewlocki, R., W. Lason, et al. (1987). "The influence of chronic stress on multiple opioid peptide systems in the rat: pronounced effects upon dynorphin in spinal cord." Brain Res **413**(2): 213-9.

Quintero, L., M. Moreno, et al. (2000). "Long-lasting delayed hyperalgesia after subchronic swim stress." Pharmacol Biochem Behav **67**(3): 449-58.

Raphael, K. G., M. N. Janal, et al. (2004). "Comorbidity of fibromyalgia and posttraumatic stress disorder symptoms in a community sample of women." Pain Med **5**(1): 33-41.

Riley, J. L., 3rd, M. E. Robinson, et al. (1999). "A meta-analytic review of pain perception across the menstrual cycle." Pain **81**(3): 225-35.

Roveroni, R. C., C. A. Parada, et al. (2001). "Development of a behavioral model of TMJ pain in rats: the TMJ formalin test." Pain **94**(2): 185-91.

Ryan, S. M. and S. F. Maier (1988). "The estrous cycle and estrogen modulate stress-induced analgesia." Behav Neurosci **102**(3): 371-80.

Schmidt, B. L., C. H. Tambeli, et al. (2002). "mu/delta Cooperativity and opposing kappa-opioid effects in nucleus accumbens-mediated antinociception in the rat." Eur J Neurosci **15**(5): 861-8.

Smith, M. S., M. E. Freeman, et al. (1975). "The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy." Endocrinology **96**(1): 219-26.

Spornitz, U. M., C. D. Socin, et al. (1999). "Estrous stage determination in rats by means of scanning electron microscopic images of uterine surface epithelium." Anat Rec **254**(1): 116-26.

Stoffel, E. C., C. M. Ulibarri, et al. (2003). "Gonadal steroid hormone modulation of nociception, morphine antinociception and reproductive indices in male and female rats." Pain **103**(3): 285-302.

Tall, J. M. and T. Crisp (2004). "Effects of gender and gonadal hormones on nociceptive responses to intraplantar carrageenan in the rat." Neurosci Lett **354**(3): 239-41.

Terner, J. M., L. M. Lomas, et al. (2005). "Influence of estrous cycle and gonadal hormone depletion on nociception and opioid antinociception in female rats of four strains." J Pain **6**(6): 372-83.

Torres, I. L., G. D. Gamaro, et al. (2001). "Effect of acute and repeated restraint stress on glucose oxidation to CO2 in hippocampal and cerebral cortex slices." Braz J Med Biol Res **34**(1): 111-6.

Vincler, M., W. Maixner, et al. (2001). "Estrous cycle modulation of nociceptive behaviors elicited by electrical stimulation and formalin." Pharmacol Biochem Behav **69**(3-4): 315-24.

Watkins, L. R., D. A. Cobelli, et al. (1982). "Opiate vs non-opiate footshock-induced analgesia (FSIA): the body region shocked is a critical factor." Brain Res **242**(2): 299-308.

Yamada, K. and T. Nabeshima (1995). "Stress-induced behavioral responses and multiple opioid systems in the brain." Behav Brain Res **67**(2): 133-45.

Yunus, M. B. (2002). "Gender differences in fibromyalgia and other related syndromes." J Gend Specif Med **5**(2): 42-7.

Zimmermann, M. (1983). "Ethical guidelines for investigations of experimental pain in conscious animals." Pain **16**(2): 109-10.

CAPÍTULO 3

Article

Effects of different periods of restraint stress on anxiety in female rats

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Running head: Stress's effects on female rats' anxiety

Abstract

A. P. BOTELHO, R. COSTA, F. K. MARCONDES AND M. C. F. A. VEIGA. *Effects of different periods of restraint stress on anxiety in female rats*. PHYSIOL BEHAV 00(0) 000-000, 2009. — The aim of this study was to evaluate the effect of estrous cycle and stress on anxiety levels. Female rats in proestrus or estrus phase, were submitted to acute restraint stress (15; 30 or 60 min), or exposed to sub-chronic (3 days—1 h/day) or chronic stress (40 days—1 h/day). Immediately after the last stress session, the animals were killed to collect blood for corticosterone radioimmunoassay determinations or submitted to the elevated plus-maze. All stress protocols significantly raised the levels of corticosterone, without any difference in relation to the phases of estrous cycle. After various protocols of stress, there were no significant differences in the percentage of entries into the open arms and in the number of entries into the end of open arms. Acutely stressed rats (60 min) presented a decreased in the percentage of time into the open arms and chronically stressed rats showed an increase in the number of entries into the closed arms. Since the percentage of times into the open arms of the plus-maze is inversely related to the level of anxiety, we conclude that the higher levels of anxiety in female rats are modulated by the exposure to 60 minutes of restraint stress.

Keywords: Stress, Anxiety, Estrous cycle, Corticosterone.

Introduction

The influence of gender, reproductive cycle, and gonadal hormones on many functions unrelated to reproduction has been observed in humans and laboratory animals [1], especially on the physiological responses to stressors [2, 3]. In humans, gonadal hormones have been implicated in the development and maintenance of several mental disorders including anxiety, depression, and schizophrenia [4]. Evidence in animal models supports a role of estrogen in mood disorders. In both male and female rodents, estrogens have been shown to decrease depressive and anxiety-related behavior [5, 6]. Therefore, the reported prevalence of depression in women is approximately twice that of men [7-9] and variations in the ovarian hormones appear to contribute to the etiology of these disorders in women [4, 10]. These differences are probably underlined by sexual dimorphisms observed in the hypothalamic–pituitary–adrenal (HPA) axis activity/response to stress [11].

Being the stress one of the most important factor that influences the behavior, particularly on cognition and emotion [12], it can trigger a cascade of events leading to activation of the hypothalamo-pituitary- adrenal (HPA) axis [13-15]. Stress-activated inputs to the parvocellular division of the paraventricular nucleus of the hypothalamus (pPVN) release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) [16]. CRH and AVP act at the anterior pituitary to release adrenocorticotropin (ACTH) [17], which in turn stimulates the release of corticosterone from the adrenal cortex [18], including in rats [14].

While HPA axis activation is generally adaptive, hyper-activation may be maladaptive and has been associated with increased indices of anxiety and depression in both humans and rodents [4, 19, 20].

The basal level of anxiety may influence the perception of the challenge posed by a stressor [21], modifying the stimulation extent of the limbic system, sympathetic nerves, and hypothalamic–pituitary–adrenal axis, depending on the gender and on the estrous cycle phase [2] in animals submitted to anxiety-related behavior tests.

In the field of experimental anxiety research, the elevated plus-maze is a widely used behavioral paradigm, which presumably measures fear-motivated avoidance behavior [22, 23] and anxiety-related behavior [9, 11], based on the natural fear of open and elevated alleys [24]. During a typical plus-maze test, animals will spend most of the time in the closed arms of the maze [25]. In this context, it was observed that anxiolytic drugs increase the number of entries onto and the time spent on open arms [24], whereas anxiogenic agents do the opposite[23].

Based on the fact that the estrous cycle can influence some rodent behaviors, that sex steroid levels vary during the estrous cycle, and that steroid hormones can modulate neuronal function, we measured basal and stress-induced corticosterone and anxiety levels following exposure to distinct restraint stress protocols, in female rats in high (proestrus) and low (estrus) ovarian hormonal levels.

Methods

1. Animals

Three-month-old female Wistar rats (n =10/group) weighing 250–300 g at the beginning of the experiment, and in different stages of their estrous cycles were studied. The rats were obtained from Centro Multidisciplinar de Investigação Biológica (CEMIB) - UNICAMP, Brazil and were housed in groups of five and maintained in a temperature-controlled room (23±1°C) with a 12/12 h light–dark cycle (lights on at 6:00 am) and

were given free access to food and water. The rats were handled prior to behavioral testing. Procedures were performed between 08:00 am and 1:00 pm. The study was conducted in accordance with the Brazilian College of Experimentation Guidelines and approved by the Committee on Animal Research of the University of Campinas (protocol 938-1).

2. Estrous phase determination

Proestrus and estrus phases were determined by daily microscope examination of vaginal smears taken by gentle lavage, between 8:00 and 10:00 a.m. Proestrus was identified by the predominance (> 70%) of nucleated epithelial cells and estrus by anucleated cornified cells, in rats with at least two consecutive regular 4-day cycles [26, 27]. These phases were chosen because they represent phases of high and low ovarian hormonal level, respectively [28].

3. Stress exposure

All animals were stressed by restraint. In the acute model, the animals were stressed during 15 min, 30 min or 1 h for a single exposure [29]. In the sub-chronic model the animals were stressed 1 h daily for 3 days [30], and females started the procedures of sub-chronic restraint stress two phases of estrous cycle (two days) before the pre-determined phases. In the chronic model, 1 h daily, 5 days per week for 40 days [31, 32], females were submitted to estrous phase determination in the thirtieth second day of restraint stress until the fortieth second day. The experiments were carried out between the thirty-eighth and fortieth second day, because it is not possible to predict the exactly estrous phase in the fortieth day.

Restraint stress protocols were carried out by placing the animal in a plastic restraint device (adjustable in size depending on the animal's weight). The area of the tube could be adjusted individually and the tube was held firmly in place with adhesive tape. There was a 1-cm hole in the far end for breathing. The control group was not submitted to restraint. The restraint procedure was carried out in a separate quiet room between 9:00 and 11:00 a.m.

4. Hormonal assay

Blood of proestrus and estrus females were collected by decapitation under basal conditions (within 30 s of removal from the home cage; also referred to as the 0 min time point) or collected at the end of each restraint (within 30 s of removal from the restrainer). Rats were decapitated, and the blood was collected in tubes containing heparin. The blood was centrifuged at 2500 rpm, 4ºC for 10 min. The serum was collected and frozen at -20°C until use. Plasma corticosterone levels were determined by radioimmunoassay [33].

5. Evaluation of anxiety level

The elevated plus-maze test was used to assess the anxiety level induced by different stress protocols and different estrous cycle phases. The plus-maze apparatus used in these experiments was made of plywood and consisted of two open arms (50 cm long *x* 10 cm wide) and two closed arms (50 cm long *x* 10 cm wide, with 40 cm high walls) that extended from a central platform elevated 50 cm above the floor [2]. Rodents avoid the open arms of the plus maze so that decreases in time spent in and entries into the open arms are thought to reflect enhanced measures of anxiety [18]. Briefly, rats were placed in the central square facing an enclosed arm [34], and allowed to freely explore

the elevated plus-maze for 5 min [24]; each rat was tested only once. All tests were made immediately after the last stress session of the various protocols [15, 30, 60 min, 3 days (1 h/day) and 40 days (1 h/day) – n =10/group] and separated into proestrus and estrus phases, the controls were not previously stressed. Before the next rat was introduced, the maze was cleaned with a solution of 20% ethanol and dried [33]. A video-camera was located above the centre of the maze for monitoring the rats. The parameters evaluated was: 1) number of entries into the close arms, 2) percentage of entries into the open arms (100 X open/total), 3) number of entries into the end of the open arms and 4) percentage of time spent in the open arms (100 X open/total). These parameters evaluate: 1) locomotor activity, 2) locomotor activity and anxiety, 3) and 4) anxiety. All data were analyzed by the software EthoVision® (version 3.1 for windows) by Noldus, Netherlands— licensed to Fernanda Klein Marcondes.

6. Statistical analysis

Statistical analyses of plasma corticosterone data were made using the analysis of variance based on ranks (ANOVA-R) using SYSTAT 12 by Hearne Scientific Sofyware., Chicago, IL, USA. The comparison of anxiety-related behavior groups was made by analysis of variance based on ranks (ANOVA-R) for randomized trials with factorial arrangement of the levels of the factors phase of the estrous cycle and type of stress. When necessary, data were previously transformed to square-root or log, as indicated by the program SAS. The values for hormonal assays and anxiety behavior are expressed as mean ± standard error of the mean (S.E.M.). A level of 5% was taken as evidence of statistical significance. Anxiety data were analyzed using SAS (version 9.1 for windows) by Institute Inc., Cary, NC, USA.

Results

1. Effects of stress procedures on plasma corticosterone

The hormone levels in female rats during proestrus and estrus phases of the estrous cycle are shown in Fig. 1 (A – acute stress; B – sub-chronic stress and C – chronic stress). There was a significant increase in plasma corticosterone levels after various stress protocols when compared to control ($p < 0.001$), independent of the phase of the estrous cycle.

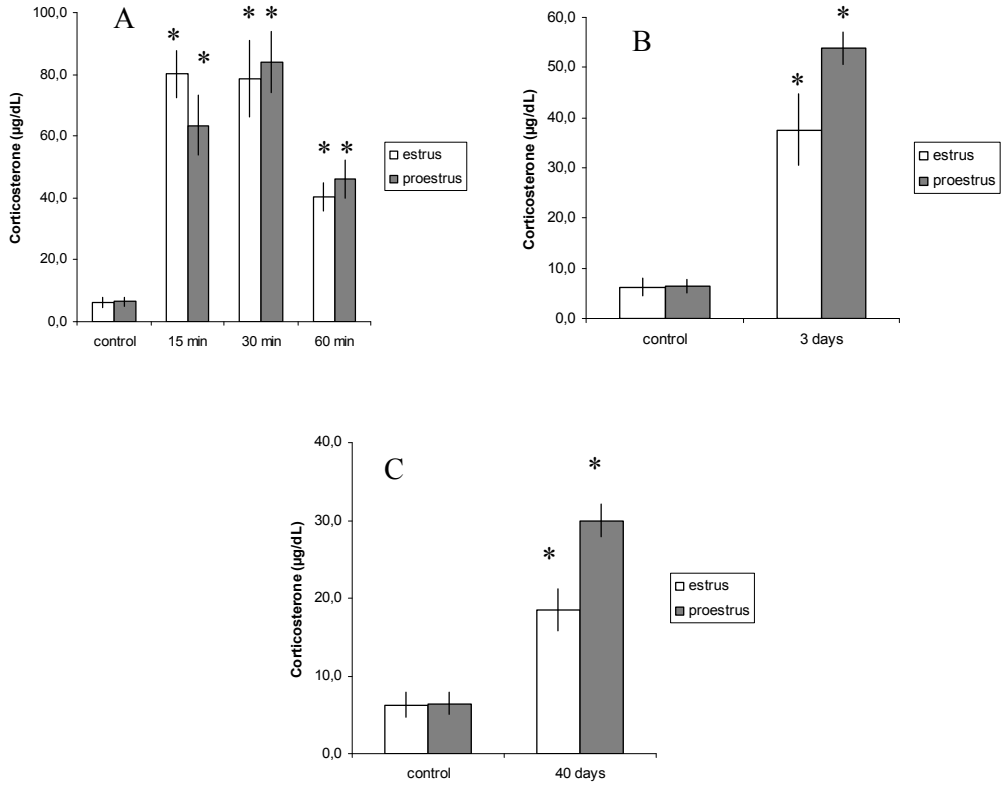

Fig 1. Basal and stress-induced plasma corticosterone levels after the various stress procedures in proestrus and estrus phases of estrous cycle [A – acute stress (control; 15; 30 and 60 min); B – sub-chronic stress (control; 3 days – 1 h/day) and C – chronic stress (control; 40 days – 1 h/day)]. Each column represents the mean and error bars indicate the standard error of the mean (S.E.M.). Number of subjects was set as n =8/group. (*) Indicates significant difference when compared to controls (ANOVA - $p < 0.001$).

2. Effects of stress on the anxiety levels (elevated plus-maze)

There were no differences in the performance of proestrus and estrus female rats in the plus-maze test. After different protocols of acute stress (15; 30 and 60 min) there were no significant differences in the number of entries into the closed arms (Fig 2.A), in the percentage of entries into the open arms (Fig 2.B) and in the number of entries into the end of the open arms (Fig 2.C). The sixty minutes stress protocol induced a decrease on the percentage of times into the open arms when compared to controls ($p \leq 0.05$, ANOVA-R, Fig 2.D).

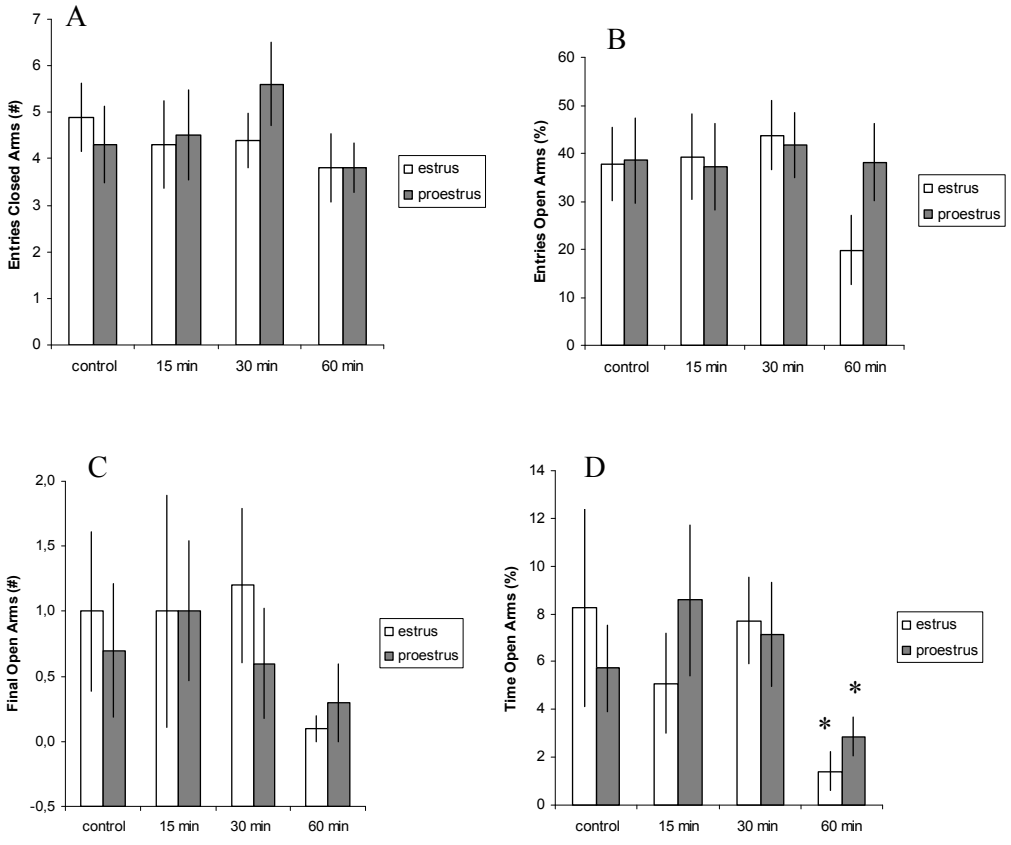

Figure 2. Effects of the various acute stress procedures in female in proestrus and estrus phases. Each column represents the mean. Error bars indicate the S.E.M. Number of subjects was set as n =10/group. A) number of entries into the closed arms; B) percentage of entries into the open arms; C) number of entries into the end of the open arms and D) percentage of times into the open arms. (*) Indicates a significant difference when compared to controls ($p \leq 0.05$, ANOVA-R).

The sub-chronic protocol of stress (3 days – 1 h/day) showed no significant difference when compared to controls in females in proestrus and estrus phases in all parameters evaluated (Fig 3. A, B, C, D).

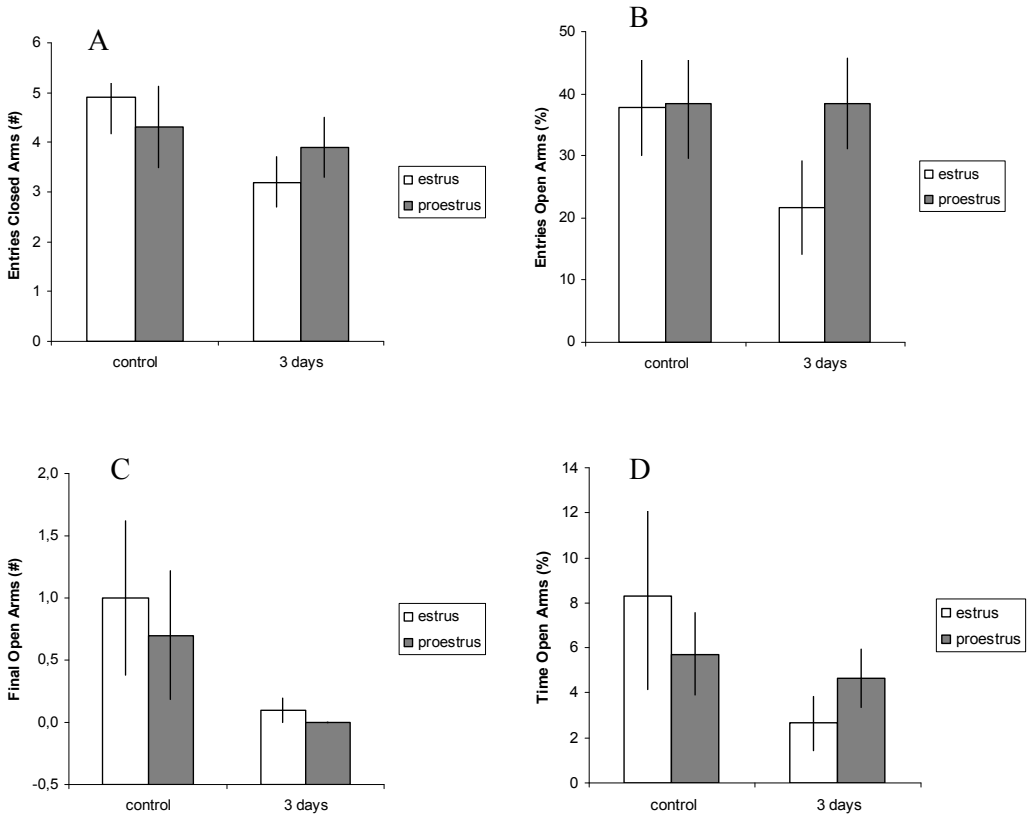

Fig 3. Effects of the sub-chronic stress procedure (3 days – 1 h/day) in female in proestrus and estrus phases. Each column represents the mean. Error bars indicate the S.E.M. Number of subjects was set as n =10/group. A) number of entries into the closed arms; B) percentage of entries into the open arms; C) number of entries into the end of the open arms and D) percentage of times into the open arms.

Chronically stressed rats (40 days – 1 h/day) in estrus and proestrus phases presented a significant increase in the number of entries into the closed arms when compared to controls ($p < 0.05$, ANOVA-R, Figure 4. A). There were no significant differences in the percentage of entries into the open arms (Fig 4.B), in the number of entries into the end of the open arms (Fig 4.C) and in the percentage of times into the open arms (Fig 4.D).

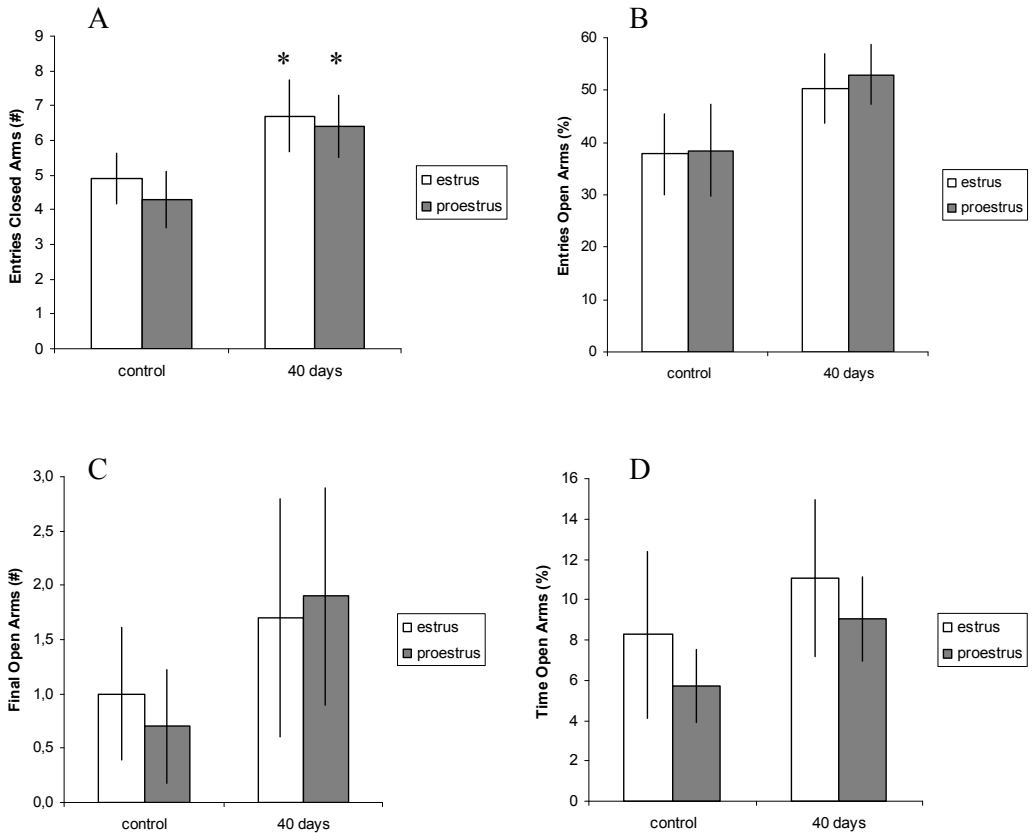

Fig 4. Effects of the chronic stress procedure (40 days – 1 h/day) in female in proestrus and estrus phases. Each column represents the mean. Error bars indicate the S.E.M. Number of subjects was set as n =10/group. A) number of entries into the closed arms; B) percentage of entries into the open arms; C) number of entries into the end of the open arms and D) percentage of times into the open arms.(*) Indicates a significant difference when compared to controls ($p < 0.05$, ANOVA-R).

Discussion

It has been described in the literature the effect of the estrous cycle on the anxiety level, in this study was not observed any significant difference related to estrous cycle. In some articles was observed a significant increase in the time spent as well as in the percentage of entries into the open arms during proestrus and estrus compared to diestrus and metestrus [35]. However, the paper by Nomikos and Spyraki [36] shows no significant difference between proestrus and diestrus [2].

In the opposite way, considering the corticosterone (CORT) level as an effective indication of stress [37], all restraint procedures, in this study, were able to induce stress. Plasma corticosterone levels have become important to assess the effects of different times of restraint stress procedures on the experimental animals. In this context, we have measured the plasma corticosterone to assess the anxiety level after different stress procedures. A significant increase in plasma corticosterone level was observed after acute (15 min, 30 min, 60 min), sub-chronic (3 days) and chronic (40 days) restraint stress sessions. Moreover, as expected, the increase in corticosterone levels was lower after chronic and sub-chronic stress when compared to acute protocols.

Sex differences in normal HPA activity are well established in animal models, with enhanced CORT secretion in response to physical and psychological stressors observed in females when compared to males [38-41]. This study presented similar results related to corticosterone levels when compared to previous study in our laboratory using male rats and the same stress protocols [33].

We chose the elevated plus-maze to evaluate anxiety, because such test is based on the natural neophobia of rodents [18, 42] and is used for studying anxiolytic stress and neurobiological mechanisms of anxiety [23]. A previous study had found that

intracerebroventricular administration of a 5-HT-3A receptor antagonist not blocked corticosterone responses to footshock or restraint [43]. However, this study was conducted in rats and suggests that the 5-HT-3A receptor normally has a more general stimulatory effect on acute stress-induced HPA activity [18].

Previous study in our laboratory, analyzed males rats using the same stress protocols, it was observed that males submitted to acute, sub-chronic and chronic restraint stress showed an increased on anxiety level (decreased percentage of time into the open arms and decreased percentage of entries on open arms) when compared to controls [33]. In this study female rats submitted to acute restrain stress (60 min) showed an increase on anxiety level, observed only a decreased on percentage of time into the open arms, this findings is not in accordance to another papers showing that female rats spend more time in the open arms of the plus-maze test than male rats, indicating a lower level of anxiety in females [2, 44], and showing that female rats are generally more active in such paradigms [45], like forced swimminig, showing increased signs of struggle compared to males [37].

Liang et al. (2008) [9], observed that females rats submitted to chronic mild stress and given a mixed diet presented an increase on anxiety level (percentage of time into the open arms) when compared to females given lab chow dietary. This study presented a decreased on percentage of time into the open arms on female rats submitted to acute stress (60 min), but no significant difference on submitted to chronic stress. On the other hand, experiments realized in male rats using chronic mild stress and chronic unpredictable stress presented decrease or ineffectiveness on anxiety levels, respectively [46, 47].

In a study using male rats, chronic immobilization stress (10 days – 2 h/day) reduced open-arm activity in the elevated plus-maze [48]. In another study, using female rats

stressed by chronic immobilization (10 days – 2 h/day), it was not observed significant difference in anxiety-like behavior. In our study, chronic stress was ineffective in induce anxiety, but increased locomotor activity in females which suggested that spontaneous locomotor activity largely reflects exploratory behavior [49].

Since the percentage of time of entries into the open arms of the plus-maze is inversely related to the level of anxiety and the number of entries into the closed arms is directly related to locomotor activity. We conclude that female rats did not presented any difference related to the estrous cycle; all stress protocols significantly raised the levels of corticosterone; acutely restraint stressed females (60 min) presented an increase on anxiety level and chronically restraint stressed females showed an increased on locomotor activity. To sum up, the increase on anxiety levels in female rats are modulated by the exposure to 60 min of restraint stress. Although we did not investigate the mechanism involved on increase of anxiety on acutely restrain stressed through the anxiolytic drug responses.

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References

1. Seeman, M.V., *Psychopathology in women and men: focus on female hormones.* Am J Psychiatry, 1997. **154**(12): p. 1641-7.
2. Marcondes, F.K., et al., *Estrous cycle influences the response of female rats in the elevated plus-maze test.* Physiol Behav, 2001. **74**(4-5): p. 435-40.
3. Viau, V. and M.J. Meaney, *Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat.* Endocrinology, 1991. **129**(5): p. 2503-11.
4. Zuloaga, D.G., et al., *Mice with the testicular feminization mutation demonstrate a role for androgen receptors in the regulation of anxiety-related behaviors and the hypothalamic-pituitary-adrenal axis.* Horm Behav, 2008. **54**(5): p. 758-66.
5. Frye, C.A. and E.H. Lacey, *Posttraining androgens' enhancement of cognitive performance is temporally distinct from androgens' increases in affective behavior.* Cogn Affect Behav Neurosci, 2001. **1**(2): p. 172-82.
6. Walf, A.A. and C.A. Frye, *Antianxiety and antidepressive behavior produced by physiological estradiol regimen may be modulated by hypothalamic-pituitary-adrenal axis activity.* Neuropsychopharmacology, 2005. **30**(7): p. 1288-301.
7. Dalla, C., et al., *Chronic mild stress impact: are females more vulnerable?* Neuroscience, 2005. **135**(3): p. 703-14.
8. Kendler, K.S., L.M. Thornton, and C.O. Gardner, *Stressful life events and previous episodes in the etiology of major depression in women: an evaluation of the "kindling" hypothesis.* Am J Psychiatry, 2000. **157**(8): p. 1243-51.
9. Liang, S., D.M. Byers, and L.N. Irwin, *Sex and diet affect the behavioral response of rats to chronic mild stressors.* Physiol Behav, 2008. **93**(1-2): p. 27-36.
10. Yazici, K., et al., *[The effects of hormone replacement therapy in menopause on symptoms of anxiety and depression].* Turk Psikiyatri Derg, 2003. **14**(2): p. 101-5.
11. Gronli, J., et al., *Effects of chronic mild stress on sexual behavior, locomotor activity and consumption of sucrose and saccharine solutions.* Physiol Behav, 2005. **84**(4): p. 571-7.
12. Selye, H., *Stress and distress.* Compr Ther, 1975. **1**(8): p. 9-13.
13. McEwen, B.S., *Effects of adverse experiences for brain structure and function.* Biol Psychiatry, 2000. **48**(8): p. 721-31.
14. Metz, G.A., N.M. Jadavji, and L.K. Smith, *Modulation of motor function by stress: a novel concept of the effects of stress and corticosterone on behavior.* Eur J Neurosci, 2005. **22**(5): p. 1190-200.
15. Vreugdenhil, E., et al., *Genetic dissection of corticosterone receptor function in the rat hippocampus.* Eur Neuropsychopharmacol, 2001. **11**(6): p. 423-30.
16. Antoni, F.A., *Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor.* Endocr Rev, 1986. **7**(4): p. 351-78.
17. Antoni, F.A., *Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age.* Front Neuroendocrinol, 1993. **14**(2): p. 76-122.
18. Bhatnagar, S., et al., *Changes in anxiety-related behaviors and hypothalamic-pituitary-adrenal activity in mice lacking the 5-HT-3A receptor.* Physiol Behav, 2004. **81**(4): p. 545-55.

19. Landgraf, R., et al., *Hyper-reactive hypothalamo-pituitary-adrenocortical axis in rats bred for high anxiety-related behaviour.* J Neuroendocrinol, 1999. **11**(6): p. 405-7.
20. Lund, T.D., et al., *Novel actions of estrogen receptor-beta on anxiety-related behaviors.* Endocrinology, 2005. **146**(2): p. 797-807.
21. Griffin, J.F., *Stress and immunity: a unifying concept.* Vet Immunol Immunopathol, 1989. **20**(3): p. 263-312.
22. Handley, S.L. and J.W. McBlane, *An assessment of the elevated X-maze for studying anxiety and anxiety-modulating drugs.* J Pharmacol Toxicol Methods, 1993. **29**(3): p. 129-38.
23. Pellow, S., et al., *Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat.* J Neurosci Methods, 1985. **14**(3): p. 149-67.
24. Cruz, A.P., F. Frei, and F.G. Graeff, *Ethopharmacological analysis of rat behavior on the elevated plus-maze.* Pharmacol Biochem Behav, 1994. **49**(1): p. 171-6.
25. Ho, Y.J., J. Eichendorff, and R.K. Schwarting, *Individual response profiles of male Wistar rats in animal models for anxiety and depression.* Behav Brain Res, 2002. **136**(1): p. 1-12.
26. Smith, M.S., M.E. Freeman, and J.D. Neill, *The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy.* Endocrinology, 1975. **96**(1): p. 219-26.
27. Marcondes, F.K., F.J. Bianchi, and A.P. Tanno, *Determination of the estrous cycle phases of rats: some helpful considerations.* Braz J Biol, 2002. **62**(4A): p. 609-14.
28. Butcher, R.L., W.E. Collins, and N.W. Fugo, *Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat.* Endocrinology, 1974. **94**(6): p. 1704-8.
29. Gamaro, G.D., et al., *The effects of acute and repeated restraint stress on the nociceptive response in rats.* Physiol Behav, 1998. **63**(4): p. 693-7.
30. Quintero, L., et al., *Long-lasting delayed hyperalgesia after subchronic swim stress.* Pharmacol Biochem Behav, 2000. **67**(3): p. 449-58.
31. da Silva Torres, I.L., et al., *Long-lasting delayed hyperalgesia after chronic restraint stress in rats-effect of morphine administration.* Neurosci Res, 2003. **45**(3): p. 277-83.
32. Ely, D.R., et al., *Effect of restraint stress on feeding behavior of rats.* Physiol Behav, 1997. **61**(3): p. 395-8.
33. Gameiro, G.H., et al., *Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress.* Physiol Behav, 2006. **87**(4): p. 643-9.
34. Stemmelin, J., et al., *Stimulation of the beta3-Adrenoceptor as a novel treatment strategy for anxiety and depressive disorders.* Neuropsychopharmacology, 2008. **33**(3): p. 574-87.
35. Diaz-Veliz, G., et al., *Ketanserin and anxiety levels: influence of gender, estrous cycle, ovariectomy and ovarian hormones in female rats.* Pharmacol Biochem Behav, 1997. **58**(3): p. 637-42.
36. Nomikos, G.G. and C. Spyraki, *Influence of oestrogen on spontaneous and diazepam-induced exploration of rats in an elevated plus maze.* Neuropharmacology, 1988. **27**(7): p. 691-6.
37. Bielajew, C., et al., *Strain and gender specific effects in the forced swim test: effects of previous stress exposure.* Stress, 2003. **6**(4): p. 269-80.

38. Carey, M.P., et al., *The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat.* J Endocrinol, 1995. **144**(2): p. 311-21.
39. Figueiredo, H.F., C.M. Dolgas, and J.P. Herman, *Stress activation of cortex and hippocampus is modulated by sex and stage of estrus.* Endocrinology, 2002. **143**(7): p. 2534-40.
40. Handa, R.J., et al., *Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis.* Horm Behav, 1994. **28**(4): p. 464-76.
41. Sliwowska, J.H., et al., *Effects of prenatal ethanol exposure on regulation of basal hypothalamic-pituitary-adrenal activity and hippocampal 5-HT1A receptor mRNA levels in female rats across the estrous cycle.* Psychoneuroendocrinology, 2008. **33**(8): p. 1111-23.
42. Rex, A., et al., *Pharmacological evaluation of a modified open-field test sensitive to anxiolytic drugs.* Pharmacol Biochem Behav, 1998. **59**(3): p. 677-83.
43. Saphier, D., G.E. Farrar, and J.E. Welch, *Differential inhibition of stress-induced adrenocortical responses by 5-HT1A agonists and by 5-HT2 and 5-HT3 antagonists.* Psychoneuroendocrinology, 1995. **20**(3): p. 239-57.
44. Johnston, A.L. and S.E. File, *Sex differences in animal tests of anxiety.* Physiol Behav, 1991. **49**(2): p. 245-50.
45. Barros, H.M. and M. Ferigolo, *Ethopharmacology of imipramine in the forced-swimming test: gender differences.* Neurosci Biobehav Rev, 1998. **23**(2): p. 279-86.
46. Kompagne, H., et al., *Chronic mild stress generates clear depressive but ambiguous anxiety-like behaviour in rats.* Behav Brain Res, 2008. **193**(2): p. 311-4.
47. Matuszewich, L., et al., *The delayed effects of chronic unpredictable stress on anxiety measures.* Physiol Behav, 2007. **90**(4): p. 674-81.
48. Vyas, A., et al., *Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons.* J Neurosci, 2002. **22**(15): p. 6810-8.
49. Borta, A. and R.K. Schwarting, *Inhibitory avoidance, pain reactivity, and plus-maze behavior in Wistar rats with high versus low rearing activity.* Physiol Behav, 2005. **84**(3): p. 387-96.

CONCLUSÃO

De acordo com os resultados do presente trabalho, conclui-se que:

- Os animais submetidos ao estresse agudo (1 h) apresentaram diminuição das respostas comportamentais nociceptivas (analgesia) nas fases de estro e proestro quando submetidos ao teste da formalina na ATM, sendo o efeito analgésico mais efetivo em fêmeas na fase de proestro.

- A ativação do receptor κ-opióide está envolvida na analgesia induzida pelo estresse em ratas na fase proestro. A injeção local de nor-BNI reverteu parcialmente a analgesia.

- Todos os protocolos de estresse independente da fase do ciclo estral aumentaram significativamente os níveis de corticosterona.

- O estresse agudo (1 h) aumenta o nível de ansiedade em fêmeas nas fases de estro e de proestro.

REFERÊNCIAS*

Aghabeigi, B. The pathophysiology of pain. Br Dent J 1992;173:91-97.

Aloisi, AM, Ceccarelli, I, Lupo, C. Behavioural and hormonal effects of restraint stress and formalin test in male and female rats. Brain Res Bull 1998;47:57-62.

Bandura, A, O'Leary, A, Taylor, CB, Gauthier, J, Gossard, D. Perceived self-efficacy and pain control: opioid and nonopioid mechanisms. J Pers Soc Psychol 1987;53:563-571.

Barlow, DH, Chorpita, BF, Turovsky, J. Fear, panic, anxiety, and disorders of emotion. Nebr Symp Motiv 1996;43:251-328.

Clemente, JT, Parada, CA, Veiga, MC, Gear, RW, Tambeli, CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neurosci Lett 2004;372:250-255.

Droste, C, Greenlee, MW, Schreck, M, Roskamm, H. Experimental pain thresholds and plasma beta-endorphin levels during exercise. Med Sci Sports Exerc 1991;23:334-342.

Fillingim, RB, Ness, TJ. Sex-related hormonal influences on pain and analgesic responses. Neurosci Biobehav Rev 2000;24:485-501.

Gamaro, GD, Xavier, MH, Denardin, JD, Pilger, JA, Ely, DR, Ferreira, MB, Dalmaz, C. The effects of acute and repeated restraint stress on the nociceptive response in rats. Physiol Behav 1998;63:693-697.

Gameiro, GH, Andrade Ada, S, de Castro, M, Pereira, LF, Tambeli, CH, Veiga, MC. The effects of restraint stress on nociceptive responses induced by formalin injected in rat's TMJ. Pharmacol Biochem Behav 2005;82:338-344.

Gameiro, GH, Gameiro, PH, Andrade Ada, S, Pereira, LF, Arthuri, MT, Marcondes, FK, Veiga, MC. Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress. Physiol Behav 2006;87:643-649.

Girardot, MN, Holloway, FA. Intermittent cold water stress-analgesia in rats: cross-tolerance to morphine. Pharmacol Biochem Behav 1984;20:631-633.

Hunskaar, S, Hole, K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 1987;30:103-114.

King, CD, Devine, DP, Vierck, CJ, Rodgers, J, Yezierski, RP. Differential effects of stress on escape and reflex responses to nociceptive thermal stimuli in the rat. Brain Res 2003;987:214-222.

King, TE, Joynes, RL, Meagher, MW, Grau, JW. Impact of shock on pain reactivity: II. Evidence for enhanced pain. J Exp Psychol Anim Behav Process 1996;22:265-278.

Lapo, IB, Konarzewski, M, Sadowski, B. Effect of cold acclimation and repeated swimming on opioid and nonopioid swim stress-induced analgesia in selectively bred mice. Physiol Behav 2003;78:345-350.

Le Bars, D, Gozariu, M, Cadden, SW. Animal models of nociception. Pharmacol Rev 2001;53:597-652.

LeResche, L. Epidemiology of temporomandibular disorders: implications for the investigation of etiologic factors. Crit Rev Oral Biol Med 1997;8:291-305.

LeResche, L, Mancl, L, Sherman, JJ, Gandara, B, Dworkin, SF. Changes in temporomandibular pain and other symptoms across the menstrual cycle. Pain 2003;106:253-261.

Locker, D, Slade, G. Prevalence of symptoms associated with temporomandibular disorders in a Canadian population. Community Dent Oral Epidemiol 1988;16:310-313.

Mogil, JS, Sternberg, WF, Balian, H, Liebeskind, JC, Sadowski, B. Opioid and nonopioid swim stress-induced analgesia: a parametric analysis in mice. Physiol Behav 1996;59:123-132.

Roveroni, RC, Parada, CA, Cecilia, M, Veiga, FA, Tambeli, CH. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain 2001;94:185-191.

Snow, AE, Dewey, WL. A comparison of antinociception induced by foot shock and morphine. J Pharmacol Exp Ther 1983;227:42-50.

Suvinen, TI, Hanes, KR, Gerschman, JA, Reade, PC. Psychophysical subtypes of temporomandibular disorders. J Orofac Pain 1997;11:200-205.

Teng, CJ, Abbott, FV. The formalin test: a dose-response analysis at three developmental stages. Pain 1998;76:337-347.

Terman, GW, Morgan, MJ, Liebeskind, JC. Opioid and non-opioid stress analgesia from cold water swim: importance of stress severity. Brain Res 1986;372:167-171.

Tjolsen, A, Berge, OG, Hunskaar, S, Rosland, JH, Hole, K. The formalin test: an evaluation of the method. Pain 1992;51:5-17.

Wiedenmayer, CP, Barr, GA. Mu opioid receptors in the ventrolateral periaqueductal gray mediate stress-induced analgesia but not immobility in rat pups. Behav Neurosci 2000;114:125-136.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Commitee of International Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

APÊNDICE

FIGURAS

1 – Lavado Vaginal

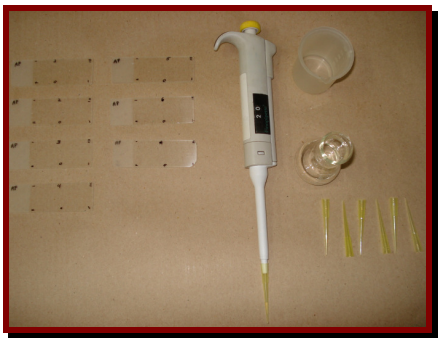


Fig. 1.1 – Material utilizado para realizar o lavado vaginal lâminas histológicas, pipeta (20µl), ponteiras e soro fisiológico.

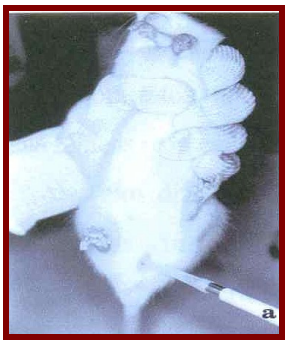


Fig. 1.2 – Procedimento de coleta do lavado vaginal.

2 – Estresse por Contenção

Fig. 2.1 – Material plástico ajustável para realizar estresse por contenção.

Fig. 2.2 – Técnica de contenção em diversos animais.

3 – Injeção de Formalina na ATM

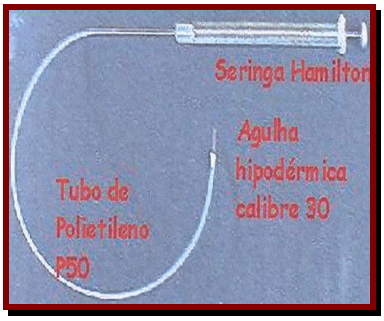


Fig. 3.1 – Seringa Hamilton preparada para aplicação de formalina na ATM.

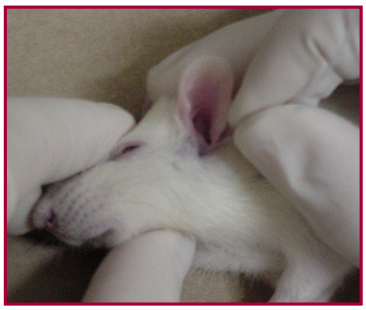


Fig. 3.2 – Local da punção da injeção na ATM.

4 – Registro das Respostas Comportamentais Nociceptivas

Fig. 4.1 – Material utilizado para quantificar respostas comportamentais nociceptivas.

Fig. 4.2 – Câmara de observação (30 cm^3, laterais fundo e base espelhada e frente em vidro).

5 – Labirinto de Cruz Elevado

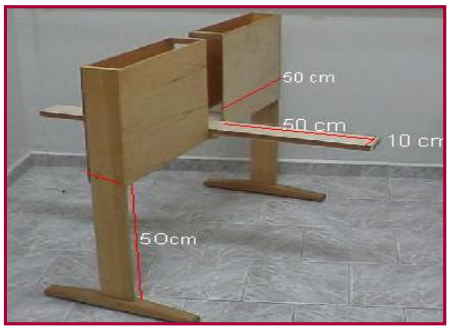


Fig. 5.1 – Labirinto de cruz elevado, utilizado para avaliação da ansiedade.

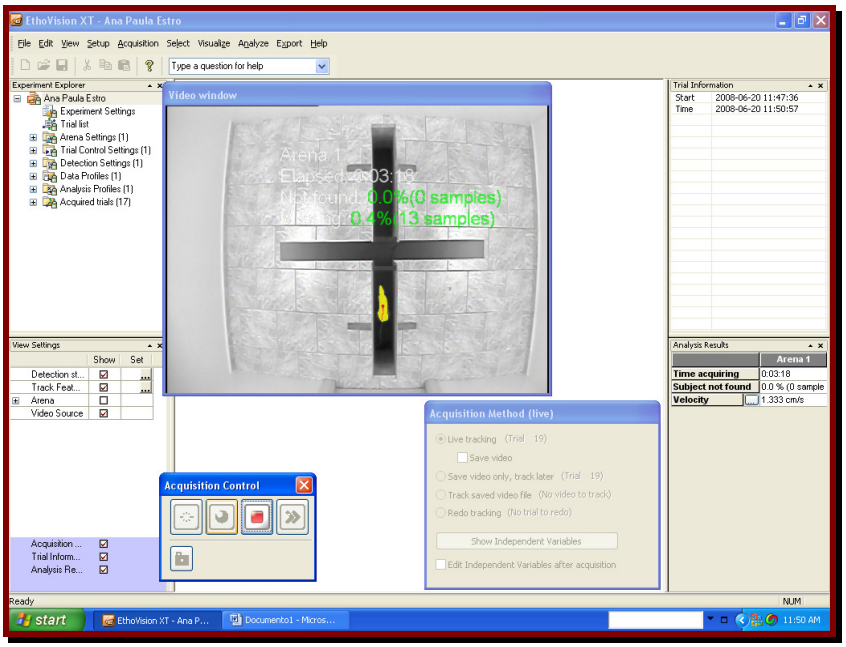


Fig. 5.2 – Software Ethon Vision, durante captação dos dados do Teste do labirinto em cruz elevado (em amarelo, rata explorando labirinto).

TABELAS REFERENTES AOS VALORES INDIVIDUAIS

Tabela 1 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 15 minutos, 30 minutos, 1 hora, 3 dias (1 h/dia) e 40 dias (1 h/dia) em fêmeas na fase de estro.

Soma dos Comportamentos (CO+LC) - Fêmeas em Estro						
Animal	**Controle**	**15 min.**	**30 min.**	**1 hora**	**3 dias**	**40 dias**
1	170,25	111,83	119,18	117,23	138,57	166,14
2	133,00	137,08	96,54	84,96	72,16	91,99
3	137,16	157,86	154,94	123,15	203,79	65,44
4	146,10	148,16	173,84	71,79	126,48	136,31
5	160,35	201,21	60,92	88,85	79,93	109,47
6	132,12	69,23	107,25	126,59	78,28	117,54
Média ± SD	146,50±15,70	137,56±44,51	118,78±40,78	102,10±23,06	116,54±50, 93	114,48±34,90

Tabela 2 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 15 minutos, 30 minutos, 1 hora, 3 dias (1 h/dia) e 40 dias (1 h/dia) em fêmeas na fase de proestro.

Soma dos Comportamentos (CO+LC) - Fêmeas em Proestro						
Animal	**Controle**	**15 min.**	**30 min.**	**1 hora**	**3 dias**	**40 dias**
1	123,77	46,33	104,25	31,09	91,58	78,99
2	116,72	98,24	41,03	44,04	131,23	115,25
3	118,30	148,79	47,48	73,35	225,88	61,44
4	113,19	60,22	94,95	72,12	45,90	117,51
5	115,35	71,23	85,52	35,12	63,25	83,52
6	109,02	88,00	142,20	64,90	142,20	216,67
Média ± SD	116,06±4,96	85,47±36,20	85,91±37,62	53,44±18,97	116,67±65,24	112,23±55,59

Tabela 3 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 1 hora em fêmeas na fase de estro após prévia (24 h) injeção de salina ou nor-BNI (200 μg/ 25 μl) na ATM.

Soma dos Comportamentos (CO+LC) - Fêmeas em Estro				
Animal	**Salina Controle**	**Nor-BNI Controle**	**Salina 1 hora**	**Nor-BNI 1hora**
1	168,34	168,31	115,56	97,78
2	134,15	137,23	83,72	84,48
3	145,20	139,19	120,81	122,20
4	158,35	141,15	72,15	100,51
5	121,14	148,28	84,48	112,73
6	142,80	159,12	125,20	109,07
Média ± SD	145,00 ± 16,82	148,88 ± 12,34	100,32 ± 22,76	104,46 ± 13,14

Tabela 4 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO) + levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 1 hora em fêmeas na fase de proestro após prévia (24 h) injeção de salina ou nor-BNI (200 μg/ 25 μl) na ATM.

Soma dos Comportamentos (CO+LC) - Fêmeas em Proestro				
Animal	**Salina Controle**	**Nor-BNI Controle**	**Salina 1 hora**	**Nor-BNI 1hora**
1	114,15	123,14	71,10	79,62
2	115,90	118,48	34,43	70,77
3	109,25	119,24	81,96	108,50
4	106,30	115,25	45,10	71,45
5	108,60	121,14	61,31	76,81
6	102,10	111,27	30,10	64,41
Média ± SD	109,38 ± 5,07	118,08 ± 4,26	59,00 ± 20,79	78,59 ± 15,57

Tabela 5 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre o número de entradas nos braços fechados, durante o teste do labirinto em cruz elevado em fêmeas na fase de estro.

Número de Entradas nos Braços Fechados* - Fêmeas em Estro						
Animal	**Controle**	**15 min.**	**30 min.**	**60 min.**	**3 dias**	**40 dias**
1	4	3	2	4	2	4
2	5	2	1	3	6	7
3	3	8	3	2	1	3
4	3	2	5	4	5	11
5	7	3	5	2	1	11
6	4	3	5	5	1	5
7	8	5	6	8	5	4
8	4	11	5	2	4	3
9	2	3	5	7	4	10
10	9	3	7	1	3	9
Média ± SD	4,9 ± 2,3	4,3 ± 2,9	4,4 ± 1,8	3,8 ± 2,3	3,2 ± 1,9	6,7 ± 3,3

*Avaliação da atividade locomotora.

Tabela 6 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre o número de entradas nos braços fechados, durante o teste do labirinto em cruz elevado em fêmeas na fase de proestro.

Número de Entradas nos Braços Fechados* - Fêmeas em Proestro						
Animal	**Controle**	**15 min.**	**30 min.**	**60 min.**	**3 dias**	**40 dias**
1	8	5	11	1	3	3
2	1	7	8	4	3	7
3	3	11	5	5	2	7
4	3	5	4	5	2	8
5	4	1	5	7	4	6
6	3	3	5	3	5	10
7	2	1	4	3	5	5
8	4	6	2	4	3	11
9	6	4	9	2	7	3
10	9	2	3	4	5	4
Média ± SD	4,3 ± 2,5	4,5 ± 3,1	5,6 ± 2,8	3,8 ± 1,7	3,9 ± 1,6	6,4 ± 2,8

*Avaliação da atividade locomotora.

Tabela 7 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre a porcentagem de entradas nos braços fechados, durante o teste do labirinto em cruz elevado em fêmeas na fase de estro.

% Entradas nos Braços Fechados* - Fêmeas em Estro						
Animal	**Controle**	**15 min.**	**30 min.**	**60 min.**	**3 dias**	**40 dias**
1	64	0	78	50	33	76
2	0	0	0	0	33	30
3	40	62	67	0	0	40
4	0	71	44	43	0	31
5	30	50	17	0	0	61
6	33	0	44	50	0	44
7	67	44	33	33	44	69
8	64	50	55	0	56	75
9	50	57	50	22	0	17
10	31	57	50	0	50	59
Média ± SD	37,9 ± 24,2	39,1 ± 28,0	43,8 ± 22,8	19,8 ± 22,4	21,6 ± 23,8	50,2 ± 20,7

* Avaliação da atividade locomotora e ansiedade.

Tabela 8 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre a porcentagem de entradas nos braços fechados, durante o teste do labirinto em cruz elevado em fêmeas na fase de proestro.

% Entradas nos Braços Fechados* - Fêmeas em Proestro						
Animal	**Controle**	**15 min.**	**30 min.**	**60 min.**	**3 dias**	**40 dias**
1	53	71	52	0	40	77
2	0	42	38	50	0	36
3	40	27	71	50	33	42
4	0	64	69	29	71	53
5	50	0	55	36	50	45
6	0	0	38	63	44	44
7	67	0	33	67	0	44
8	71	45	0	0	50	31
9	54	64	36	67	36	82
10	50	60	25	20	58	73
Média ± SD	38,5 ± 28,0	37,3 ± 28,7	41,7 ± 21,2	38,2 ± 25,6	38,2 ± 22,9	52,7 ± 18,1

* Avaliação da atividade locomotora e ansiedade.

Tabela 9 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre o número de vezes no final do braço aberto, durante o teste do labirinto em cruz elevado em fêmeas na fase de estro.

Final do Braço Aberto* - Fêmeas em Estro						
Animal	**Controle**	**15 min.**	**30 min.**	**60 min.**	**3 dias**	**40 dias**
1	6	0	2	0	0	0
2	0	0	0	0	1	3
3	0	9	0	0	0	0
4	0	0	0	1	0	0
5	2	0	0	0	0	3
6	0	0	4	0	0	0
7	2	0	0	0	0	0
8	0	1	1	0	0	0
9	0	0	5	0	0	0
10	0	0	0	0	0	11
Média ± SD	1 ± 1,9	1 ± 2,8	1,2 ± 1,9	0,1 ± 0,3	0,1 ± 0,3	1,7 ± 3,5

* Avaliação da ansiedade.

Tabela 10 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre o número de vezes no final do braço aberto, durante o teste do labirinto em cruz elevado em fêmeas na fase de proestro.

Final do Braço Aberto* - Fêmeas em Proestro						
Animal	**Controle**	**15 min.**	**30 min.**	**60 min.**	**3 dias**	**40 dias**
1	0	5	0	0	0	1
2	0	1	0	3	0	1
3	0	1	4	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	9
9	5	3	2	0	0	2
10	2	0	0	0	0	6
Média ± SD	0,7 ± 1,6	1 ± 1,7	0,6 ± 1,3	0,3 ± 0,9	0 ± 0	1,9 ± 3,1

* Avaliação da ansiedade.

Tabela 11 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre a porcentagem de tempo no braço aberto, durante o teste do labirinto em cruz elevado em fêmeas na fase de estro.

% Total nos Braços Abertos* - Fêmeas em Estro						
Animal	**Controle**	**15 min.**	**30 min.**	**60 min.**	**3 dias**	**40 dias**
1	42	14	5	2	0	5
2	0	0	0	0	11	10
3	0	0	0	0	0	5
4	0	0	0	0	0	3
5	6	2	1	0	0	29
6	0	0	0	0	0	5
7	15	5	2	1	5	5
8	14	5	2	1	6	9
9	4	1	0	0	0	1
10	1	0	0	0	5	38
Média ± SD	8 ± 13,1	3 ± 4,4	1 ± 1,5	0 ± 0,5	3 ± 3,7	11 ± 12,3

* Avaliação da ansiedade.

Tabela 12 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre a porcentagem de tempo no braço aberto, durante o teste do labirinto em cruz elevado em fêmeas na fase de proestro.

% Total nos Braços Abertos* - Fêmeas em Proestro						
Animal	**Controle**	**15 min.**	**30 min.**	**60 min.**	**3 dias**	**40 dias**
1	4	32	10	3	1	11
2	0	10	2	1	0	5
3	6	6	22	7	7	9
4	0	16	14	5	10	10
5	4	0	8	3	1	1
6	0	0	2	1	7	6
7	9	0	2	1	0	4
8	5	3	0	0	2	10
9	18	15	5	2	5	10
10	10	4	6	2	12	25
Média ± SD	6 ± 5,7	9 ± 9,9	7 ± 6,9	2 ± 2,3	5 ± 4,3	9 ± 6,7

* Avaliação da ansiedade.

Tabela 13 – Valores individuais do nível de corticosterona plasmática (µg/dL) das fêmeas em estro submetidas aos diferentes protocolos de estresse.

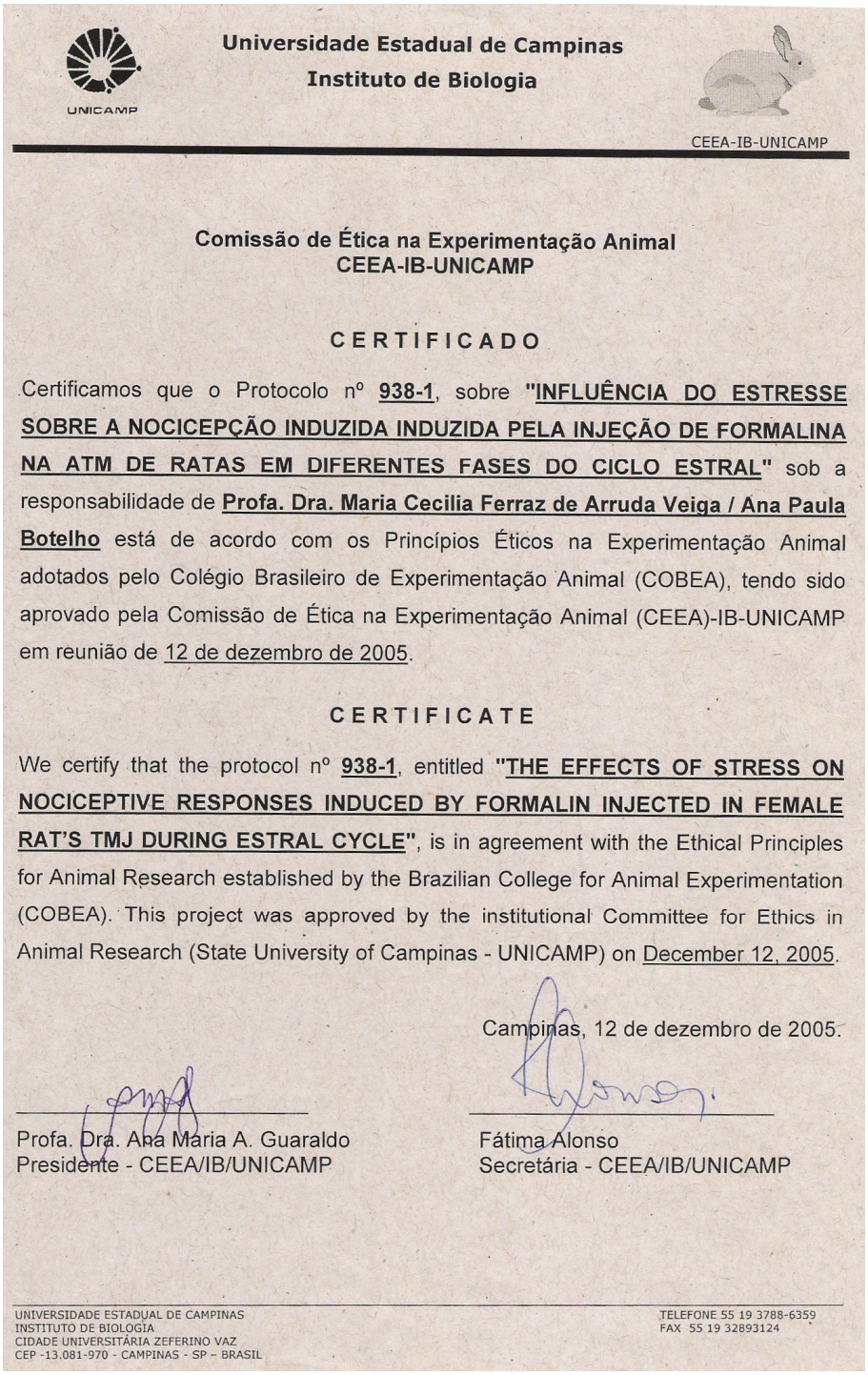
Corticosterona Plasmática (µg/dL) - Fêmeas em Estro						
Animal	**Controle**	**15 min.**	**30 min.**	**1 hora**	**3 dias**	**40 dias**
1	6,4	75,0	53,0	37,8	24,9	19,9
2	5,6	88,0	57,0	36,4	26,7	31,9
3	3,2	39,7	64,0	28,2	71,0	19,5
4	6,8	62,5	14,9	64,0	37,2	20,10
5	7,1	95,0	113,0	33,9	34,2	21,4
6	8,7	87,5	122,0	36,4	27,5	17,4
7	5,2	94,5	111,0	69,0	85,5	20,0
8	7,3	83,5	34,8	37,7	34,6	25,4
Média ± SD	6,3 ± 1,6	78,2 ± 18,9	71,2 ± 39,6	42,9± 14,6	42,7 ± 22,7	22,0 ± 4,6

Tabela 14 – Valores individuais do nível de corticosterona plasmática (µg/dL) das fêmeas em proestro submetidas aos diferentes protocolos de estresse.

Corticosterona Plasmática (µg/dL) - Fêmeas em Proestro						
Animal	**Controle**	**15 min.**	**30 min.**	**1 hora**	**3 dias**	**40 dias**
1	8,3	57,0	58,0	65,5	30,2	29,9
2	7,6	59,0	97,5	28,1	31,8	27,7
3	6,9	71,5	113,5	66,5	29,3	20,8
4	7,3	47,5	82,5	36,5	19,4	38,9
5	4,1	78,0	80,5	62,0	37,9	22,1
6	6,5	32,7	77,0	29,2	74,0	28,4
7	4,8	86,5	64,5	76,5	35,7	26,6
8	6,2	32,7	76,0	39,5	34,2	35,9
Média ± SD	6,5 ± 1,4	58,1 ± 20,0	81,2 ± 17,6	50,5 ± 19,1	36,6 ± 16,1	28,8 ± 6,2

ANEXO 1

Certificado do Comitê de Ética



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C E R T I F I C A D O

Certificamos que o Protocolo nº **938-1**, sobre **"INFLUÊNCIA DO ESTRESSE SOBRE A NOCICEPÇÃO INDUZIDA INDUZIDA PELA INJEÇÃO DE FORMALINA NA ATM DE RATAS EM DIFERENTES FASES DO CICLO ESTRAL"** sob a responsabilidade de **Profa. Dra. Maria Cecilia Ferraz de Arruda Veiga / Ana Paula Botelho** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 12 de dezembro de 2005.

C E R T I F I C A T E

We certify that the protocol nº **938-1**, entitled **"THE EFFECTS OF STRESS ON NOCICEPTIVE RESPONSES INDUCED BY FORMALIN INJECTED IN FEMALE RAT'S TMJ DURING ESTRAL CYCLE"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on December 12, 2005.

Campinas, 12 de dezembro de 2005.

Profa. Dra. Ana Maria A. Guaraldo
Presidente - CEEA/IB/UNICAMP

Fátima Alonso
Secretária - CEEA/IB/UNICAMP

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ANEXO 2

Confirmação de Publicação do Artigo – Capítulo 1

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September 9, 2008

Ref.: Ms. No. 382
Influence of sex on temporomandibular disorder pain: a review of occurrence and development.
Brazilian Journal of Oral Sciences

Dear Dr Veiga,

I am pleased to tell you that your work has now been accepted for publication in the Brazilian Journal Of Oral Sciences.

Thank you for submitting your work to this journal.

With kind regards

JF Höfling
Editor
Brazilian Journal of Oral Sciences

ANEXO 3

Carta de Submissão do Artigo – Capítulo 2

Title: The estrous cycle modulate the stress-induced analgesia via k-opioid mechanism Corresponding Author: Ms.C Ana Paula Botelho
Authors: Gustavo H Gameiro, Ph.D; Maria Cecília Ferraz A Veiga, Ph.D

Dear Ana Paula,

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