Effect of Food Deprivation on Formalin-Induced Nociceptive Behaviors and Beta-Endorphin and Sex Hormones Concentration in Rats

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ABSTRACT

Background: The present study examined the possible role of endogenous opioidergic system in effect of food deprivation on formalin-induced nociceptive behaviors in male and female rats. Also, we investigated the effect of food deprivation on the plasma level of beta-endorphin and sex hormones. **Methods:** Food was withdrawn 48 h prior to performing the formalin test, but water continued to be available *ad libitum*. The formalin was injected into hind plantar paw. **Results:** There is significant difference between male and female control rats during phase 2B. Following 48-h food deprivation, both male and female rats exhibited enhanced nociceptive behavior in response to formalin. Food deprivation for 12 and 24 h increased and for 48 h decreased beta-endorphin level in male and female rats. Food deprivation for 24 h decreased testosterone level in male, while it had no significant effect on female rats and food deprivation for 48 h decreased testosterone level in both sexes. Food deprivation for 24 h increased estradiol level in female and that for 48 h had no significant effect on male and female rats. **Conclusions:** The present study demonstrates the existence of food deprivation for 48 h causes enhancement of nociception in the formalin test in male and female rats that has correlation with decrease in plasma beta-endorphin and testosterone levels. *Iran. Biomed. J. 18 (2): 107-113, 2014*

INTRODUCTION

It is well-known that in rodents, females present more behavioral responses to noxious than males in the most of pain models. Pain thresholds in response to experimentally induced pain are generally lower in women than men, and women are more likely than men to experience a variety of chronic pain conditions and have more frequent pain-related disability [1, 2]. Both short-term and intermittent food deprivation have antinociceptive effectbecause of neuromodulatory systems such as endogenous opioid system and adrenocortical hormones [3, 4]. In addition, the pronociceptive effect of 48-h food deprivation was much greater in female than male rats [5].

Furthermore, the role of sex hormones in different aspects of formalin test has been studied and indicated that female rats show more formalin-induced nociceptive responses compared to male rats [6]. Also, systemic injection of estradiol in male rats increases nociceptive behavior that blocks with naloxone [7]. In addition, administration of testosterone has anti-nociceptive effect on formalin test [8]. It is widely known that estrogen modulates nociceptive responses during interphase, while testosterone decreases nociceptive responses during phases 1 and 2 of formalin test [9].

Our previous observation on the effect of 48-hour food deprivation on formalin test showed that nociceptive behaviors in male and female rats were increased, and the second phase was finished with a delay [10]. Naloxone administration blocked the pronociceptive effect of the 48-hour food deprivation in the interphase of male rats and in the first phase of female rats. Based on Gaumond's previous studies [9, 11], female sex hormones modulated only the interphase, while testosterone acted in phases 1 and 2 without affecting the interphase. We hypothesized that 48-h food deprivation in a sex-dependent manner modulated formalin-induced nociceptive behaviors in

phase 1, interphase, and phase 2. To test these hypotheses, formalin tests were performed following the 48-h food deprivation in both male and female rats. We also investigated the effect of food deprivation on the plasma level of sex hormones including estradiol and testosterone.

MATERIALS AND METHODS

Subjects. All experiments were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and approved by the Research and Ethics Committee of Qazvin University of Medical Sciences, Qazvin, Iran. Efforts were made throughout the experiments to minimize the animal discomfort and to reduce the number of animals used. Adult male and female Sprague-Dawley rats (220-300 g), which were purchased from Razi Institute for Serums and Vaccines (Hesarak Karaj, Iran), were housed in groups of three in a temperature controlled room, under a 12 h light-dark cycle with lights on at 7:00 to 19:00. Food and water were provided *ad libitum*. During the experiments, attention was strictly paid to the regulations of local authorities for handling laboratory animals.

Food deprivation. Food was withdrawn 48 h prior to performing the formalin test, but water continued to be available *ad libitum*. Control rats had free access to both food and water.

Formalin-induced nociceptive behavior. Rats were moved to the test room at least 1 h before the commencement of the experiment. The rats were first acclimatized in an acrylic observation chamber (30 cm in diameter and in height) for 30 minutes, and then 50 µL 2% formalin was injected subcutaneously into the plantar surface of the right hind paw with a 30-gauge needle. Each rat was then immediately returned to the observation box, and the behavioral recording commenced. A mirror was placed at 45-degree angle beneath the box to permit the observation of behaviors without moving the box. Pain behaviors was scored as follows: 0, the injected paw was not favored; 1, the injected paw had little or no weight placed on it; 2, the injected paw was elevated and not in contact with any surface; and 3, the injected paw was licked or bitten. Scores were continuously observed for duration of the experiment (90 minutes). The nociceptive behavior score for each 3-minute interval was calculated as the number of seconds spent in each nociceptive behavioral condition from the start of the experiment. The scores were recorded in normal rats as well as in

those who received food deprivation. In each group, the behavioral responses of each rat during the first phase (1-7 minutes), interphase (8-14), and the second phase (15-90) were separately evaluated [12].

Blood sampling and beta-endorphin and sex hormone measurement. Under deep anesthesia, blood was collected from the heart of rats (n = 6 for each group). First from the control group and then from food deprived groups. Bloods were allowed to clot, and sera were separated using centrifugation at 2,100 ×g for 5 min and stored at -80ºC until use. Total serum level of beta-endorphin was measured using ELISA kit (Glory Science Co., USA), and those of estradiol and testosterone were measured by radioimmunoassay kits (Immunotech, France). Test principle of ELISA kits was based on a double-antibody sandwich ELISA to assay beta-endorphin level. In radioimmunoassay kits, there was a competition between analysis in samples and 125I-labeled reagent in antibody-coated tubes. Six calibrators were used for preparing the calibration curve and calculation of unknown samples. All 3 control samples were within their ranges. The performance characteristics of the assay were: within-run precision: 8%, limit of quantification: 1 µmol/L, and linearity: up to 50 µmol/L.

Data analysis. Data were presented as mean ± S.E.M. and analyzed by one-way analysis of variance and t-test among the groups. The mean nociceptive score in each phase (phase 1, interphase, and phase 2) of the formalin test was analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Phase 1 (1-7 minutes), the interphase (8-14 minutes), and the phase 2 (2A: 15–60 and 2B: 61–90 minutes) of the formalin test were analyzed separately. The defined level for statistical significance was $P<0.05$.

RESULTS

Effect of 48-h food deprivation on formalin-induced nociceptive behaviors. In control group that received no food deprivation, formalin produced typical biphasic pain responses. The first and second phases were separated by a brief interphase where little to no nociceptive behaviors were observed in control group. There was a significant difference between male and female control rats during phase 2B. Although interphase in male rats was more than female ones, but there was not any significant difference in interphase ($T(1,18) = 0.379$, $P = 0.546$), and the phase 2B was more in female than male rats, and phase 2 was finished with a delay ($T(1,18) = 5.595$, $P<0.05$). Food-
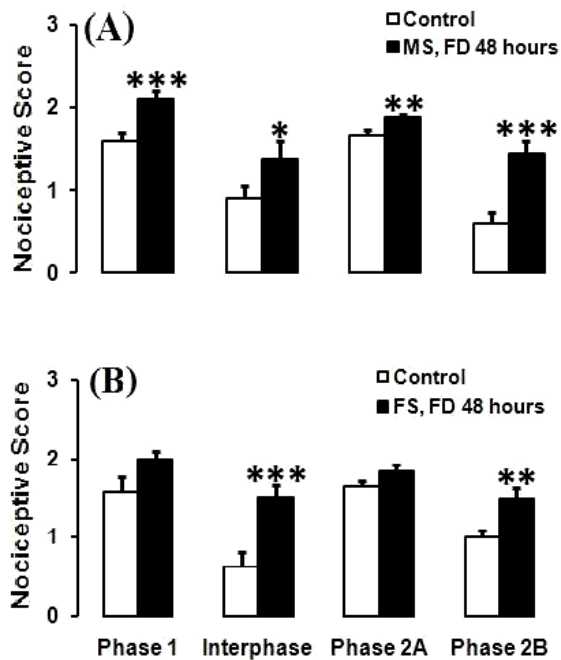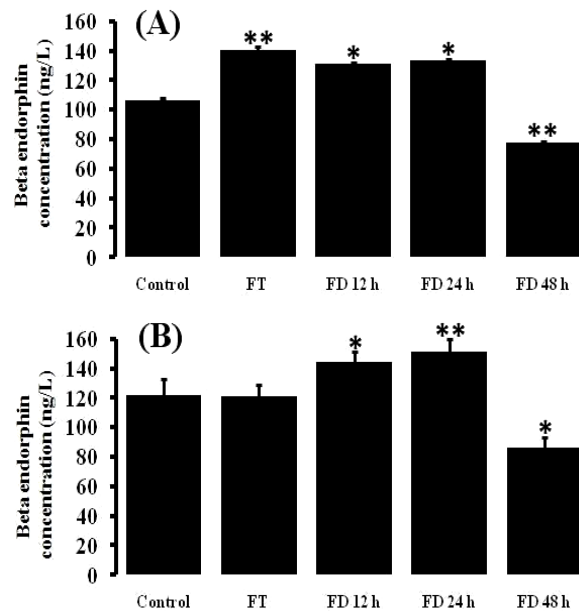
Fig. 1. Effect of food deprivation (FD) on formalin-induced nociceptive behaviors. The columns represent the mean of nociceptive score in each phase for male and female Sprague (MS and FS) in control and food-deprivation condition (**A** and **B**). *$P<0.05$, **$P<0.01$, ***$P<0.001$ food deprivation group in comparison with control one in male and female rats. MS, male Sprague; FS, female Sprague

deprived male and female rats were compared with non-food-deprived controls to determine if the food deprivation could induce pronociceptive effect on formalin test. Following a short-term food deprivation (48 h), both male and female rats exhibited enhanced nociceptive behavior in response to formalin. There were significant differences in behavioral response between food-deprived female (n = 8) and non-fasted (n =10) control rats during phase 1 (T(1,16) = 2.484, P = 0.135), the interphase (T(1,16) = 18.882, $P<0.001$), phase 2A (T(1,16) = 3.938l, P = 0.065), and phase 2B (T(1,16) = 7.668, $P<0.01$) (Fig. 1). Also, there were significant differences in behavioral response between food-deprived male (n = 10) and non-fasted (n = 12) control rats during phase 1 (T(1,20) = 20.375, $P<0.001$), the interphase (T(1,20) = 6.577, $P<0.05$), phase 2A(T(1,20) = 7.984, $P<0.01$), and phase 2B (T(1,20) = 24.648, $P<0.001$). Furthermore, there were significant differences in behavioral response between food-deprived male (n = 10) and female rats (n = 8) during interphase after 48-h food deprivation (T(1,16) = 5.485, $P<0.05$) (Fig. 1).
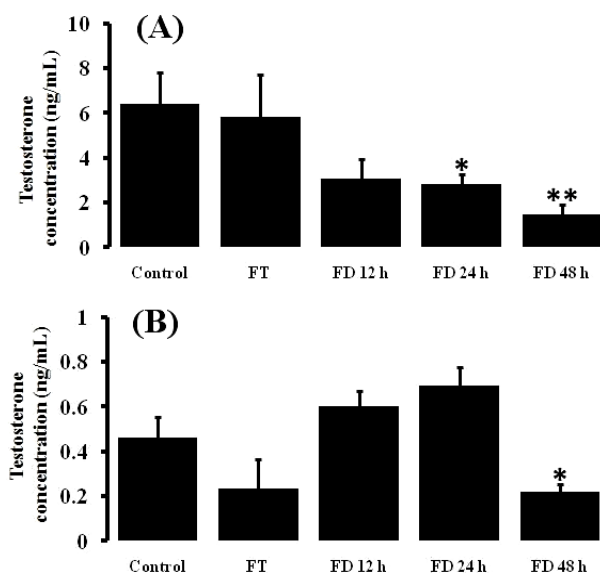
Effect of food deprivation on plasma beta-endorphin level. The effect of food deprivation and

formalin test on plasma beta-endorphin levels (mean ± SEM) of both sexes have been shown in Figure 2. The formalin injection in the female rats (n = 6) had no significant effect on the concentration of plasma beta-endorphin [T(1, 14) = -0.302, P = 0.769], while formalin injection in the male rats (n = 6) increased the concentration of plasma beta-endorphin as compared with control groups [T(1, 14) = -2.834, $P<0.01$]. Food deprivation for 12 and 24 h increased beta-endorphin level in male [T(1, 18) = -2.539, $P<0.05$ and T (1, 18) = -2.734, $P<0.05$, respectively] and female [T(1, 18) = -2.582, $P<0.05$ and T(1, 18)=-2.624, $P<0.01$, respectively] rats as compared with control. In addition, food deprivation for 48 h decreased beta-endorphin level in male [T(1, 18) = 2.793, $P<0.01$] and female [T (1, 18) = -2.143, $P<0.05$] rats as compared with control [both for male and for female.

Effect of food deprivation on testosterone and estradiol levels in male and female rats. The effect of food deprivation and formalin test on plasma sex hormone levels (mean ± SEM) of both sexes have been shown in Figure 3. The formalin test in the male (n = 6) and the female rats (n = 6) had no significant effect on the concentration of plasma testosterone [for male: T(1, 14) = 0.869, P = 0.399 and for female: T(1, 14) = 1.591, P = 0.163]. Food deprivation for 12 h in male [n = 10; T(1, 18) = 1.231, P = 0.235] and female rats [n = 10; T(1, 18) = 0.870, P = 0.398)] had no significant

Fig. 2. Effect of food deprivation (FD) on plasma beta-endorphin. Beta-endorphin of male (**A**) and female (**B**) rats that had free access to their diets or were food-deprived for 12, 24, and 48 h (n = 6 for each group). *$P<0.05$ and **$P<0.01$ compared to control group. FT, formalin test

Fig. 3. Effect of food deprivation (FD) on plasma testosterone. Testostrone level of male **(A)** and female **(B)** rats that had free access to their diets or were food-deprived for 12, 24, and 48 h (n = 6 for each group). *$P<0.05$ and **$P<0.01$compared to control group. FT, formalin test

effect on the concentration of plasma testosterone. Also, food deprivation for 24 h decreased testosterone level in male, while it had no significant effect on testosterone concentration in female rats as compared with control [T(1, 18) = 1.366, P = 0.190] and food deprivation for 48 h decreased testosterone level in male [T(1, 18) = 3.162, $P<0.01$] and female [T(1, 18) = -2.211, $P<0.05$] rats as compared with control. The formalin injection in the male (n = 6) and female rats (n = 6) significantly increased the level of plasma estradiol [for male: T (1, 14) = 7.207, $P<0.001$ and for female: T(1, 14) = 2.933, $P<0.0$]. Food deprivation for 12 h had no significant effect on the concentration of plasma estradiol in the male and female rats [for male (n = 10): T(1, 18) = 0.762, P = 0.456 and for female (n = 10): T(1, 18) = 0.971, P = 0.347]. Also, food deprivation for 24 h increased estradiol level in female [T(1, 18) = 2.306, $P<0.05$], while had no significant effect on estradiol level in male rats as compared with control [T(1, 18) = 1.245, P = 0.299] and food deprivation for 48 h had no significant effect on estradiol level in male [T(1, 18) = 0.048, P = 964] and female [T(1, 18) = 0.222, P = 830] rats as compared with control (Fig. 4).

DISCUSSION

In this study, we found that there is a significant difference between male and female control rats during

phase 2B that has correlation with increases in beta-endorphin level after formalin test in male comparing to female rats. Following food deprivation (48 h), both male and female rats exhibited enhanced nociceptive behavior in response to formalin. Food deprivation for 12 and 24 h increased beta-endorphin level, and food deprivation for 48 h decreased beta-endorphin level in male and female rats. Also, food deprivation for 24 h decreased testosterone level in male, while it had no significant effect on testosterone concentration in female rats, and food deprivation for 48 h decreased testosterone level in male and female rats. Food deprivation for 24 h increased estradiol level in female, and food deprivation for 48 h had no significant effect on estradiol level in male and female rats.

Food deprivation and nociception are physiological conditions associated with homeostatic functioning and have bidirectional effect. The magnitude of the analgesia was caused by food and/or water-deprivation consistent on the duration of the deprivation and also correlated with the increasing of the beta-endorphin level in the blood plasma [13]. It seems that the duration of food deprivation plays an important role in the effect of fasting on nociceptive behaviors. In consistent with our study, Khasar *et al.* [5] showed 48-h fasting increased formalin-induced nociceptive behavior. Also, they suggested that food deprivation-induced effect on nociception appears to be mediated by the vagus nerve, since it is prevented by sub-
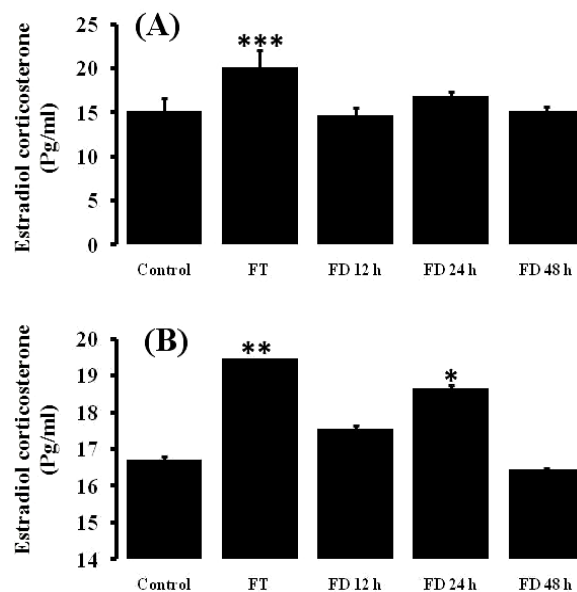
Fig. 4. Effect of food deprivation (FD) on plasma estradiol. Estradiol level of male **(A)** and female **(B)** rats that had free access to their diets or were food-deprived for 12, 24, and 48 h (n = 6 in each group). *$P<0.05$ and **$P<0.01$, ***$P<0.001$ compared to control group. FT, formalin test;

diaphragmatic vagotomy. Khasar *et al.* [5] observed formalin-induced nociceptive behaviors during 60 min, and fasting male rats induced pronociceptive effect only during second part of phase 2, which did not discuss in that study. Increase of beta-endorphin following 12- and 24-h food deprivation was in consistent with the mild food deprivation-induced analgesia which endogenous opioidergic system might be involved [4, 14, 15].

The duration or intensity of fasting has a correlation with the level of beta-endorphin that is in the circulation. In consistent with our finding, Majeed *et al.* [16] showed that 24-h food deprivation increased beta-endorphin in hypothalamus and pituitary gland, suggesting that the levels of opioid peptides in these regions may be differentially and independently affected by alteration of the ingestive behavior. However, Knuth and Friesen [17] indicated that beta-endorphin concentrations were decreased in the preoptic suprachiasmatic area after 4 days of starvation. In other study, Kim *et al.* [18] demonstrated that restricting food intake or 48-h food deprivation significantly decreased mRNA levels of pro-dynorphin and pro-enkephalin in arcuate nucleus. Following 1-4 days of food deprivation, the pituitary and different regions of brain were analyzed for level of opioid peptides such as beta-endorphin. Vaswani and Tejwani [19] indicated that food deprivation in rats affects the opioid peptide levels in a differential manner. Also, chronic food restriction decreases gene expression of arcuate pro-opiomelanocotin, as precursor of beta-endorphin [20]. Barnes *et al.* [21] revealed that 48-h food deprivation resulted in a significant increase in the mRNA expression of μ-opioid receptors in the ventral medial hypothalamus and arcuate nucleus and the lateral hypothalamus, but not following 12 or 24 h of food deprivation. They also suggested that beta-endorphin elevation activates the μ-opioid receptors. However, following 48-h food deprivation, beta-endorphin concentration decreased, and consequently the population of μ-opioid receptors increased [21]. We hypothesize that our observations of an increase in beta-endorphin following the 12- or 24-h food deprivation and a decrease in beta-endorphin after 48-h food deprivation have correlation with effect of fasting duration on formalin-induced nociceptive behaviors. The endogenous opioidergic system might participate in analgesic effect of food deprivation and through descending inhibitory system change formalin induced nociceptive behaviors.

Increase in estradiol level following 24-h food deprivation in our study is in consistent with evidence that plasma estrogen levels are increased in food-deprived hamsters [22] and up-regulation of estrogen receptors after 48-h food deprivation in the paraventricular nucleus [23]. In opioidergic mechanisms, the study of Sandner-Kiesling and Eisenach [24] indicated that estrogen reduced the inhibitory effect of morphine and suggested that estrogen might influence the action of opioids. Moreover, estrogens induce μ-opioid receptor internalization that is blocked by the opioid antagonist naltrexone [25]. In line with our result that formalin increased beta-endorphin level in male rats, a pervious study showed that hind paw formalin injection significantly increased mean preproenkephalin mRNA content per ventromedial nucleus of the hypothalamus in rats, and also ovarian steroid hormones were able to increase the mean preproenkephalin mRNA in this region [26].

Some studies suggested that the interphase of the formalin test was the result of endogenous pain-suppressing mechanisms [27, 28], and the results of this study clearly indicated that pain inhibitory mechanisms during the interphase of the formalin test was related to opioidergic systems. Therefore, we suggest that the extended fasting time might produce tolerance in endogenous opioid system that involves in the pronociceptive effect of the food deprivation during the interphase, which was reversed by naloxone administration.

In a study, Gaumond *et al.* [29] demonstrated that the endogenous opioid system was involved in interphase of formalin test in female rat as completely inhibited by naloxone, and it produced only a slight blockade in males. They also suggested that opioids played a major role in the pain inhibitory mechanism of the interphase in females, while a non-opioid mechanism seemed to be responsible for this inhibitory pathway in males [29].

Furthermore, role of sex hormones in different aspects of formalin test has been studied [9, 11] and indicated that they have a role in modulating nociceptive responses during the formalin test. In agreement with our result that shows phase 2 increases beta-endorphin level after formalin test in male comparing to female rats, Aloisi *et al.* [6] stated that female rats showed more nociceptive responses in formalin test as compared to the male rats. They also showed that intracerebroventricular injection of estradiol to the male rats increased licking behavior, which was mediated supraspinally. In addition, this effect of estrogens was mediated with endogenous opioid system and was sex-related [7]. In another studies, the same team showed that administration of testosterone had antinociceptive effect [8, 30]. Gaumond *et al.* [11] demonstrated that female rats showed more nociceptive responses than male rats in all phases of formalin test, and gonadectomy of female rats eliminated this difference. In 2005, Gaumond and co-workers [9] showed that female sex hormones (estrogen and progesterone) modulated responses

during interphase of formalin test, while testosterone decreased nociceptive responses during phases 1 and 2 of formalin test. Food deprivation for 48 h decreased beta-endorphin and testosterone level in male and female rats, while it had no significant effect on estradiol level in male and female rats. Previous studies [9, 11] indicated that estrogen had pronociceptive effect on interphase, and testosterone had hypoalgesia in phases 1 and 2. We hypothesize that the difference between females and males in the interphase of the formalin test may implicate an endogenous opioidergic system in a sex-dependent manner.

Based on our previous study, we suggests that decreased beta-endorphin concentration and decreased testosterone to estradiol ratio could be considered for pronociceptive effect of fasting in the formalin test and decreased testosterone to estradiol ratio might be dependent on opioid receptor [10].

Loyd and Murphy [31] showed sexually dimorphic in the periaqueductal gray matter projection to rostral ventromedial medulla as inhibitory modulatory pathway, which have important role in pain modulation. Then, we considered this circuit could be a part of anatomical substrate for fasting-induced pronociceptive effect on formalin test and beta-endorphin as well as sex hormones has a modulatory effect on this pathway.

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