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Title: Tramadol Reverses the Effects of Neuropathic Pain on Oocyte Maturation and Copulation Ratio in Mice

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Abstract

Objective: Neuropathic pain (NP) is an inescapable stressor that significantly affects nervous and endocrine system functions. In this study we investigated the effect of neuropathic pain on female reproductive function – using the number of oocytes as an index – and the copulation ratios of female mice, with and without males. We also examined whether these symptoms stopped after injecting the opioid analgesic tramadol.

Materials and Methods: Tight ligation of the partial sciatic nerve was performed to produce neuropathy, and allodynia was assessed in the cold-plate test. A superovulation protocol was applied to control, sham, neuropathy, and neuropathy + tramadol groups. Each group was divided into two subgroups according to two housing conditions: female alone or female with a male. After induction, oocytes/zygotes were isolated from the ampulla of

female mice. Total oocyte number, oocyte maturation, and the copulation ratio were determined.

Results: The results showed that allodynia, which is a prominent NP symptom was detected in all neuropathic mice and tramadol (50 mg/kg, ip) reversed these effects. The results also showed that NP decreased oocyte the maturation and copulation rates of mice and tramadol reversed all of these effects.

Conclusion: In conclusion, we suggest that NP affects reproductive performance by altering regulation neuroendocrine mechanisms. Prospective studies that determine levels of cortisol, fertility hormone, cytokine, and other potential endogenous substances in NP animals are needed to clarify the roles in reproduction of these suggested mechanisms.

Key words: Neuropathic pain, oocytes, mice, tramadol, oocytes maturity

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Introduction

Chronic pain is one of the most debilitating health problems worldwide, as it negatively affects daily activities, retards personal and social productivity, and generates hopelessness. Neuropathic pain (NP) is a particularly troubling subtype of chronic pain. NP is caused by damage to the somatosensory nervous system and its management is unsatisfactory as a consequence of the complex underlying mechanism[1]. Similar to other types of chronic pain, NP is an inescapable stressor that significantly affects quality of life according to changes in the physical and mental functioning of patients. NP and chronic pain lead to permanent changes in brain structure and function, which can affect brain processes not directly connected with the pain itself[2]. The endocrine system is particularly affected by NP[3]. NP activates the hypothalamic–pituitary–adrenal–thyroid–gonadal system, which controls the stress mechanism[4]. The relationships between chronic pain (and NP) and stress have been discussed in a number of recent reviews that investigated the relevance of the hypothalamo-pituitary-adrenal (HPA), hypothalamic-pituitary-gonadotropic (HPG), and corticotrophin-releasing hormone (CRH) axes[5, 6]. As a consequence of interactions of these axes, chronic pain can affect reproductive function. Although there exits one study on the effects of stress on fertility in dairy cows[7], we haven't found any experimental studies that analyse effects of neuropathic pain on fertility.

In this study, we used an experimental neuropathy model to investigate whether NP affects reproductive parameters in mice by examining the number and maturity of oocytes and the copulation ratio. We also investigated whether tramadol, which has been shown to be an effective agent to treat NP in preclinical and clinical studies – and produces an anti-allodynic effect in sciatic nerve-ligated mice[8, 9] – modified the effect of peripheral neuropathy on the oocyte count, maturity and the copulation ratio of female mice.

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Materials and Methods

Animals

Adult female and male Balb/c mice obtained from Çukurova University, Health Sciences Experimental Application and Research Centre, Turkey were housed in standard cages (10/cage) with *ad libitum* access to food and water. The mice were maintained in a laboratory under a controlled temperature of 21 ± 1°C and a 12 h light/dark cycle; they were assigned randomly to experimental groups after a 2-week habituation period. Behavioral tests were performed during the light cycle. Mice were 4–8 weeks of age at the time of the experiments. All procedures were conducted in accordance with protocols approved by the Çukurova University Institutional Laboratory Animal Care and Use Committee and the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP).

Peripheral nerve injury procedure

Tight ligation of the partial sciatic nerve was performed to produce neuropathy, as described previously in rats by Seltzer et al.[10]. Briefly, the mice were anaesthetised with ketamine (80 mg/kg, intramuscularly [i.m.]) and xylazine (2.5 mg/kg, i.m.). The skin was prepared and an incision was made to expose the sciatic nerve and its three terminal branches at the upper-thigh level. A tight ligature with an 8-0 silk suture was made around one-third to one-half of the diameter of the sciatic nerve. The muscle/fascia layer and skin layer were closed separately with 4-0 silk sutures[11]. The nerve was exposed, but not ligated, in sham-operated mice. The cold-plate (CP) test was applied 2 or 4 weeks after surgery.

Cold-plate test

The CP test was used to evaluate allodynia, which is a prominent NP symptom. Allodynia and hyperalgesia are symptoms and signs that index pain and thus contribute to improved delineation of NP[12]. Allodynia refers to pain elicited by a stimulus that normally does not cause pain, according to the IASP. Mechanical (light touch) and heat stimuli (hot/cold) are usually used to measure allodynia. Clinical and animal studies have shown that a cold stimulus is more effective to measure allodynia[12]. We used the CP test to

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measure allodynia and track the development of NP. The CP test was carried out using a hot/CP apparatus (Ugo Basile Biological Research Apparatus, Varese, Italy). The temperature of the CP was kept at 5 ± 0.5°C. Each mouse was placed on the metal surface of the apparatus. The latency to first withdrawal behaviour (lifting, shaking, licking of one hind paw, or jumping) was measured and recorded as cold-plate latency (CPL). Each mouse was used in one experiment. A maximum cut-off time of 20 sec was used to prevent tissue damage at the low temperature[13].

Isolation of oocytes: Isolation of metaphase I (MI) and metaphase II (MII) phase oocytes

Human chorionic gonadotropin (5 IU hCG) (CG-5; Sigma, St Louis, MO, USA) was injected intraperitoneally (i.p.) into female mice 48 h after and injection of pregnant mare serum gonadotropin (PMSG, 5 IU, i.p.). Oocytes were collected from the ampulla under a stereo zoom microscope (SDZ; Kyowa Optical, Nagano, Japan) 18–20 h post-hCG injection. Cumulus cells were removed by hyaluronidase (75 mg/mL) treatment. The oocytes were washed three times with HEPES-buffered KSOM + bovine serum albumin (KFHM + BSA). Oocytes were classified using a phase-contrast microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan). Oocytes with homogeneous cytoplasm and no polar body were classified as MI. Oocytes with homogeneous cytoplasm and one polar body were classified as MII. Oocytes with fragmented cytoplasm were classified as degenerated.

Zygotes: Female mice induced with hCG 48 h post-PMSG injection were placed in a cage with a male for copulation. The ampulla of a female mouse with positive vaginal plaque was removed 22 h after the hCG injection. Cumulus cells of oocytes/zygotes were removed using hyaluronidase (75 mg/mL), and washed three times with KFHM + BSA. The oocytes were classified as described above. Oocytes containing two polar bodies and/or double pronucleus (PN) were classified as zygotes.

Experimental groups

Female mice were divided into Control (C), Sham (Sh), Neuropathy (NP) and Neuropathy + Tramadol (NP+TR) groups. Ten animals were used in each group. The C group did not undergo any surgical intervention. The Sh and NP groups were incised after

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anesthesia. Following the incision, the sciatic nerve was ligated, as described above, in the NP group but not in the Sh group. The superovulation protocol was performed 2 weeks after sciatic nerve ligation. Each group was divided into two subgroups: female alone or female with a male. One day after the superovulation protocol was performed, vaginal plaque control was made in the female mice caged with male mice. Then, the CP test was performed in all groups. Oocytes obtained from single female mice, and those caged with males, were classified as MI, MII, degenerated, or zygote according to their morphology. The sum of the MII oocytes and zygotes was considered as mature. Oocytes obtained from the single mice were classified as MI, MII, or degenerated, and MII-phase oocytes were considered as mature. The percentages of oocytes/zygotes were calculated. The CP test and superovulation protocol were performed in the groups 2 weeks after surgery to investigate the effect of NP and tramadol on oocytes. Tramadol (50 mg/kg, ip) was injected 30 min before the CP test. Tramadol dose was chosen according to similar previous publications generating in our laboratory in similar conditions not to use large number of animals for ethical reasons[14]. The results by experimental group and protocol are summarised in Table 1.

Drugs and solutions

Ketamine and xylazine were purchased from Sigma and injected i.m. for anesthesia. Tramadol hydrochloride was purchased from Sigma and injected i.p. and HEPES-buffered KFHM solution was prepared as KSOM and used to obtain oocytes and zygotes[15].

Statistical analysis

The CP latency results were analysed using one-way analysis of variance with a *post-hoc* Bonferroni test applied. The oocyte classification results were analysed using the Kruskal–Wallis and Bonferroni-corrected Mann–Whitney *U*-multiple-comparison tests. Results are expressed as means ± standard error, and $P < 0.05$ was considered significant.

Results

Cold plate latencies of the control, sham, neuropathic, and NP + Tramadol groups

As shown in Figure 1, no differences were observed between the CPL values of

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single females versus those kept with male mice, in any of the groups (SC vs. MC, SSh vs. MSh, SNP vs. MNP, and SNP + TR vs. MNP + TR). The CPL values of the NP single females and NP females kept with male mice were different from those of all other groups ($p < 0.05$). Tramadol enhanced CPL latency in NP mice, to nearly the same level as the C and Sh groups, in both the single females and females kept with males. The CPL of the SNP group was shorter than those of the SC, SSh, and SNP + TR groups (2.21 ± 0.29, 5.53 ± 0.55, 5.04 ± 0.69, and 6.37 ± 0.65, respectively, $p < 0.05$). The CPL of the MNP group was shorter than those of the MC, MSh, and MNP + TR groups (2.35 ± 0.43, 7.54 ± 1.16, 5.90 ± 0.60, and 5.55 ± 1.26 respectively, $p < 0.05$). These results show that NP induced by sciatic nerve ligation was prevented by tramadol.

Classification and number of oocytes recovered by superovulation in the experimental groups and the effects of tramadol

No differences in total oocyte numbers were observed between the single females and those kept with males, in the C, Sh, NP, or NP + TR groups (single females: 36.33 ± 4.34, 31.85 ± 3.88, 30.66 ± 5.18, and 27.85 ± 4.70; females kept with males: 30.77 ± 3.33, 33.25 ± 4.81, 30.00 ± 3.70, and 33.50 ± 4.43, respectively [Table 2]). The percentages of oocytes retrieved from all groups after superovulation are shown in Table 3 and Figure 2. The percentage of mature oocytes retrieved from MNP mice was significantly lower than that of the MC, MSh, and MNP + TR groups (30.24 ± 13.64, 94.79 ± 3.27, 86.67 ± 3.75, and 75.45 ± 6.24 respectively; $p < 0.01$). No significant change in percentage of mature oocytes was observed between the SNP, SC, SSh, and SNP + TR groups. The copulation ratio of MNP mice was lower than that of the MC, MSh, and MNP + TR groups (44.44%, 100%, 100%, and 85.71%, respectively, $p<0.05$). The percentage of MI oocytes in the MNP group was higher than that in the MC, MSh, and MNP + TR groups (66.09 ± 13.30, 5.28 ± 2.61, 4.63 ± 1.92, and 13.96 ± 6.98, respectively; $p < 0.01$). However, no significant change in percentage of immature oocytes was observed between the SNP, SC, SSh, and SNP + TR groups. The results according to housing conditions were as follows: the numbers of mature oocytes in the MC, MSh, and MNP + TR groups were higher compared to those in the SC, SSh, and SNP + TR groups, respectively; however, there was no difference between the MNP and SNP groups

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(Table 3, Fig. 2). These results show that tramadol reversed the negative effect of NP on oocyte maturation and the copulation ratio in female mice.

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Discussion

The results show that NP affected oocyte maturation and the copulation ratio of female mice and tramadol reversed the negative effects of NP in female mice. Although no significant difference was observed in the total number of oocytes in NP versus control mice, oocytes maturation rates decreased significantly in NP female mice housed with male mice compared to the control, sham, and tramadol groups.

We used the NP pain model to generate chronic pain in our experiments because of its clinical relevance, convenience of application, and reduced experimenter bias[16]. All NP models that include a manipulation are designed to produce nerve damage[16]. The most common nerve injury target is the sciatic nerve, which is readily accessible and can be easily probed because it innervates the hind limbs. We used a tight ligation of the partial sciatic nerve model because it is easy to implement, sensitive to cold stimuli, and has an appropriate time course of experimental chronic pain[17]. Our team has experience with this model, and all surgeries were performed by the same experienced individual (S.D), thereby greatly decreasing variability. Sensitivity to cold stimuli develops more robustly in these models and allodynia and hyperalgesia, which are symptoms of NP, become more apparent under cold conditions[12]. Our preliminary results were in line with other studies[18] and showed that neuropathy symptoms started during the first week. In the present study, cold allodynia was detected within the first day after the sciatic nerve was ligated and persisted for 2 weeks.

In the present study, CPLs of the Sh-operated and NP mice were lower than those of the C mice. Salo[19] showed that surgical trauma and anesthesia activated pro-inflammatory and anti-inflammatory responses. The lower CPL value of the Sh-operated mice compared with that of the C mice may due to the effect of cytokines induced by incision and anesthesia. On the other hand, no differences were found when we compared female mice housed singly or with a male (C, Sh, and NP groups; Fig 1). In other words, housing female mice with or without a male did not affect the degree of allodynia. These results show that ligating the sciatic nerve generates NP in all female mice, regardless of whether they are housed with a male.

The total numbers of oocytes retrieved after superovulation, of the C, Sh, NP, and

NP + TR mice, did not differ according to whether females were housed with or without a male (Table 2). However, the percentages of immature and mature oocytes differed in NP mice versus C, Sh, NP + TR and mice that were housed with males (MC, MSh, and NP + TR, respectively; Table 3). The percentage of mature oocytes and the copulation rate were significantly lower in the MNP group versus the MC, MSh and MNP + TR groups, but maturation was not different between the SC, SSh, SNP, and SNP + TR groups. The percentage of immature oocytes in the MNP group was higher than that in the MC, MSh and MNP + TR groups, but the difference was not significant between the SC, SSh, and SNP groups. We hypothesised that chronic pain negatively affected oocyte maturation by generating stress-induced changes. Interactions between chronic pain and stress have been reviewed previously and it was shown that stress affects pain and vice versa; however, the mechanisms underlying the effects remain unclear[5, 6]. Continuous pain decreases quality of life and limits physical and sexual activity[20]; furthermore chronic pain can be a source of increased stress and tension. On the other hand, stress increases the severity of persistent pain. Blackburn-Munro and Blackburn-Munro[5] discussed the link between chronic pain, chronic stress, and depression based on experimental and clinical evidence, and suggested that these diseases are linked via chronic stress-induced HPA dysfunction. Nerve injury increases spinal glucocorticoid (GC) receptors[6, 21]. Thus, the potential for stress-induced GCs to exert effects on spinal cord neurons or glia is enhanced by nerve injury. Stress and GCs may exacerbate NP by modulating neuroplasticity[22].

Additional underlying mechanisms have been suggested with respect to the relationship between stress and pain. Bravo et al.[23] proposed a locus coeruleus-related mechanism in the chronic pain/depression relationship. They suggested that mild stress triggers several modifications in locus coeruleus-noradrenergic transmission that are exacerbated by co-morbid chronic pain[23]. Several clinical and preclinical studies have shown that chronic pain and stress affect each other, via the HPA axis and several other complex neuroendocrine mechanisms. Chapman et al.[24] reported that physical injury or wounding generates a complex stress response that extends beyond the nervous system and contributes to the experience of pain. Chronic pain can lead to permanent changes in brain structures and functions, which in turn can affect brain processes not directly connected

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with the pain itself[2]. It has been suggested that chronic pain alters immune and endocrine functions[21-25].

On the other hand, stress-induced changes, for example with respect to endocrine, immune, and mechanical factors, reduce fertility, while psychological factors such as depression, anxiety, and stress affect fertility in women [26]. All of these factors are related to the HPA axis, which is an important mediator of infertility involved in the secretion of CRH, adrenocorticotrophic hormone (ACTH), and cortisol. Changes in diurnal cortisol secretion patterns accompany mental stress and mediate downregulation of the HPG axis. This effect could involve inhibitory mechanisms at the pituitary level, through a reduction in the release of follicle stimulating hormone (FSH) and luteinising hormone (LH) caused by gonadotropin releasing hormone (GnRH). Furthermore, the effect of cortisol on the HPG axis is dependent on the endocrine status of the ovary at different stages within the menstrual cycle. It has also been suggested that stress might alter cortisol-secretion patterns during the menstrual cycle, which would ultimately affect the hormonal profile during critical stages of fertilisation. The number of oocytes retrieved from patients undergoing IVF treatment who have a high stress score is lower than that of those with a low stress score[26]. Stress-related factors induced by pain negatively affect the HPA and decrease GnRH and FSH/LH release[27]. Severe pain has serious physiological effects on the endocrine system caused by stimulation of the HPA system, which in turn results in elevated serum ACTH, cortisol, and pregnenolone levels. If pain persists for too long, the hormonal system is unable to tolerate the stress of pain, and hormone production may decrease, thereby causing serum hormone levels to drop below normal[3]. We argue that NP persisting for 2 weeks caused severe stress such that hormone levels and oocyte maturation rates decreased in the mice in our study.

Decreases in the copulation ratio and oocyte maturation in female mice housed with male mice may be explained by the pheromone hypothesis. Pheromones are chemicals released by an animal that change the behavior of another animal of the same species [28]. These substances are secreted to trigger many types of behaviors, including sexual arousal. Pheromones released by female, and perceived by male, mice enhance libido and cause the male to exhibit mating behavior. In the present study, discrepancies between the C, Sh, and neuropathy subgroups of female mice housed with male mice may have been due to the

negative effects of stress on pheromone secretion. Female mice stressed due to neuropathy may not have been able to release pheromones, which may explain why the copulation rates of our MNP mice were lower than those of the MC and MSh groups. A simpler explanation that declined copulation rate may be because of aggressive behaviour of female mice due to inflicted pain.

In our study, tramadol reversed the negative effects of NP on oocyte maturation and the copulation ratio in female mice. Tramadol is an effective treatment for NP that exerts analgesic activity by inhibiting neuronal uptake of norepinephrine and serotonin and activating opioid receptors[29]. Another possible mechanism underlying tramadol-induced analgesia is attenuation of thermal and mechanical hyperalgesia and allodynia through its effect on pro-inflammatory cytokines. Nerve injury increases the expression and secretion of pro-inflammatory cytokines, such as tumour necrosis factor-α, interleukin (IL)-1b, IL-6, and interferon (INF)-g, all of which are required for pain hypersensitivity. Pro-inflammatory cytokines levels are high in patients with NP, whereas levels of anti-inflammatory cytokines, such as IL-10, are low. Tramadol decreases the release of pro-inflammatory cytokines during NP[30]. On the other hand, some cytokines affect female fertility. Women with reproductive failure have increased IL-10 and INF-g levels. IL-12 is positively correlated with fertilisation rate[31]. In our study we could not investigate the possible mechanisms of our results. Further research is needed to elucidate the mechanisms of of NP and tramadol on oocyte maturation and the copulation ratio in female mice.

In conclusion, we suggest that NP affects reproductive performance by altering various endogenous mechanisms such as regulation of the HPA axis, noradrenergic/serotonergic or cytokine-related pathways. Prospective studies that determine levels of cortisol, fertility hormones, cytokines, and other potential endogenous substances in neuropathic animals are needed to clarify the roles of possible mechanisms in reproduction.

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Conflict of Interest

The authors have no conflict of interest to disclose.

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Table 1. Experimental groups and applied protocols

Group	Housing	Surgical procedure		SO*	CP	Oocyte (O & C)	Drug (TR) / SP **	
		Incision	Ligation				Drug	SP
SC,	Single	-	-	+	+	+	-	+
MC,	With male	-	-	+	+	+	-	+
SSh	Single	+	-	+	+	+	-	+
MSh,	With male	+	-	+	+	+	-	+
SNP,	Single	+	+	+	+	+	-	+
MNP,	With male	+	+	+	+	+	-	+
MNP+TR	Single	+	+	+	+	+	+	-
MNP+TR	With male	+	+	+	+	+	+	-

SC: single female control, MC: female with male control, SSh: single female sham, MSh: female with male sham, SNP: single neuropathic female, MNP: neuropathic female housed with male, SO: superovulation, oocyte (O & C): oocyte collected and classification, TR: tramadol, SP: serum physiological, *: superovulation protocol was performed 2 or 4 weeks after the experiments to investigate the effects of peripheral neuropathy on oocyte features and fertility (drug-free experiments), **: drug or SF application and superovulation protocol was performed only 2 weeks after the experiments that investigated the effects of tramadol.

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Table 2. Total numbers of oocytes in the SC, SSh, SNP, SNP+TR and MC, MSh, MNP, MNP+TR groups

	Control	Sham	NP	NP+TR
Single	36,33±4,34 (n: 6)	31,85±3,88 (n:7)	30,66±5,18 (n:6)	27,85±4,70 (n:7
With male	30,77±3,33 (n:9)	33,25±4,81 (n: 6)	30,00±3,70 (n:6)	33,50±4,43 (n:6)

SC: single female control, SSh: single female sham, SNP: single neuropathic female, MC: female with male control, MSh: female with male sham, MNP: neuropathic female housed with male, TR: tramadol.

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Table 3. Percent oocytes values obtained from all groups of mice in the experiments that evaluated the effect of tramadol

% Values	Control		Sham		NP		NP + TR	
	Single	With male	Single	With male	Single	With male	Single	With male
MI	57.55 ± 5.79	5.28 ± 2.61[a]	57.67 ± 8.13	4.63 ± 1.92[a]	73.61 ± 7.29	66.09 ± 13.30*	67.06 ± 7.86	13.96 ± 6.98[a]
MII	32.76 ± 4.31	20.12 ± 9.67	39.93 ± 7.46	19.45 ± 7.35	20.95 ± 6.22	25.28 ± 12.70	27.64 ± 7.74	15.95 ± 6.11
PN	-	74.67 ± 10.70	-	67.22 ± 7,77	-	4.88 ± 2.16	-	59.50 ± 12.94
Degenerated	9.57 ± 3.41	1.63 ± 0.96	2.40 ± 1.66	8.33 ± 2.12	4.38 ± 2.79	3.90 ± 1.46	6.84 ± 2.96	3.30 ± 2.58
Mature	32.76 ± 4.31	94.79 ± 3.27[a]	39.93 ± 7.46	86.67 ± 3.75[a]	20.95 ± 6.22	30.24 ± 13.64*	27.64 ± 7.74	75.45 ± 6.24[a]
Copulation (%)	-	100	-	100	-	44,44**	-	85.71

*, Different from MC, MSh, and MNP+TR groups ($p<0.01$); **, Different from MC, MSh, and MNP+TR groups ($p<0.05$); a, different from related single groups ($p<0.05$)

MC: female with male control, MSh: female with male sham, MNP: neuropathic female housed with male, TR: tramadol.

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