行政院國家科學委員會專題研究計畫　成果報告

嗎啡的免疫調理作用機轉　與細胞凋亡和 μ受體的關係　第二年

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中文摘要
神經病變性疼痛是由於神經受到外傷或疾病導致的傷害所引起的疼痛，為最困難治療的疼痛之一。臨床上，對此種疼痛仍然沒有一絕對的治療方式。單獨使用嗎啡類藥物或合併其他藥物治療神經病變性神經性疼痛在動物實驗和人體上，已證實有效。使用嗎啡治療神經性疼痛的病人需長期服藥，免疫功能的變化極為重要。我們對嗎啡、神經病變性疼痛、及免疫功能的相關作用之研究已投注不少心力，建立穩定之神經病變性疼痛動物及免疫功能測試之模式，結果顯示以系統方式給予嗎啡控制神經病變性疼痛雖然可達到止痛效果但對免疫功能的調理作用有負面的影響，且止痛效果尚未達最大時，免疫功能已受相當之抑制。於88年度所提出之研究計劃“嗎啡治療神經病變性疼痛的免疫調理作用及機轉”探討嗎啡影響免疫功能的機轉是抑制中樞神經的作用亦或抑制周邊免疫系統所造成，以及不同作用機轉之止痛藥物tramadol 對神經病變性疼痛及免疫功能之作用，也得到正面的成果。已有研究顯示免疫細胞上opioid 受體的存在，其在嗎啡對免疫功能的調理作用扮演重要角色並可能影響免疫細胞的凋亡。急性給予heroin 會引起老鼠明顯的免疫功能改變，長期服用heroin之成癮者容易有細菌感染，這些作用和heroin 減弱macrophage 的phagocytosis 有關，而heroin也改變老鼠白血球的數目並且引起細胞之凋亡。嗎啡雖是heroin 的活性代謝產物，兩者對免疫功能的作用仍有所差別，由transforming growth factor-β 調控。嗎啡對免疫系統的抑制作用來自中樞系統及周邊系統的影響，若以脊椎內給藥方式則免疫功能之抑制如何，來自周邊系統的抑制作用消失後是否可以排除脊椎上中樞系統的影響。延續前二次研究計劃之結果，繼續探討在神經病變性疼痛的動物模式下，給予嗎啡治療免疫細胞和 μ 受體之關係的作用，並試著研究在CCI大白鼠身上椎管內嗎啡對自然殺手細胞活性與 T 淋巴球功能之影響。在雄性Sprague-Dawley大白鼠的右後肢坐骨神經進行CCI的七天前，預先植入椎管內導管，以測量對熱刺激的腳掌縮回延遲時距來確定已產生熱痛覺過敏反應。藉由傑克豆球蛋白(ConA)與植物性血球凝集素(PHA)誘發之脾細胞增生來評估 T 淋巴球功能。以乳酸去氫酵素(LDH)釋放試驗來測量自然殺手細胞活性。經由椎管內導管給予生理食鹽水或嗎啡(分別給予 30、60、90 µg)，必要時以食鹽水稀釋到總體積為 10 µl。給藥後 60、120、180、240 分鐘後評估 PWL。給藥後 16 小時將動物犧牲以進行免疫研究。經CCI處理之大白鼠全部產生熱痛覺過敏反應，且椎管內嗎啡注射對此呈現劑量相關的反轉效果。椎管內嗎啡治療可顯著抑制 ConA 與 PHA 誘發之自然殺手細胞活性與脾細胞增生，且此抑制與劑量有關。經CCI處理之大白鼠給予椎管內嗎啡，可引起自然殺手細胞活性與脾細胞增生之抑制，且此抑制與劑量有關。上述結果暗示脊髓上的鴉片類途徑可能與椎管內嗎啡的免疫調理有關。

關鍵詞：神經病變性疼痛，嗎啡，免疫力，μ 受體。

Abstract
Neuropathic pain, resulting from nerve injury due to trauma or disease, is one of the most difficult challenge to pain management. All these painful conditions share some characteristics such as spontaneous pain, hyperalgesia, dysesthesia and allodynia. Research has revealed several possible mechanisms. However, there was no absolute effective therapeutic modality clinically. Recently, using morphine-like drugs to control neuropathic pain has proved to be effective in both animal and human studies. Opioids are also among the most widely prescribed drugs for individuals suffering from pain. Accordingly, the issue on immunomodulation of morphine in patients with neuropathic pain is very important. Although opioids may have deleterious effects on immune function, the evidence that neuropathic pain may suppress immune function emphasized the need for aggressive control of pain state. In our previous study, the data showed that the analgesic effects of morphine in neuropathic pain modol are dose-dependent, and so is the immune modulation, but the suppression of immune function developed before reach of the maximal analgesic effects. The findings
evoked us the studies for the mechanisms about immunomodulation of morphine and the immune effects of the potent analgesics with different analgesic mechanisms. Morphine modulates the immune function on both central and peripheral immune systems. By intrathecal administration of equipotent analgesic dosage of morphine as systemic route, morphine has less depression on immunity. Therefore, central immunomodulation of morphine may result from supraspinal mechanisms. Studies have shown the evidence for opioid receptors on cells involved in host defense and the immune system, which may play an important role in the immunomodulation of morphine and affect the apoptosis of immune cells. Clinical evidence indicates that heroin addicts are prone to infection. Morphine inhibits T cell proliferation and suppresses natural killer cell activity in dose dependent manner. Chronic pain and opioids are linked with suppression of immune function. We have demonstrated that systemic morphine therapy can cause a dose-dependent inhibition of NK cell activity and splenocyte proliferation in rats with chronic constriction injury (CCI) of the sciatic nerve. The role of spinal opioid systems in modulation of immune system have not been evaluated yet. This study attempts to investigate the effects of intrathecal morphine on NK cell activity and T lymphocyte function in CCI rats.

Intrathecal catheters were implanted in male Sprague-Dawley rats 7 days before CCI of the sciatic nerve over the left hind limb. Paw withdrawal latencies (PWL) to heat stimulation were measured to assure the development of thermal hyperalgesia. T lymphocyte function was evaluated based on concanavalin-A (ConA) - and phytohemagglutinin (PHA)-induced splenocyte proliferation. NK cell activity was measured by lactic acid dehydrogenase (LDH) release assay. Either normal saline or morphine at doses of 30, 60, and 90 µg, respectively, was administered through intrathecal catheter in each groups. All drugs were diluted with saline if needed to a total volume of 10 µl. PWLs were evaluated 60, 120, 180, and 240 min after drug administration. Animals were killed for immune study 16 hours after drug administration. All rats who received CCI developed thermal hyperalgesia, which was dose-dependently reversed after intrathecal injection of morphine. NK cell activity and splenocyte proliferation induced by ConA and PHA were significantly suppressed by intrathecal morphine treatment in a dose-related manner. These data suggest that intrathecal morphine can cause a dose-dependent inhibition of NK cell activity and splenocyte proliferation in CCI rats. This may imply that supraspinal opioid pathways are involved in the immunomodulation of intrathecal morphine.

Keywords: Neuropathic pain, morphine, immunity, apoptosis

1. Introduction
Chronic pain and opioids are linked with suppression of immune function. We have demonstrated that systemic morphine therapy can cause a dose-dependent inhibition of NK cell activity and splenocyte proliferation in rats with chronic constriction injury (CCI) of the sciatic nerve. The role of spinal opioid systems in modulation of immune system have not been evaluated yet. This study attempts to investigate the effects of intrathecal morphine on NK cell activity and T lymphocyte function in CCI rats.

2. Material and Methods

1. Animals
This study will adhere to the Ethical Guidelines of the International Association for the Study of Pain (Zimmermann, 1983). Male Sprague-Dawley rats weighing between 250 to 300 g will be obtained from the Animal Center of the National Cheng Kung University (Tainan, Taiwan). The animals will be housed at a controlled temperature of 21 ± 0.5°C in wire-mesh cages, with free access to food and water. The vivarium will be maintained automatically on a 12:12-h light/dark cycle with lights on at 07:00 h.

2. Surgery
Rats will be anesthetized with a 60-mg/kg intraperitoneal injection of sodium pentobarbital. An incision will be made from the left sciatic notch to the distal thigh using standard aseptic techniques. The subcutaneous tissue will be bluntly dissected under the skin to expose the biceps femoris muscle. The sciatic nerve will be freed from its investing fascia. Four ligatures (4-0 chromic) will be placed approximately 1 mm apart around the sciatic nerve and tightened until the suture barely indented the nerve. The muscle and skin will be then closed. To observe the effect of chronic constriction injury (CCI)-induced pain on apoptosis and µ-opioid receptor regulation, a group of rats will receive sham surgery consisting of exposure of the sciatic nerve in the same way but without ligation in the left limb.

3. Paw withdrawal latency (PWL) test
Thermal hyperalgesia will be assessed by
measurement of PWLs to radiant heat as described previously (Hargreaves et al., 1988). A plantar test apparatus will be used (Ugo Basile, Comerio, Italy). Briefly, the rats will be placed in a clear plastic chamber and left to acclimatize for at least 10 minutes before testing. The radiant heat source is a high intensity lamp, which is projected through a round aperture at the base of the plastic chamber and thereby delivered to the plantar surface of the rat’s hindpaw. The time taken for the animal to withdraw its hindpaw will be measured.

4. Pharmacological experiments
Morphine chloride will be purchased from the Narcotics Control Bureau of Taiwan. Ten animals will be used for each treatment group. PWLs will be obtained one day before surgery (day 0) and the 4th day after surgery (day 5) to confirm the development of thermal hyperalgesia. On day 6, either normal saline or morphine will be subcutaneously administered at doses of 7.5 and 15 mg/kg respectively in the acute treatment groups, and PWLs will be measured 60 minutes after treatment. The animals will be killed by cervical dislocation 60 min after treatment. In the chronic treatment groups, two doses of morphine will be decided according to the antinociceptive effects and duration of morphine in the acute groups. Continuous systemic administration of morphine or normal saline will be provided for 7 days at a uniform rate of 10 µl/hr via an osmotic minipump (Alza, Model 2ml1), which will be implanted subcutaneously in the rat’s lower back. PWLs were evaluated on the first, third, fifth and seventh days of treatment (day 6, 8, 10, and 12, respectively). The animals will be killed on day 12.

5. Preparation of spleen cells
All spleens will be aseptically removed and teased on a steel mesh immersed in chilled RPMI-1640 (Gibco BRL, Grand Island, NY) in a plastic dish. The cells that pass through the mesh will be washed twice with RPMI-1640. The erythrocytes will be lysed with double distilled water, while the remaining cells will be resuspended in Dulbecco’s Modified Eagle medium (DMEM, Gibco BRL) with 10% fetal bovine serum (FBS, Gibco BRL) containing 2 mM glutamine (Sigma Chemical, St. Louis, MO), 100 IU/ml penicillin G, and 100 µg/ml streptomycin.

6 Measurement of the mRNA level of µ-opioid receptor
a. RNA extraction
The spleen cells will be washed twice with phosphate-buffered saline (PBS) and detached with 0.05 % trypsin with 0.02% EDTA. This suspension will be then collected in sterile tubes, and centrifuged at 2000 r.p.m. for 5 min. The pellet was resuspended in 2 ml of an RNA extraction buffer containing guanidinium isothiocyanate and homogenized. Total cellular RNA will be extracted with phenol and precipitated with ethanol using a procedure that results in a recovery of RNA that averages 77 ± 7.2% (S.D.). 20 µm of glycogen (Boehringer-Mannheim, GmbH, Germany) will be used during the extraction procedure as a carrier. Poly (A) + RNA was prepared from 1 mg of total RNA and 0.1 mg of oligo dT cellulose (Sigma) as described by Sambrook (1989).

b. Northern blot analysis
RNA samples from spleen cells will be denatured in 1 M glyoxal/50% (v/v) dimethyl sulphoxide for 60 min at 50℃, fractionated by horizontal electrophoresis at room temperature in agarose gel containing 0.01 M sodium phosphate at pH 7.1 (with buffer recirculation) transferred to nitrocellulose filter in the presence of 20 x standard saline citrate, and baked in a vacuum oven at 80℃ for 4 h. Filters will be washed three times in 1 x SSC (0.15M NaCl and 0.015 M sodium citrate) at room temperature for 1 h and air-dried before hybridization. Following hybridization, the filters will be washed for 1 h in 0.1 x SSC at 65℃ and then exposed to Kodak X-AR film for three to five days at -70℃. The size of each hybridization band will be estimated by use of an unlabelled RNA marker. The developed films will then be scanned using the Adobe Photoshop software and the image will be printed on photographic paper. The presence of multiple bands for mRNA will be confirmed by densitometry analysis of the computer scans using the IP Lab Gel software.

7. Statistical analysis
PWLs will be expressed as a percentage of the day 0 value. Statistical analysis of the data on PWLs, and apoptosis markers in acute and chronic treatment groups will be performed by one-way analysis of variance (ANOVA), followed by a post hoc Tukey’s test for multiple comparisons. Data will be presented as the means ± SEM. A P value of less than 0.05 will be considered significant.

Results
Fig. 1. Effect of intrathecal morphine treatment at doses of 0, 30, 60, and 90 µg on PHA-induced (vehicle, 2, and 4 µg/ml) splenocyte proliferation. Values are mean ± SEM. **P<0.01 vs. saline group, #P<0.01 vs. 30µg group, !P<0.05 vs. 30µg group.

Fig. 2. Effect of intrathecal morphine treatment at doses of 0, 30, 60, and 90 µg on ConA-induced (vehicle, 1, and 2 µg/ml) splenocyte proliferation. Values are mean ± SEM. *P<0.05 vs. saline group, **P<0.01 vs. saline group, #P<0.01 vs. 30µg group, !P<0.05 vs. 60µg group.

Fig. 3. Effect of intrathecal morphine treatment at doses of 0, 30, 60, and 90 µg on natural killer cell activity (effector: target = 1.25 :1, 2.5:1, 5:1, 10:1). Values are mean ± SEM. **P<0.01 vs. saline group, #P<0.01 vs. 30µg group.

There were no significant differences in the weights of rats used in this study. As of the 4th day following surgery (day 5), all rats with loose ligation of the sciatic nerve had developed neuropathic pain syndrome as previously described by Bennett and Xie (1988), and thermal hyperalgesia indicated by a significant reduction in PWLs of the CCI limb (P<0.001) compared to the preoperative (day 0) value.

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4. Discussion
The results of this study demonstrate that thermal hyperalgesia in a rat model of neuropathic pain can be relieved with intrathecal morphine treatment. When administered acutely, intrathecal morphine significantly depressed T cell functions, as indicated by the inhibition of ConA and PHA induced proliferation. Moreover, it inhibited the activity of NK cells, a subset of lymphocytes capable of mediating spontaneous cytotoxicity against tumor cells (Whiteside and Herberman, 1989), which appear to have an important role in immune defense against viral infections (Biron et al., 1989). This study also found that both the reversal of thermal hyperalgesia by
in the CCI neuropathic pain model and the immunosuppressive effects were dose-dependent.

Considerable evidence suggests that opioids interact with the immune system. Opioid anesthesia suppresses natural killer cell activity (Beilin et al., 1992). Immunological dysfunction developed in heroin addicts (Brown et al., 1974). Chronic opioid administration results in depressed lymphocyte proliferative responses and reduced expression of cell surface antigens specific to T-lymphocytes and T-helper/inducer (CD4+) and T-suppressor/cytotoxic (CD8+) lymphocyte subsets in mice (Kimes et al., 1992). Morphine–exposed rats demonstrate suppression of NK cell cytotoxicity (Shavit et al., 1984). Morphine inhibits spontaneous and cytokine-enhanced natural killer cell cytotoxicity at doses within the range of analgesic use in volunteers (Yeager et al., 1995).

The mechanism of morphine’s effects on immunomodulation is still not completely understood. Studies have suggested that morphine’s immunomodulatory effects may not be mediated via mechanisms similar to those which are responsible for morphine’s antinociceptive effects. Morphine injected into the third cerebral ventricle or the periaqueductal gray mater (PAG) of the mesencephalon in rats results in a marked suppression of lymphocyte proliferation and NK cell activity (Weber and Pert, 1989; Hernandez et al., 1993). Dissociation of immunosuppressive effects with antinociceptive effect was noted when morphine was injected into the anterior hypothalamus (Hernandez et al., 1993). These findings suggest that supraspinal opioid pathways are involved in the immunosuppressive effects of morphine and these pathways may be distinct from those participating in opioid-induced analgesia. Hamra and Yaksh (1996) found that subcutaneous morphine, but not equianalgesic doses of intrathecal morphine, inhibited mitogen-induced lymphocyte proliferation, decreased splenic lymphocyte number, and altered phenotypic expression of cell surface markers. The lack of effect of spinally administered morphine indicates that spinal opioid receptors are not involved in morphine immunomodulation. Moreover, direct effects of morphine by stimulating opiate receptors on the immune cells may play a role as well. Morphine might also act on immune function via non-opiate mechanisms (Peterson et al., 1993).

Opioid-immune interactions may have important clinical implications. Treatment of neuropathic pain with opioids may require a higher dosage relative to non-neuropathic pain (Cherny et al., 1994). In the present study, although the analgesic effects of intrathecal morphine in the neuropathic pain model and the immunomodulation were both dose dependent, the immunosuppressive effects seemed late sensitive to intrathecal morphine treatment. Further clarification of the mechanisms responsible for the interaction between intrathecal and supraspinal morphine’s immunologic and analgesic effects in the neuropathic pain state may provide information which is beneficial in a clinical setting. The decision to use intrathecal morphine therapy for the treatment of neuropathic pain should be made cautiously, especially for immunocompromised patients.

In conclusion, the present study suggests that intrathecal morphine can cause a dose-dependent inhibition of NK cell activity and splenocyte proliferation in CCI rats. This may imply that supraspinal opioid pathways are involved in the immunomodulation of intrathecal morphine.

References:


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執行單位：成大醫學院麻醉學科

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