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Endomorphins and Ohmefentanyl in the Inhibition of Immunosuppressant Function in Rat Peritoneal Macrophages: An Experimental In Vitro Study

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ABSTRACT

BACKGROUND: The potential immunosuppressant effects of opioids might have clinical implications. The effects of endomorphins (EMs) and ohmefentanyl (OMF) on cultured rat peritoneal macrophages remain unclear.

OBJECTIVE: The aim of this study was to investigate the immunosuppressant effects of EMs and OMF on cultured rat peritoneal macrophages in vitro.

METHODS: Purified rat peritoneal macrophages, from healthy adult male Sprague-Dawley rats, were cultured with EM-1 (EM-1 group), EM-2 (EM-2 group), OMF (OMF group), and saline (saline group). We measured the concentrations of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in supernatant when macrophages were cultured with 10^{-6} mol/L of EM-1, EM-2, OMF, or saline for 0, 6, 12, and 24 hours (time-effect relationship) or with 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} mol/L of these substances for 24 hours (concentration-effect relationship). We also determined the phagocytic and bactericidal activities of macrophages using isotope markers when macrophages were cultured with 10^{-6} mol/L of EM-1, EM-2, OMF, or saline for 24 hours.

RESULTS: Compared with the saline group, TNF-α concentration decreased significantly in the OMF, EM-2, and EM-1 groups at 12 hours ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) and at 24 hours ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). Compared with the saline group, IL-1β concentration decreased significantly in the OMF, EM-2, and EM-1 groups at 12 hours ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) and at 24 hours ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). Decreased TNF-α and IL-1β concentrations were observed in the supernatant at 24 hours when cultured with 10^{-8}, 10^{-7}, and 10^{-6} mol/L in the OMF and EM-2 groups (all, $P < 0.05$) and in the EM-1 group (all, $P < 0.01$). Compared with the saline group, macrophage phagocytic activity (all, $P < 0.05$) and macrophage bactericidal activity (all, $P < 0.01$) were significantly lower in the 3 experimental groups compared with the saline group.

CONCLUSION: In this in vitro experiment, EM-1, EM-2, and OMF inhibited the immunosuppressant function of cultured rat peritoneal macrophages, including decreasing TNF-α and IL-1β concentrations and phagocytic and bactericidal activities. (*Curr Ther Res Clin Exp.* 2008;69:56–64) © 2008 Excerpta Medica Inc.

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KEY WORDS: endomorphins, ohmefentanyl, macrophage, immunomodulator.

INTRODUCTION

Pharmacologic studies have reported the effects of opioids on the immune system, with their effects on μ-, Δ-, and κ-opioid receptors being particularly well documented.[1] Some studies suggested that endogenous opioid peptides and opioid receptors exist widely in both the nervous system and the immune system and may play an important role in the immune system.[2–4] Endomorphins (EMs) are potent and selective endogenous agonists for μ-opioid receptors (MORs),[5–7] while ohmefentanyl (OMF) is a selective exogenous agonist for MORs with high analgesic potency.[8]

Studies have found that the ligands for MORs, including endogenous opioid peptides and exogenous opiates, might not only produce powerful analgesia in treating various types of pain but might also interfere with the immunoresponse that usually manifests as immunosuppression, especially cellular immunodepression.[9–13] Acute and chronic use of morphine, a typical exogenous ligand with high affinity and specificity for MORs, could impair host innate immunoresponse and increase susceptibility to bacteria, virus, and cancer metastasis.[9–11] EM-1 and EM-2, endogenous ligands for MORs, are tetrapeptides located within the mammalian central nervous system and immune system.[2–4] After a literature review, Jessop[12] concluded that EMs might exert potent anti-inflammatory effects in both acute and chronic peripheral inflammation.

EM-1 and EM-2 have high specificity and affinity for MORs. They have different amino acid compositions at the third amino acid[5,14,15]; this difference may allow the formation of different 3-dimensional structures, which may lead to different affinity and selectivity of MORs.[15]

A preliminary study completed by Sedqi et al[16] suggested that EM-1 and EM-2 at 10^{-8} to 10^{-6} mol/L concentration dependently inhibited oxyradical formation and the oxidative burst caused by neutrophils. They also found that these effects could be reversed by β-funaltrexamine, but not by the antagonist of κ-opioid, which suggests that EM affected the immune system through the action of μ-opioids.

The effects of EMs and OMF on cultured rat peritoneal macrophages in vitro remain unclear. The aim of this study was to investigate the immunosuppressant effects of EMs and OMF on cultured rat peritoneal macrophages in vitro.

MATERIALS AND METHODS

Animals

The experimental protocol of the present trial was reviewed and approved by the Animal Investigation Ethics Committee of Jinling Hospital. The Animal Center of Jinling Hospital provided healthy adult male Sprague-Dawley rats for the study.

Collection and Cultivation of Rat Peritoneal Macrophages

After the rats were anesthesized with phenobarbital (40–50 mg/kg), their abdomens were punctured and peritoneal macrophages were washed out using citrate-phospho buffer (0.15 mol/L, pH 7.2). After centrifugation at 1000 rpm for 10 minutes, adherent

cells were cultured in RPMI 1640 (Promega Co., Madison, Wisconsin) supplemented with 10% bovine serum (Xuzhou Huamei Co. Ltd., Jiangsu, China). A concentration of 1×10^7 cells/mL was obtained and then cultured on 6-well plates in 5% carbon dioxide (CO_2) at 37°C for 2 hours. Nonadherent cells were then removed by washing 3 times with RPMI 1640. The viability of macrophages determined by trypan blue was >98%.

A concentration of purified macrophages of 1×10^7 cells/mL was obtained using RPMI 1640 and 10% bovine serum. The macrophage solution was then placed in 96-well plates. The procedure was performed by the same researcher (W.-Y.L.) under the same conditions for each group.

STUDY PROTOCOL

The purified rat peritoneal macrophages were divided into 4 groups: EM-1 group (Sagon Co., Shanghai, China), EM-2 group (Sagon Co.), OMF group (Sigma-Aldrich Co., St. Louis, Missouri), and saline group. There were 10 wells per group. The supernatant was stored at –20°C. The immunosuppressant functions of the macrophages were evaluated by determining concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and phagocytic and bactericidal activities.

TUMOR NECROSIS FACTOR-α AND INTERLEUKIN-1β CONCENTRATIONS

TNF-α and IL-1β concentrations were measured by double antibody sandwich enzyme-linked immunosorbent assay using the procedure specified in the cytokine kits (Sigma-Aldrich Co.). Concentrations were measured when the macrophages were cultured with EM-1, EM-2, OMF, or saline 10^{-6} mol/L for 0, 6, 12, and 24 hours (time-effect relationship) or with 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} mol/L for 24 hours (concentration-effect relationship). These concentrations were selected according to the results of our pilot study.

MACROPHAGE PHAGOCYTIC ACTIVITY

We studied macrophage phagocytic and bactericidal activities using isotope markers. The method was modified according to the report from Schroit and Gallily.[17] After the macrophages were cultured with 10^{-6} mol/L for 24 hours, they were added to *Candida albicans* at the ratio of 5:100 and 5 μCi/mL tritiated thymidine (3H-TdR) (radiation intensity was 23 Ci/mmol). The macrophages were then cultured with RPMI 1640 plus 10% bovine serum in 5% CO_2 at 37°C. New *C albicans* would uptake 3H-TdR, and macrophage radiation activity was measured after 30 minutes. A liquid scintillation counter was used to evaluate macrophage radiation activity by measuring count/minute (cpm). Macrophage phagocytic activity was evaluated as the change in cpm.

MACROPHAGE BACTERICIDAL ACTIVITY

The same method used to evaluate macrophage phagocytic activity was used to determine macrophage bactericidal activity, except that macrophage cpm was measured at 60 minutes. The method was modified according to the report from Schroit and Gallily.[17]

Bactericidal activity was measured as the sterilization percentage, which was calculated as follows:

$$\text{sterilization percentage} = \frac{(\text{30-min cpm value} - \text{60-min cpm value})}{\text{30-min cpm value} \times 100\%}$$

STATISTICAL ANALYSIS

After a test for homogeneity of related variances, comparisons were made using 1-way analysis of variance followed by the Student-Newman-Keuls test for post hoc multiple comparisons. Differences were considered to be significant at $P < 0.05$. Data are expressed as mean (SD). Statistical analysis was performed using SPSS software, version 11.0 (SPSS Inc., Chicago, Illinois).

RESULTS

TUMOR NECROSIS FACTOR-α AND INTERLEUKIN-1β CONCENTRATIONS

Compared with the saline group, TNF-α concentration decreased significantly in the OMF, EM-2, and EM-1 groups at 12 hours (242 vs 196 pg/mL, $P < 0.05$; 242 vs 179 pg/mL, $P < 0.05$; and 242 vs 156 pg/mL, $P < 0.01$, respectively) and at 24 hours (238 vs 170 pg/mL, $P < 0.05$; 238 vs 132 pg/mL, $P < 0.01$; and 238 vs 110 pg/mL, $P < 0.01$). Compared with hour 0, TNF-α concentration also decreased significantly in the OMF, EM-2, and EM-1 groups at 12 hours (249 vs 196 pg/mL, $P < 0.05$; 242 vs 179 pg/mL, $P < 0.05$; and, 246 vs 156 pg/mL, $P < 0.01$) and at 24 hours (249 vs 170 pg/mL, $P < 0.05$; 242 vs 132 pg/mL, $P < 0.01$; and 246 vs 110 pg/mL, $P < 0.01$) (Figure 1). The

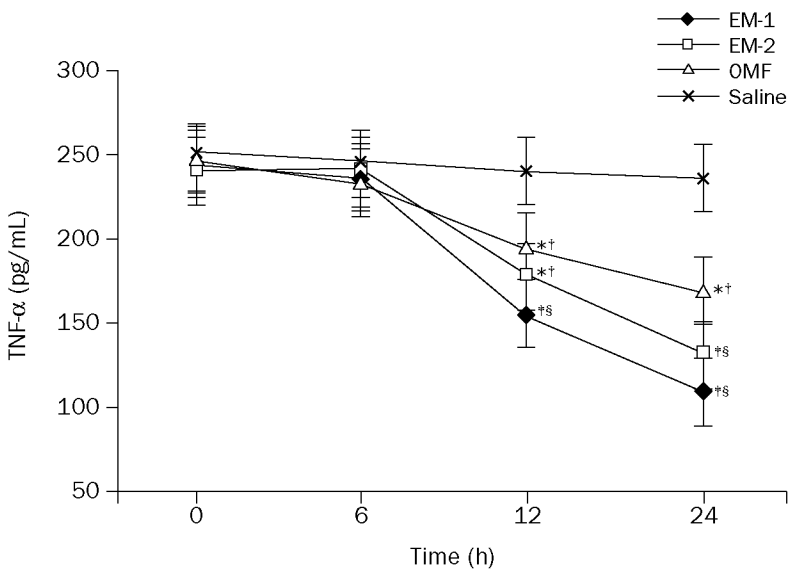

Figure 1. Time-effect relationship of experimental rat macrophage-derived tumor necrosis factor (TNF)-α concentration measured when the macrophages were cultured with 10^{-6} mol/L of endomorphin (EM)-1, EM-2, or ohmefentanyl (OMF) for 0, 6, 12, and 24 hours (n = 10 wells/group). *$P < 0.05$ versus saline group; †$P < 0.05$ versus hour 0; ‡$P < 0.01$ versus saline group; §$P < 0.01$ versus hour 0.

percentage decreases in TNF-α concentration at 12 and 24 hours in the EM-1 group (37% and 55%, respectively) were significantly higher than those in the EM-2 and the OMF groups (both, $P < 0.05$).

Compared with the saline group, IL-1β concentration decreased significantly in the OMF, EM-2, and EM-1 groups at 12 hours (48 vs 41 pg/mL, $P < 0.05$; 48 vs 39 pg/mL, $P < 0.05$; and 48 vs 34 pg/mL, $P < 0.01$, respectively) and at 24 hours (47 vs 35 pg/mL, $P < 0.05$; 47 vs 32 pg/mL, $P < 0.01$; and 47 vs 26 pg/mL, $P < 0.01$). Compared with hour 0, IL-1β concentration also decreased significantly in the OMF, EM-2, and EM-1 groups at 12 hours (51 vs 41 pg/mL, $P < 0.05$; 51 vs 39 pg/mL, $P < 0.05$; and 52 vs 34 pg/mL, $P < 0.01$) and at 24 hours (51 vs 35 pg/mL, $P < 0.05$; 51 vs 32 pg/mL, $P < 0.01$; and 52 vs 26 pg/mL, $P < 0.01$) (**Figure 2**). The percentage decreases in IL-1β concentration at 12 and 24 hours in the EM-1 group were significantly higher than those in the EM-2 and OMF groups (both, $P < 0.05$).

TNF-α and IL-1β concentrations decreased in the supernatant at 24 hours when cultured with 10^{-8}, 10^{-7}, and 10^{-6} mol/L in the OMF and EM-2 groups (all, $P < 0.05$) and with 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} mol/L in the EM-1 group (all, $P < 0.05$) (**Figures 3** and **4**).

MACROPHAGE PHAGOCYTIC ACTIVITY

Macrophage phagocytic activity was significantly lower in the OMF, EM-2, and EM-1 groups than that in the saline group (5.3, 5.2, 4.5 vs 6.0 cpm, respectively; all,

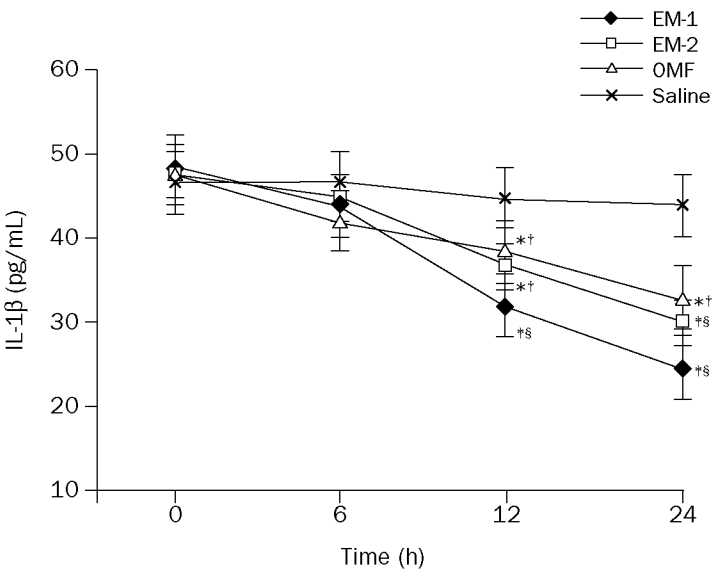

Figure 2. Time-effect relationship of experimental rat macrophage-derived interleukin (IL)-1β concentration measured when the macrophages were cultured with 10^{-6} mol/L of endomorphin (EM)-1, EM-2, or ohmefentanyl (OMF) for 0, 6, 12, and 24 hours (n = 10 wells/group). *$P < 0.05$ versus saline group; †$P < 0.05$ versus hour 0; *$P < 0.01$ versus saline group; §$P < 0.01$ versus hour 0.

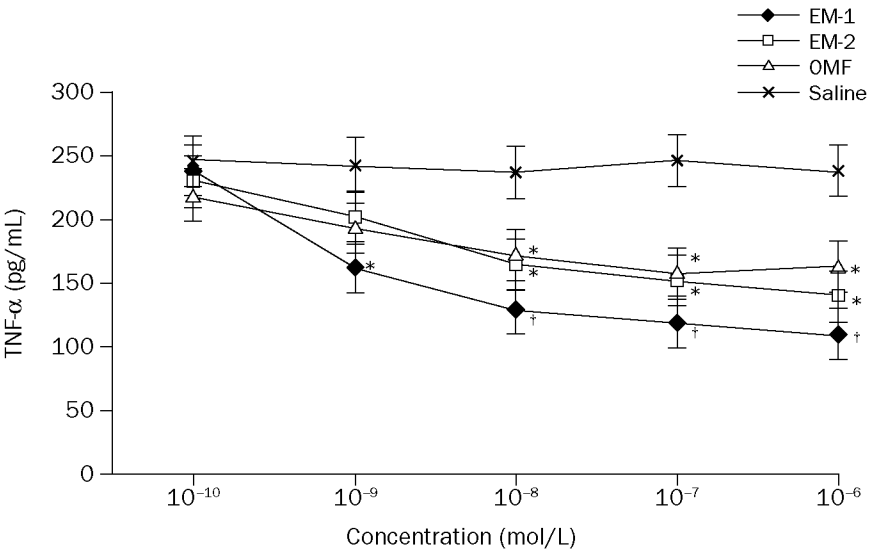

Figure 3. Concentration-effect relationship of experimental rat macrophage-derived tumor necrosis factor (TNF)-α concentration measured when the macrophages were cultured with 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} mol/L of endomorphin (EM)-1, EM-2, or ohmefentanyl (OMF) for 24 hours (n = 10 wells/group). *$P < 0.05$ versus saline group; †$P < 0.01$ versus saline group.

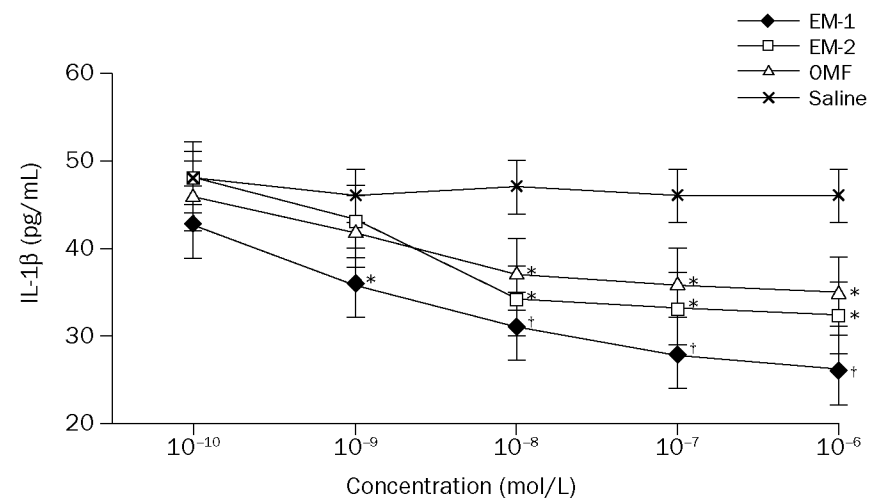

Figure 4. Concentration-effect relationship of experimental rat macrophage-derived interleukin (IL)-1β concentration measured when the macrophages were cultured with 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} mol/L of endomorphin (EM)-1, EM-2, or ohmefentanyl (OMF) for 24 hours (n = 10 wells/group). *$P < 0.05$ versus saline group; †$P < 0.01$ versus saline group.

$P < 0.05$) (Figure 5). There were no significant between-group differences among the 3 experimental groups in macrophage phagocytic activity.

MACROPHAGE BACTERICIDAL ACTIVITY

Similar to phagocytic activity, macrophage bactericidal activity was significantly lower in the OMF, EM-2, and EM-1 groups than that in the saline group (21%, 22%, 20% vs 39%, respectively; all, $P < 0.01$) (Figure 6). There were no significant between-group differences among the 3 experimental groups in macrophage bactericidal activity.

DISCUSSION

TNF-α and IL-1β concentrations decreased in both a time-dependent and a concentration-dependent manner in the supernatant when cultured with 10^{-6} mol/L of EM-1, EM-2, or OMF for 12 hours or with 10^{-8}, 10^{-7}, or 10^{-6} mol/L of EM-1, EM-2, or OMF for 24 hours in the present study. These results suggest that EM-1 and EM-2 might alter macrophage functions (eg, cytokine production and functions related to natural host defense), as has been reported by other researchers.[7,18–20] EM-1, EM-2, and OMF also inhibited macrophage phagocytic and bactericidal activities, indicating suppressed macrophage cytoimmunity.[7,18–20]

Azuma et al performed a series of trials[7,19,20] to study the immune effect of EM-1 and EM-2. They reported[19,20] that EM-1 and EM-2 inhibited TNF-α, IL-10, and IL-12 production by macrophages stimulated with both lipopolysaccharide (LPS) or phorbol-12-myristate-13-acetate (PMA), but potentiated IL-1β production by macrophages stimulated with PMA. Moreover, phagocytosis of *Escherichia coli* by macrophages was not altered by EM-1,[19] but was suppressed by EM-2.[20] Their findings were not totally

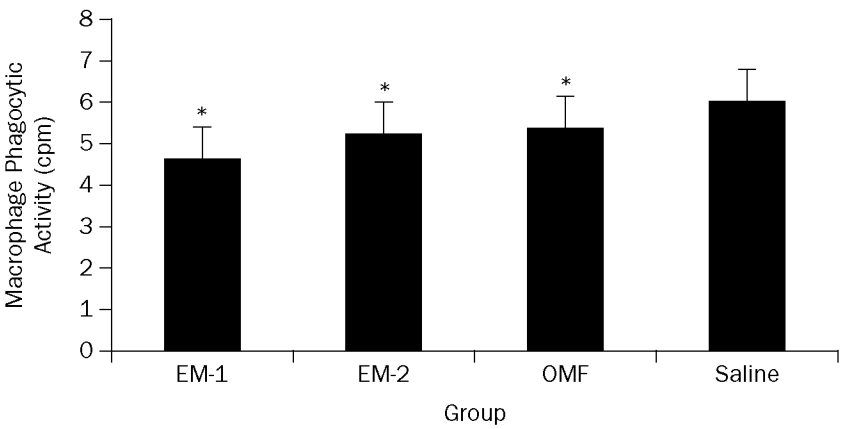

Figure 5. Experimental rat macrophage phagocytic activity measured using isotope marker when the macrophages were cultured with 10^{-6} mol/L of endomorphin (EM)-1, EM-2, or ohmefentanyl (OMF) for 24 hours (n = 10 wells/group). (Liquid counter was used to assay the counts per minute [cpm] value of the macrophages.) *$P < 0.05$ versus saline group.

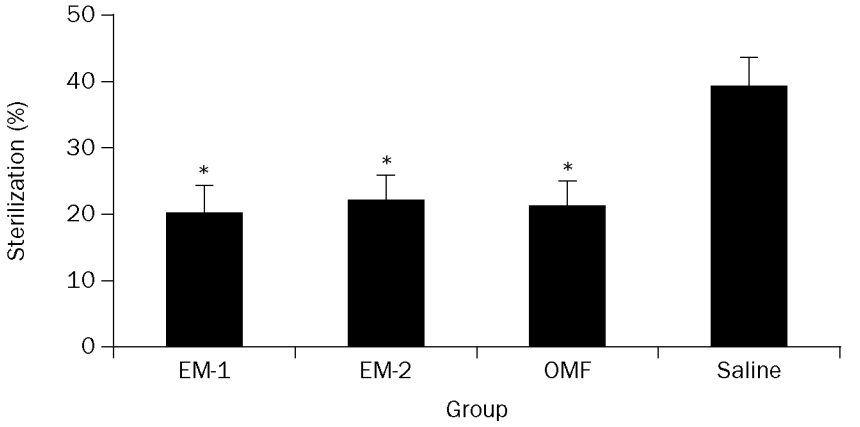

Figure 6. Experimental rat macrophage bactericidal activity measured using isotope marker when the macrophages were cultured with 10^{-6} mol/L of endomorphin (EM)-1, EM-2, or ohmefentanyl (OMF) for 24 hours (n = 10 wells/group). *$P <$ 0.01 versus saline group.

supportive of our findings. Perhaps it was because macrophages were stimulated by LPS or PMA in their studies,[19,20] while no stimulation was used in the present study.

The findings of the present study must be interpreted with regard to the study's limitations. Although all procedures were performed by the same researcher under the same conditions for each group, we should have evaluated the viability of the peritoneal macrophages cultured at different times. Furthermore, we did not explore whether the EMs or OMF blocked cytokine production from the activated macrophage-stimulated cells (eg, by LPS). In addition, the sample size in the study was small and the researchers were not blinded to each study group, which might have increased study bias.

CONCLUSIONS

In this in vitro experiment, EM-1, EM-2, and OMF inhibited immune functions of cultured rat peritoneal macrophages, including decreasing TNF-α and IL-1β concentrations and phagocytic and bactericidal activities.

REFERENCES

1. Wollemann M, Benyhe S. Non-opioid actions of opioid peptides. *Life Sci.* 2004;75:257–270.
2. Brogden KA, Guthmiller JM, Salzet M, Zasloff M. The nervous system and innate immunity: The neuropeptide connection. *Nat Immunol.* 2005;6:558–564.
3. Gilmore W, Moloney M, Weiner LP. The role of opioid peptides in immunomodulation. *Ann N Y Acad Sci.* 1990;597:252–263.
4. Peterson PK, Molitor TW, Chao CC. The opioid-cytokine connection. *J Neuroimmunol.* 1998; 83:63–69.
5. Zadina JE, Hackler L, Ge LJ, Kastin AJ. A potent and selective endogenous agonist for the mu-opiate receptor. *Nature.* 1997;386:499–502.
6. Jessop DS, Major GN, Coventry TL, et al. Novel opioid peptides endomorphin-1 and endomorphin-2 are present in mammalian immune tissues. *J Neuroimmunol.* 2000;106:53–59.

7. Azuma Y, Ohura K, Wang PL, Shinohara M. Endomorphins 1 and 2 modulate chemotaxis, phagocytosis and superoxide anion production by microglia. *J Neuroimmunol.* 2001;119:51–56.
8. Gawley RE, Dukh M, Cardona CM, et al. Heptapeptide mimic of ohmefentanyl binding in the discontinuous mu-opioid receptor. *Org Lett.* 2005;7:2953–2956.
9. Wang ZY, Wang CQ, Yang JJ, et al. Which has the least immunity depression during post-operative analgesia—morphine, tramadol, or tramadol with lornoxicam? *Clin Chim Acta.* 2006;369:40–45.
10. Wang J, Barke RA, Charboneau R, Roy S. Morphine impairs host innate immune response and increases susceptibility to *Streptococcus pneumoniae* lung infection. *J Immunol.* 2005;174:426–434.
11. Sacerdote P, Bianchi M, Gaspani L, et al. The effects of tramadol and morphine on immune responses and pain after surgery in cancer patients. *Anesth Analg.* 2000;90:1411–1414.
12. Jessop DS. Endomorphins as agents for the treatment of chronic inflammatory disease. *BioDrugs.* 2006;20:161–166.
13. Molina PE. Opioids and opiates: Analgesia with cardiovascular, haemodynamic and immune implications in critical illness. *J Intern Med.* 2006;259:138–154.
14. Carrigan KA, Nelson CJ, Lysle DT. Endomorphin-1 *induces* antinociception without immunomodulatory effects in the rat. *Psychopharmacology (Berl).* 2000;151:299–305.
15. Horvath G. Endomorphin-1 and endomorphin-2: Pharmacology of the selective endogenous mu-opioid receptor agonists. *Pharmacol Ther.* 2000;88:437–463.
16. Sedqi M, Roy S, Ramakrishnan S, et al. Complementary DNA cloning of a mu-opioid receptor from rat peritoneal macrophages. *Biochem Biophys Res Commun.* 1995;209:563–574.
17. Schroit AJ, Gallily R. Quantitative in vitro phagocytic rate measurements. *J Immunol Methods.* 1977;17:123–130.
18. Jessop DS, Richards LJ, Harbuz MS. Opioid peptides endomorphin-1 and endomorphin-2 in the immune system in humans and in a rodent model of inflammation. *Ann N Y Acad Sci.* 2002;966:456–463.
19. Inui Y, Azuma Y, Ohura K. Differential alteration of functions of rat peritoneal macrophages responsive to endogenous opioid peptide endomorphin-1. *Int Immunopharmacol.* 2002;2:1133–1142.
20. Azuma Y, Ohura K. Endomorphin-2 modulates productions of TNF-alpha, IL-1beta, IL-10, and IL-12, and alters functions related to innate immune of macrophages. *Inflammation.* 2002;26:223–232.

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