

Chronic Morphine Consumption Increase Allograft Rejection Rate in Rat through Inflammatory Reactions

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ABSTRACT

Background: Although opioids suppressive effects on immune system function have been reported, this study demonstrates inflammatory reactions, such as production of pro-inflammatory cytokines and suppression of anti-inflammatory cytokines, are the main causes at organ's allotransplantation rejection in chronic morphine-treated recipients. **Methods:** 28 rats were categorized in 4 groups through intra-peritoneal administrations: control, sham, morphine treated animals (20 mg/kg injected of morphine daily until biopsy day), morphine and naloxane treated animals (20 mg/kg morphine and 2 mg/kg naloxane daily injected until biopsy day), which their donors were normal rats. The grafts were done at the 14th day of the experiment. Plasma interleukins levels (IL-6 and IL-10) in three sampling times were measured by ELISA. With almost 80% of macroscopic rejection signs in rats of one group, full thickness skin biopsy has been taken and histological parameters like perivascular infiltrates, epidermal changes, and stromal changes were detected. The statistical significance differences between the control and experimental groups were analyzed using the Kruskal-Wallis, followed by ANOVA post hoc test. **Results:** Accelerated skin allograft rejection by chronic morphine consumption can be resulted of increased IL-6 concentration and decreased IL-10. The enhancing effects of morphine on the graft inflammation were partially antagonized by Naloxane. It can illustrate the complexity of opiates and immune system connections and should be considered during organ transplantation of opiate addicts. **Conclusion:** Expansion of skin cells in recipient with chronic morphine administration history may be resulted in failure. *Iran. Biomed. J. 15 (3): 85-91, 2011*

Keywords: Morphine, Skin allograft, Inflammation, IL-6, IL-10

INTRODUCTION

For long years, both beneficial and detrimental effects of opioid compounds have been discovered. The immune-modulatory activities of morphine and heroin (diacetylmorphine) are evidently due to intricate interplay of their direct and indirect influences on immune system [1]. Opioids directly mediate immune response through μ , δ and κ opioid receptors, expressed by immune cells, including macrophages, lymphocytes, endothelial cells and others. Also, their indirect influences are resulted through participation of central nervous system and hypothalamic-pituitary-adrenal (HPA) axis, which induce the production of pro-inflammatory inter-

leukins, such as IL-2, IL-6 and IL-1 β [1-3]. In addition, the presence of μ 3 receptors on human immunocytes, which are morphine selective [4], and the production of morphine by white blood cells [5] are among the other factors that efficiently confirm the immune-modulatory activities of morphine.

Although there are various studies emphasizing immune-suppressive effects of morphine [6], such as dose-dependent suppression of NK-cells cytotoxicity, depression of lymphocyte proliferation to T and B cell mitogens [7, 8], apoptotic effects [1, 9], marked reduction in chemotaxis and phagocytosis [10] and inhibition of cellular velocity through nitric oxide (NO) release or enhanced neutral endopeptidase activity [10, 11], below are some reports supporting the immune-

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stimulatory effects of opioids. It is illustrated the concentration of pro-inflammatory cytokines IL-2, IFN- γ and IL-1 β in spleen cell supernatant are augmented by enhanced NO production in heroin-treated mice, which resulted in inflammatory and delayed hypersensitivity reactions [2, 12, 13].

Zubelewicz *et al.* [14] have shown that morphine administration into the lateral ventricle of normal and arthritis animal model can induce the production of pro-inflammatory cytokine IL-6, which can be the result of increased corticosterone plasma levels. Similarly, Peng *et al.* [8] have illustrated that morphine modulates the mRNA expression of some pro-inflammatory and anti-inflammatory cytokines in peritoneal resident macrophages, including IL-12, TNF- α and IL-10. The same results were also stated by Davorka *et al.* [15], who demonstrated that morphine mutually regulates the production of IL-10 and IL-12 by monocyte-derived human dendritic cells and suggested morphine mediates this stimulatory effect (decrease and increase in IL-10 and IL-12 production, respectively) via a P38 mitogen-activated protein kinase-dependant pathways.

Recent researches on transplantation immunity suggest that macrophages and inflammatory reactions, rather than cytotoxic T cells, are the essential parameters at allograft rejection [16]. Due to more infection rates at substance abusers the lower allograft rejection rate should be possible. Therefore, considering the morphine effects on immunocytes at pro-inflammatory and anti-inflammatory cytokines levels, we surveyed the correlation between chronic morphine administration and skin allotransplantation reactions in animal models.

MATERIAL AND METHODS

Animals and ethics. Male Sprague-Dawley rats (n = 28), weighing 200-240 g, were selected from the Laboratory Animal Center of Shiraz University of Medical Sciences (Shiraz, Iran). The animals were kept under controlled humidity and temperature condition in a 12 h light/dark cycle with free access to food and water. All the animal experiments were approved by the Animal Ethics Committee of the Shiraz University of Medical Sciences (Shiraz, Iran). Control rats were untreated and sham group were daily administrated with the same volume of saline.

Drugs and treatment. Morphine sulfate and naloxane were purchased from Daropaksh Co. (Iran). After two weeks of acclimatization, the rats were divided into four groups each including 7 animals: morphine-treated rats (test 1, 20 mg/kg/day daily),

morphine and naloxane-treated rats (test 2, 2 mg/kg naloxane and after 30 minutes 20 mg/kg morphine daily), control rats (untreated with any drug) and sham group (administrated daily with the same volume of saline). The drugs were injected i.p. for 14 days.

Skin graft. Recipients (categorized in 4 groups as mentioned above) were grafted from normal rats according to the technique of Billingham *et al.* [17], which under the sterile condition the full thickness transplantation on behind of the lumbar region of rats was done after anesthesia induction with 100 mg/kg ketamine. After skin graft operation, percentage of graft survival was measured based on daily observation of macroscopic rejection signs, such as necrosis, edema and scaling off. Daily injections of recipients were continued until the graft areas in most rats of one group were rejected.

Pathological assessment. With daily observation of graft area and assessing the macroscopic rejection signs, the percentage of normal area was registered. When almost 80% of rejection signs in most rats of one group were seen, full thickness skin biopsy from rats in each group was taken. The samples were fixed in formaldehyde and after tissue processing, 5 micrometer sections were prepared. The histological features were categorized as dyskeratotic cells (a), endothelial enlargement (b), perivascular infiltration (c), spongiosis (d) and epidermal infiltration (e). To illustrate the absence or presence of these histological parameters, they were estimated in a 0 to 3 score, respectively. A grading system based on the number of cases encountered was used: degree 0 (no rejection), no perivascular infiltration was assessed; degree 1 (intermediate rejection), up to 10% of vessels showed perivascular infiltration and no spongiosis, eosinophil, large lymphocytes and epidermal or stromal infiltration were apparent; degree 2 (mild rejection), 11% to 50% of the vessels were inflamed with small lymphocyte, mild spongiosis and epidermal infiltrate; degree 3 (moderate rejection), more than 50% of perivascular and epidermal infiltrate, spongiosis and plumping were seen and degree 4 (severe), more than 50% perivascular and epidermal infiltrate, severe spongiosis and plumping were assessed [18].

Detection of cytokine production. To detect the serum levels of IL-6 and IL-10, blood samples from each group were taken in three specified time, including 1st day of treatment, 14th day of experiment, and the day of biopsy. These time intervals were selected for comparing time-dependant morphine effects in continued morphine administration. The concentration of IL-6 (R & D system, Minneapolis, MN, USA) and

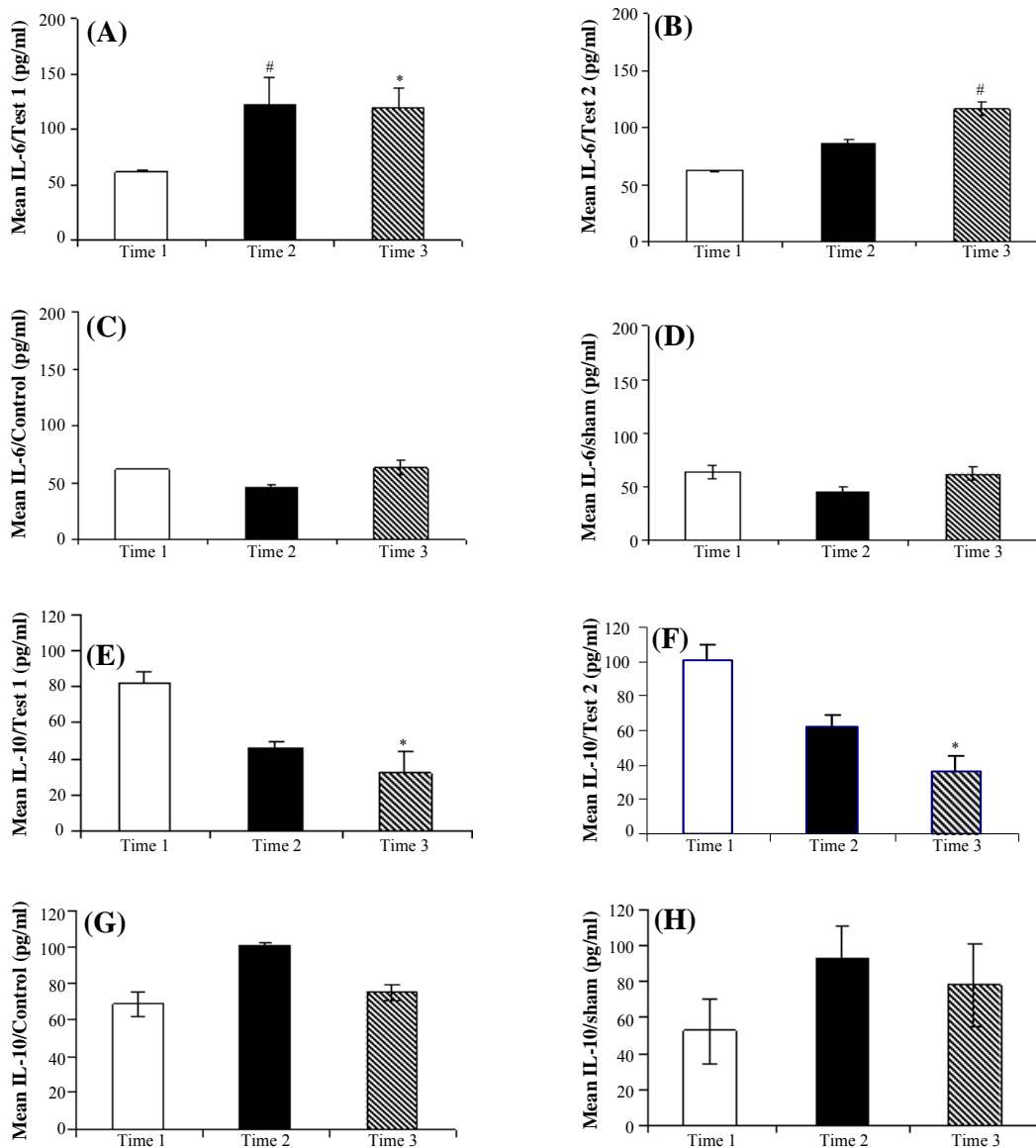


Fig. 1. Production of IL-6 (pg/ml, **A-D**) and IL-10 (pg/ml, **E-H**) in 3 different sampling times. 1st, first day of experiment; 2nd, 14th day of experiment (skin grafting); 3rd, day of taking biopsy. Each bar represents the mean \pm SD at three independent time of sampling (* $P < 0.05$ and $^{\#}P < 0.001$).

IL-10 (DRG system, GMBH, Deutschland, Germany) were determined by ELISA using sets of cytokine-specific capture and detection monoclonal antibody. Standards for IL-6 and IL-10 (both purchased from R & D and DRG, respectively) were included in all ELISA determinations.

Statistics. The statistical significance differences between the control and experimental groups were analyzed using Kruskal-Wallis, followed by ANOVA post hoc test. $P < 0.05$ was considered significant.

RESULTS

Interleukin concentration in each group. Difference among plasma cytokines of rats in 3 above-mentioned sampling times were analyzed for cytokine production. The production of IL-6 in the morphine-treated rats was significantly increased in 3th and 2nd time compared to 1st sampling time (Fig. 1C) and in morphine and naloxane-treated group; it was significantly increased in 3th time compared to the first sampling time (Fig. 1D). IL-10 was significantly

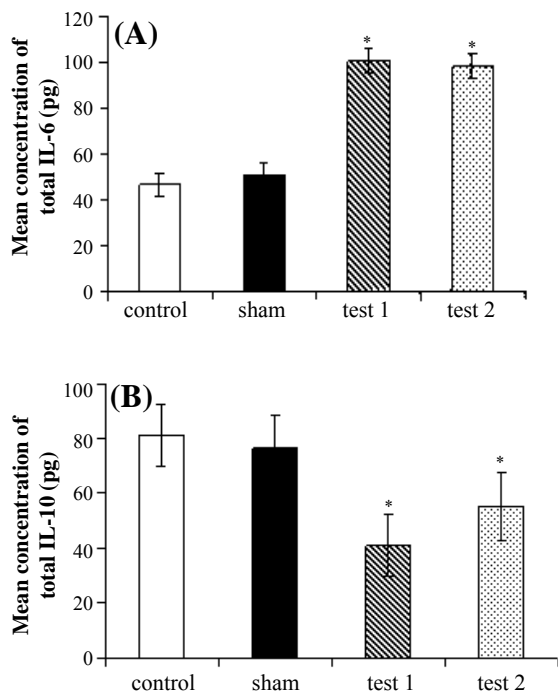


Fig 2. Mean concentration of total (A) IL-6 (pg/ml) and (B) IL-10 resulted of 3 sampling times (the first day of experiment, 14th day (when the graft was done) and the day of biopsy) in 4 groups. Each bar represents the mean \pm SD from three independent times of sampling (* $P < 0.05$).

decreased in 3th time compared to first and second time both in morphine-treated and morphine and naloxane-treated groups (Fig. 1E-1H).

Total interleukin concentration among groups. The total production of IL-6 was significantly increased in morphine-treated group in comparison with control and sham groups (Fig. 2A). The total production of IL-10 was significantly decreased in morphine-treated group comparing with control and sham groups and between test 2 and control groups (Fig. 2B).

Relation between IL-6 and IL-10 with graft survival time. Scatter plot (Fig. 3) shows a reverse relation between the increase of 3th time IL-6 and the time of 80% graft rejection in morphine-treated group at the level of $P < 0.05$ with a linear equation ($y = -0.018 \text{ IL-6} + 8.22$), but not in other groups. A direct relation is shown in Figure 4 between the decrease of third time IL-10 and the time of 80% graft rejection in morphine-treated group at the level of $P < 0.05$ with a linear equation ($y = +0.012 \text{ IL-10} + 5.9$), which was not seen in other groups.

Morphine administration and skin graft survival period. Differences between percentage of allograft

survival in the 3th, 5th and 8th days (the day when 80% of grafts was rejected in the first group) were randomly analyzed (Fig. 5). From 3th day, the rejection signs were started to promote in morphine-treated group and it became more faster in the 5th day. Approximately 80% of graft areas in most of the morphine-treated rats were rejected in the 8th day. Allograft survival percentage in morphine-treated group was significantly decreased in 5th h and 8th days compared to other three groups ($P < 0.05$). Also, the difference between naloxane-treated group and control group was significantly different in 8th day ($P < 0.05$).

Pathological parameters. One pathologist, who has expertise in transplantation pathology, examined the slides blindly. Significantly rejection degree increased with chronic morphine consumption in Test 1 group in comparison with other groups ($P < 0.05$, Fig. 6).

DISCUSSION

There are some researches emphasizing morphine induces dose-dependent suppression of NK cell cytotoxicity [7, 8, 19] and depression of lymphocyte proliferation to T and B cell mitogens [7, 8] and also has apoptotic effects [9]. It has been shown that *in vivo* treatment with morphine is resulted in a reduction at thymus and spleen weight [20, 21] and reduces resistance to oral *Salmonella typhimurium* infection [22]. In addition, opioids cause viral infections through the decrease in chemokine α and β , CCR5 and CCR3 chemoreceptor expression, INF- γ production and increase in HIV gene expression [23].

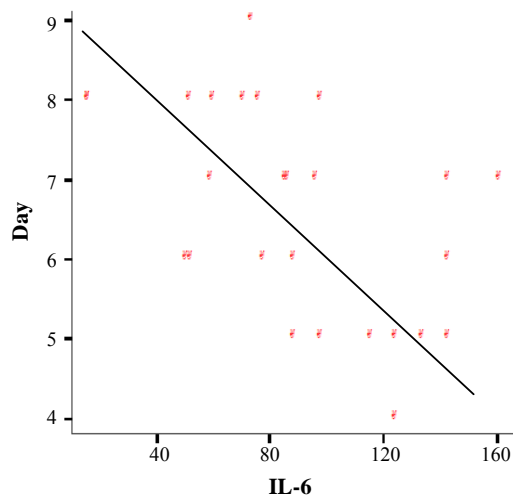


Fig. 3. Relation between increased IL-6 (pg/ml) and the day of rejection in morphine-treated group. Reverse relation between 3th time IL-6 changes and the time of 80% graft rejection at the level of significant difference ($P < 0.05$) with a linear equation $y = -0.018 \text{ IL-6} + 8.2$ is seen.

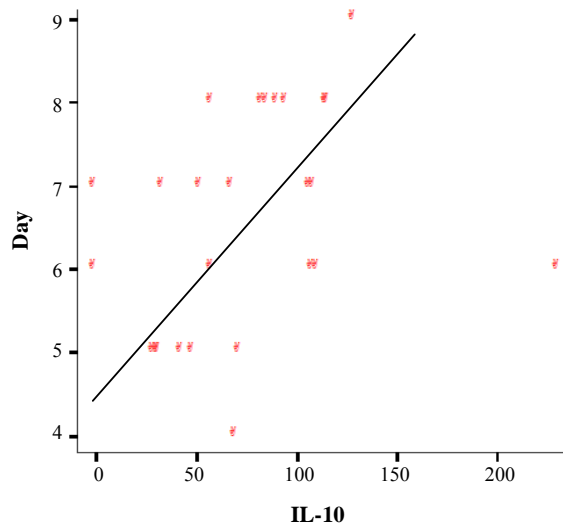


Fig. 4. Relation between decreased IL-10 and the day of rejection in morphine-treated group. Direct relation between 2nd time IL-10 (pg/ml) changes and the time of 80% graft rejection at the level of significance ($P < 0.05$) with a linear equation $y = +0.012 \text{ IL-10} + 5.9$ is seen.

Morphine can thus affect on the function of immune system either directly through opioid receptors on immunocytes or indirectly through nervous system. In this respect, it has been shown that suppression of lymphocyte proliferation by morphine is independent of the HPA axis and is resistant to adrenalectomy [24]. Moreover, production of IL-6 depends on the activity of the HPA axis and can be blocked by adrenalectomy [7, 25].

Different cytokine production mechanisms in acute versus sub-acute and chronic morphine administration have been reported [26]. At acute administration and on exciting the secretion of endogenous morphine, Ca^{+2} releases through influx and intracellular pools via the μ_3 receptors found on human immunocytes. Released NO stabilizes $\text{I}\kappa\text{-B}\alpha$, a NF- κB inhibitor, which inhibits NF- κB transcriptional activation and iNOS release [15, 27]. Therefore, the production of major mediators of inflammatory responses by macrophages, including IL-2, IL-6 and TNF- α , are suppressed [28, 29]. On the other hand, high morphine concentration and continued exposure to same doses cause lymphocyte conformational changes, which force the reestablish of its excitability threshold, owing to the emergence of tolerance [15].

Recently, some researches on organ transplantation have suggested that macrophages, delayed hypersensitivity and inflammatory reactions rather than cytotoxic T cells are the main effectors of allograft and xenograft rejection [16, 18]. The present study

confirms the results of Pacific *et al.* [30] and Holan *et al.* [2], although other cytokines in chronic morphine administration applying histological assays have been surveyed at our study. In addition, we concluded that IL-6 concentration as a cytokine of Th₁ type at 14th day of administration and at the allograft rejection was increased that could be responsible for the acceleration of allograft rejection. On the other hand, IL-10 concentration, which is an endogenous suppressor of Th1 type mediators such as IL-12, TNF- α and IFN- γ [32, 33], decreased simultaneously through mitogen-activated protein kinase-kinase signaling [15, 31].

In addition, some apoptotic features, including dyskeratotic cells, endothelium enlargement, perivascular infiltration, spongiosis and epidermal infiltration were noticeably observed at histological slides of morphine-treated group in comparison with other groups. Regarding these findings, it is illustrated that chronic morphine administration changed the allograft rejection severity (rejection type) among the four mentioned groups.

Considering the effects of morphine on immunocytes that increases pro-inflammatory cytokine levels, suppresses anti-inflammatory cytokine secretion and alters the histological parameters, the present study shows the expansion of skin cells in recipients with the history of chronic morphine administration may be resulted in failure. It can also be concluded that immune-stimulatory effects of chronic morphine consumption were antagonized by naloxane, a specific inhibitor of classic opioid receptors, and it could accelerate allograft reaction in rats.

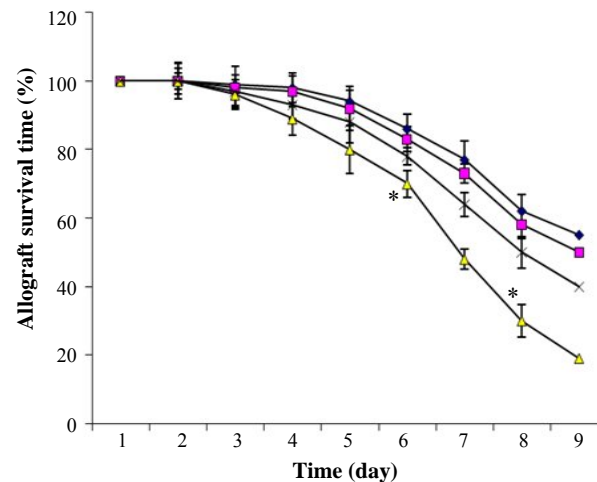


Fig. 5. Effect of chronic morphine administration on skin allograft survival. The percentage of allograft survival in morphine-treated group was significantly decreased in 5th and 8th days compared to other three groups ($*P < 0.05$). The difference between morphine and naloxane-treated and control groups were significant in the 8th day ($*P < 0.05$).

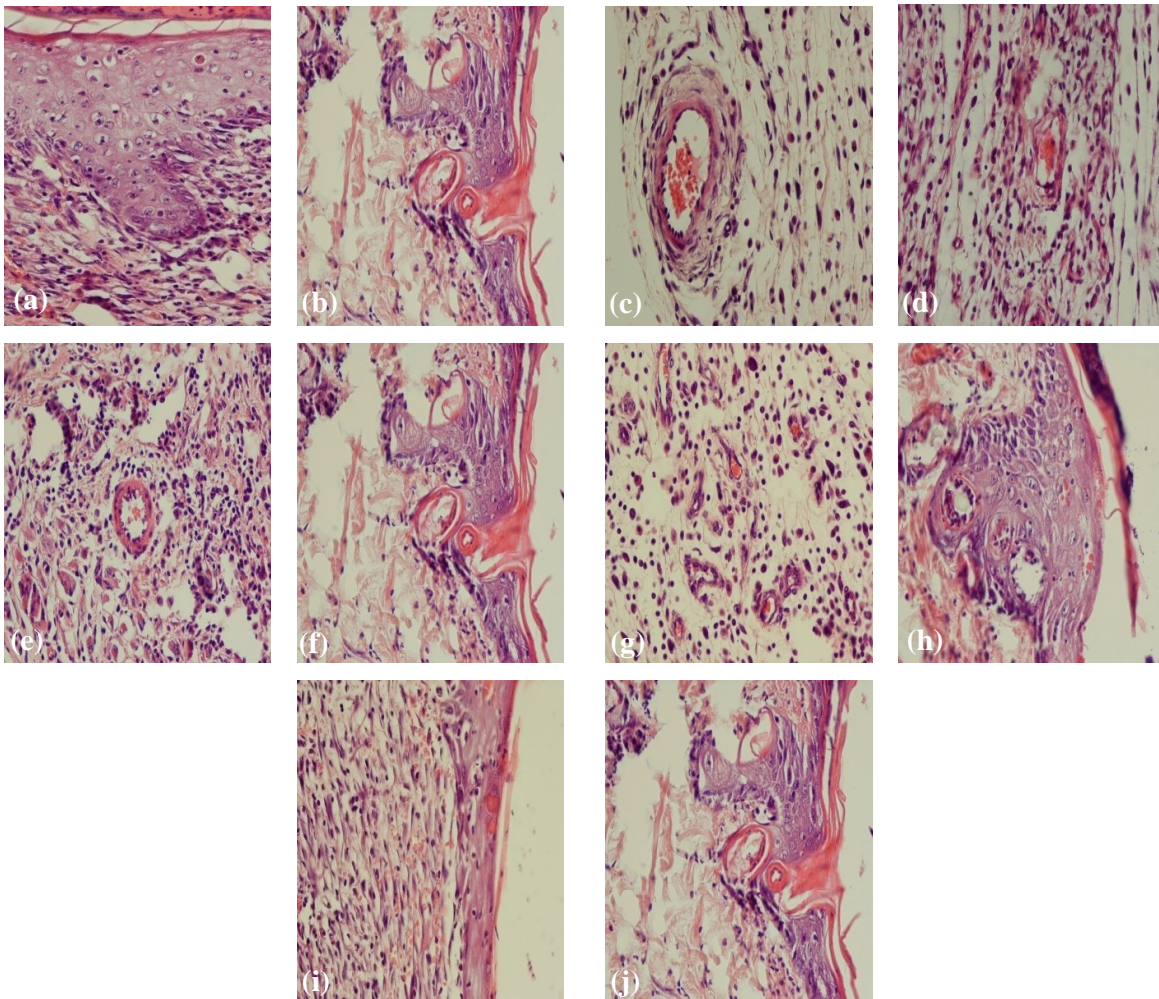


Fig. 6. Influence of chronic morphine administration on pathological parameters: dyskeratotic cells (**a** and **b**); endothelium enlargement (**c** and **d**); perivascular infiltration (**e** and **f**); spongiosis (**g** and **h**) and epidermal infiltration (**i** and **j**) in test 1 group in comparison with test 2 group ($P < 0.05$).

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REFERENCES

- Shen, C.H., Tsai, R.Y., Shih, M.S., Lin, S.L., Lo, Tai, Y.H., Chien, C.C and Wong, C.S. (2011) Etanercept restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in morphine-tolerant rats. *Anesth. Analg.* 112: 454-459.
- Holan, V., Zajicova, A., Krulova, M., Blahoutova, V. and Wilczek, H. (2003) Augmented production of proinflammatory cytokines and accelerated allotransplantation reactions in heroin-treated mice. *Clin. Exp. Immunol.* 132: 40-45.
- Kraus, J. (2009) Regulation of mu-opioid receptors by cytokines. *Front Biosci (Schol Ed).* 1: 164-170.
- Cadet, C., Mantione, K.J. and Stefano, G.B. (2003) Molecular identification and functional expression of mu3, a Novel alternatively spliced variant of the human mu opiate receptor gene. *J. Immunol.* 170: 5118-5123.
- Wei, Z., Cadet, P., Baggerman, G., Mantione, K.J. and Stefano, G.B. (2005) Human white blood cells synthesize morphine: CYP2D6 modulation. *J. Immunol.* 175: 7357-7362.
- Lin, J.A., Chen, J.H., Lee, Y.W., Lin, C.S., Hsieh, M.H., Chang, C.C., Wong, C.S., Chen, J.J., Yeh, G.C., Lin, F.Y. and Chen, T.L. (2011) Biphasic effect of curcumin on morphine tolerance: a preliminary evidence from cytokine/chemokine protein array analysis. *Evid. Based Complement Alternat. Med.* 452153:1-11.
- Odunayo, A., Dodam, J.R., Kerl, M.E. and DeClue, A.E. (2010) Immunomodulatory effects of opioids. *J.*

- Vet. Emerg. Crit. Care (San Antonio)* 20: 376-385.
8. Peng X., Mosser, D.M., Adler, M.W., Rogers, T.J., Meissler, J.J. Jr and Eisenstein, T.K. (2000) Morphine enhances interleukin-12 and the production of other pro-inflammatory cytokines in mouse peritoneal macrophages. *J. Leukoc. Biol.* 68: 723-728.
 9. Cheng, W.F., Chen, L.K., Chen, C.A., Chang, M.C., Hsiao, P.N., Su, Y.N., Lee, C.N., Jeng, H.J., Hsieh, C.Y. and Sun, W.Z. (2006) Chimeric DNA vaccine reverses morphine-induced immunosuppression and tumorigenesis. *Mol. Ther.* 13: 203-210.
 10. Shirzad, H., Shahrani, M. and Rafieian K.M. (2009) Comparison of morphine and tramadol effects on phagocytic activity of mice peritoneal phagocytes *in vivo*. *Int. Immunopharmacol.* 9: 968-970.
 11. George, B., Yannick, G., Federico, C., Patrick, C., Gregory, L.F., Christos, R., Doris, P., Dario, S., Massimo, G., Ingeborg, D.W. and Enrica, B. (2000) Endogenous morphine. *Trends Neurosci.* 23: 436-442.
 12. Muscoli, C., Doyle, T., Dagostino, C., Bryant, L., Chen, Z., Watkins, L.R., Ryerse, J., Bieberich, E., Neumann, W. and Salvemini, D. (2010) Counter-regulation of opioid analgesia by glial-derived bioactive sphingolipids. *J. Neurosci.* 30: 15400-15408.
 13. Wang, Z., Ma, W., Chabot, J.G. and Quirion, R. (2009) Cell-type specific activation of p38 and ERK mediates calcitonin gene-related peptide involvement in tolerance to morphine-induced analgesia. *FASEB J.* 23: 2576-2586.
 14. Zubelewicz, B., Muc-Wierzgon, M., Harbuz, M.S. and Brodziak, A. (2000) Central single and chronic administration of morphine stimulates corticosterone and interleukin (IL)-6 in adjuvant-induced arthritis. *J. Physiol. Pharmacol.* 51: 897-906.
 15. Messmer, D., Hatsukari, I., Hitosugi, N., Schmidt-Wolf, I.G. and Singhal, P.C. (2006) Morphine Reciprocally Regulates IL-10 and IL-12 Production by Monocyte-Derived Human Dendritic Cells and Enhances T Cell Activation. *Mol. Med.* 12: 284-290.
 16. Krulova, M., Zajícova, A., Fric, J. and Holan, V. (2002) Alloantigen-induced, T-cell-dependent production of nitric oxide by macrophages infiltrating skin allografts in mice. *Transpl. Int.* 15: 108-116.
 17. Billingham, R.E., Brent, L., Medawar, P.B. and Sparrow, E.M. (1954) Quantitative studies on tissue transplantation immunity. The survival times of skin homografts exchanged between members of different inbred strains of mice. *Proc. R. Soc. Lond. B. Biol. Sci.* 143: 43-58.
 18. Bejarano, P.A., Levi, D., Nassiri, M., Vincek, V., Garcia, M., Weppler, D., Selvaggi, G., Kato, T. and Tzakis, A. (2004) The pathology of full-thickness cadaver skin transplant for large abdominal defects: a proposed grading system for skin allograft acute rejection. *AM J. Surg. Pathol.* 28: 670-675.
 19. Saurer, T.B., Carrigan, K.A., Ijames, S.G. and Lysle, D.T. (2006) Suppression of natural killer cell activity by morphine is mediated by the nucleus accumbens shell. *J. Neuroimmunol.* 173: 3-11.
 20. Bhargava, H.N., Thomas, P.T., Thorat, S. and House, R.V. (1994) Effect of morphine tolerance and abstinence on cellular immune function. *Brain. Res.* 642: 1-10.
 21. Nguyen, T., Kramer, J., Vallejo, R., Stanton, G., Heidenreich, B.A., Benyamin, R. and Vogel, L.A. (2009) Citalopram Enhances B Cell Numbers in a Murine Model of Morphine-Induced Immunosuppression. *Pain Pract.* 9: 195-205.
 22. Breslow, J.M., Feng, P., Meissler, J.J., Pintar, J.E., Gaughan, J., Adler, M.W. and Eisenstein, T.K. (2010) Potentiating effect of morphine on oral Salmonella enterica serovar Typhimurium infection is μ -opioid receptor-dependent. *Microb Pathog.* 49:330-335.
 23. Vallejo, R., de Leon-Casasola, O. and Benyamin, R. (2004) Opioid therapy and immunosuppression: a review. *Am. J. Ther.* 11: 354-365.
 24. Mojadadi, S., Jamali, A., Khansarinejad, B., Soleimanjahi, H. and Bamdad, T. (2009) Acute morphine administration reduces cell-mediated immunity and induces reactivation of latent herpes saimplex virus type 1 in BALB/c mice. *Cell Mol. Immunol.* 6: 111-116.
 25. Wang, J., Charboneau, R., Balasubramanian, S., Barke, R.A., Loh, H.H. and Roy, S. (2001) Morphine modulates lymph node-derived T lymphocyte function. role of caspase-3, -8, and nitric oxide. *J. Leukoc. Biol.* 70: 527-536.
 26. Martínez, L.M., Cruz, S. and Espinosa, C.G. (2011) Chronic morphine administration induces tolerance to suppressive morphine effects in two canonical LPS-induced innate immunity responses in mice. *J. Immunol.* 186: 162-170.
 27. Toda, N., Kishioka, S., Hatano, Y. and Toda, H. (2009) Modulation of opioid actions by nitric oxide signaling. *Anesthesiology* 110: 166-181.
 28. Tekieh, E., Zaringhalam, J., Manaheji, H., Maghsoudi, N., Alani, B. and Zardooz, H. (2011) Increased serum IL-6 level time-dependently regulates hyperalgesia and spinal mu opioid receptor expression during CFA-induced arthritis. *EXCLI J.* 10: 23-33.
 29. Liou, J.T., Liu, F.C., Mao, C.C., Lai, Y.S. and Day, Y.J. (2011) Inflammation confers dual effects on nociceptive processing in chronic neuropathic pain model. *Anesthesiology* 114: 660-672.
 30. Pacifici, R., Di Carlo, S., Bacosi, A., Pichini, S. and Zuccaro, P. (2000) Pharmacokinetics and cytokine production in heroin and morphine-treated mice. *Int. J. Immunopharmacol.* 22: 603-614.
 31. Zaki, N.G., Osman, A., Moustafa, H. and Saad, A.H. (2006) Alterations of immune functions in heroin addicts. (2006) *Egypt. J. Immunol.* 13: 153-171.
 32. Welters, I.D., Fimiani, C., Bilfinger, T.V. and Stefano, G.B. (2000) NF-kappaB, nitric oxide and opiate signaling. *Med. Hypotheses* 54: 263-268.
 33. Ozdemir E., Bagcivan I., Durmus N., Altun A. and Gursoy S. (2011) The nitric oxide-cGMP signaling pathway plays a significant role in tolerance to the analgesic effect of morphine. *Can. J. Physiol. Pharmacol.* 89: 89-95.