

## The effects of hydromorphone on astrocytic responses in cerebral ischemia

Department of Anesthesiology and Pain Medicine, Korea University Ansan Hospital, Korea University College of Medicine, Ansan, Korea

Chang Hyung Lee, Young Cheol Park, Jae Hwan Kim, Woon Young Kim, Yoon Sook Lee, Yun Hee Kim, and Too Jae Min

**Background:** Ischemic insult during operation could cause ischemic-reperfusion injuries in brain and memory impairments. Total intravenous anesthesia (TIVA) is preferred in brain surgery to promote the use of motor evoked potential monitoring and the use of opioids is common in TIVA. However there were few studies about ischemic protective effect of opioids to astrocytes.

**Methods:** We used astrocytes, which were derived from human brain. We divided groups by conditioning period; i) pre-culture, ii) post-culture, or iii) pre + post-culture. All groups were treated 100 nM hydromorphone. We measured reactive oxygen species (ROS) by flow cytometry with 2',7'-dichlorofluorescein diacetate. Then ROS in astrocytes which treated by opioid receptor antagonist were measured after treating 100 nM hydromorphone.

**Results:** ROS was reduced in hydromorphone treated group, as compared to the control group (only tert-butyl hydroperoxide [TBH] treated). There was no difference in pre-conditioned group and post-conditioned group. However, ROS was much more reduced in pre + post-conditioned group compared to pre-conditioned only or post-conditioned only group. Furthermore each selective  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptor antagonists partially negated the effect of hydromorphone.

**Conclusions:** This study provides evidence that hydromorphone has both preconditioning and postconditioning effects on TBH-induced oxidative stress. Furthermore we proved each  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptor relates to protective mechanism of hydromorphone to astrocytes. (*Anesth Pain Med* 2016; 11: 23-27)

**Key Words:** Astrocytes, Cerebral ischemia, Hydromorphone.

Received: September 25, 2015.

Revised: October 22, 2015

Accepted: October 23, 2015.

Corresponding author: Too Jae Min, M.D., Ph.D., Department of Anesthesiology and Pain Medicine, Korea University Ansan Hospital, Korea University College of Medicine, 123, Jeokgeum-ro, Danwon-gu, Ansan 15355, Korea. Tel: 82-31-412-5297, Fax: 82-31-412-5294, E-mail: minware2@lycos.co.kr

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

### INTRODUCTION

In recent times, astrocytes are considered to represent a specialized functional unit with neurons, in addition to providing neuronal support. It is known that astrocyte dysfunction is a cause of cognitive disorder or short-term memory loss [1]. Ischemic shock during surgery could cause ischemic-reperfusion injuries in the brain and memory impairments [2]. Many studies have established that opioids demonstrate protective effects against ischemia/reperfusion in different types of cells [3,4]. However, there are few studies that discuss the protective effects of opioids on astrocytes in ischemic conditions. Although the opioids have demonstrated preconditioning effects on the astrocytes in the brain [5], it remains to be proved that they possess post-conditioning effects as well. On the other hand, recent studies showed the hypoxic post-conditioning effects on the astrocytes in cerebral ischemia [6].

At present, total intravenous anesthesia (TIVA) is the preferred technique of anesthesia in neurosurgery for motor evoked potential (MEP) monitoring [7,8] and the perioperative use of opioids is common in such settings. Therefore, it is meaningful to investigate whether opioids possess pre and post-conditioning effects on astrocytes. In this study, we tried to investigate whether hydromorphone, one of the opioids that is widely used during the perioperative period, has pre and post-conditioning effects on astrocytes. Furthermore we tried to figure out whether pre and postconditioning of hydromorphone has synergic effects or not.

### MATERIALS AND METHODS

**Chemicals:** Hydromorphone was purchased from Hanapharm Co. (Seoul, Korea), tert-butyl hydroperoxide (TBH) and

2',7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma Chemical Co. (St Louis, MO, USA), the anti-glial fibrillary acidic protein primary antibody and Alexa Fluor 488 goat anti-mouse antibody were purchased from Millipore Co. (Billerica, MA, USA) and Invitrogen Co. (Carlsbad, CA, USA) respectively, XTT (2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt, WelCount Cell Viability Assay kit) was purchased from WELLGNE Inco. (Dalseo-gu, Daegu, Republic of Korea) and the mountain solution was purchased from Vector Laboratories (Burlingame, CA, USA).

**Astrocytes:** The cells used in this study were human brain-derived progenitor astrocytes (Gibco, St. Louis, MO, USA).

**Cell viability**

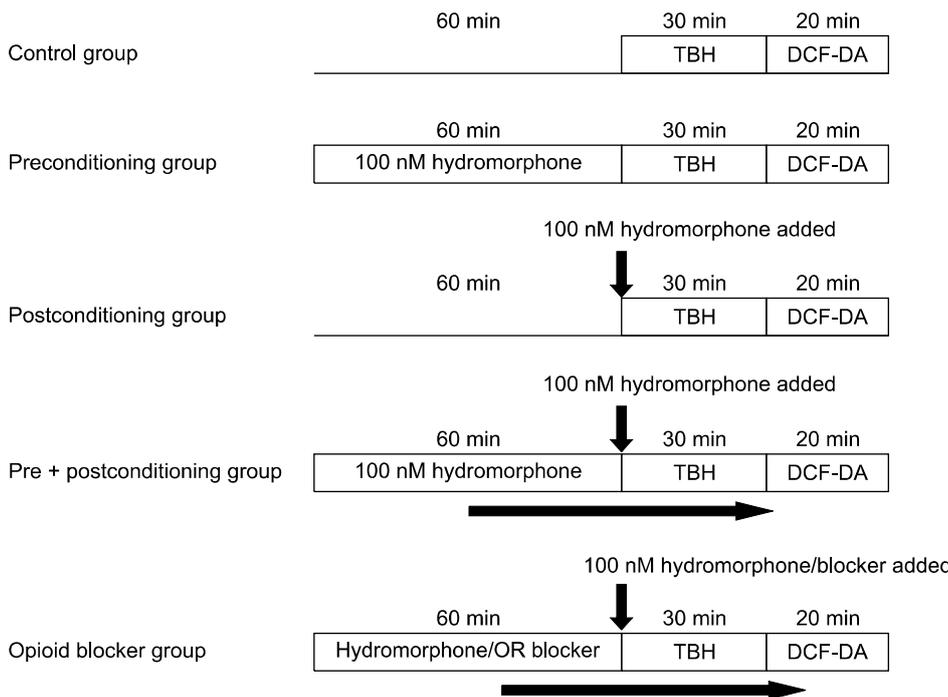
Cell viability was measured by XTT. The astrocytes were cultured in a 96-well plate. The control and TBH (100 uM) and/or hydromorphone (100 nM) treated astrocytes were placed in a new DMEM (Dulbecco's Modified Eagle Medium), containing a 5% XTT reaction mixture (50 : 1 = XTT solution: PMS [N-methyl dibenzopyrazine methyl sulfate] solution) and incubated in a CO<sub>2</sub> incubator, at a temperature of 37°C for a period of 2 hours. The XTT assay generated formazan crystals, which were measured spectro-photometrically with an ELISA reader (690 nm).

**Experimental protocol**

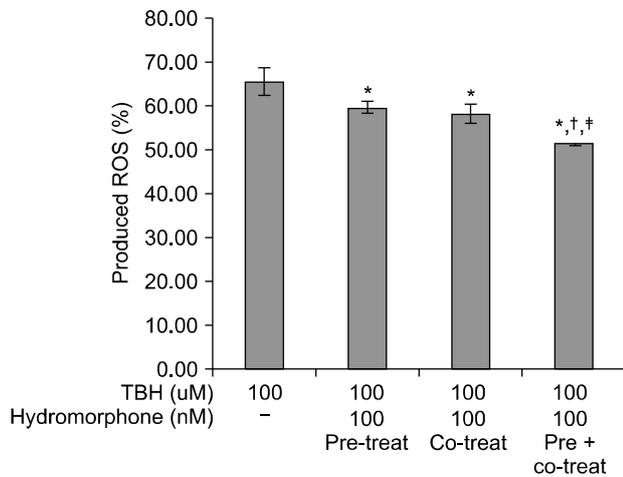
ROS-production induced by TBH was measured by flow cytometry using DCF-DA. We used the astrocytes for cell proliferation to 5 × 10<sup>4</sup> cells in each of the 6-well plates, and then transferred them to a serum-free medium, by performing overnight serum starvation. Thereafter, some of the cells were treated with hydromorphone during 3 different periods: i) pre-culture period (treating the cells with 100 nM hydromorphone for 1 hour and removing before treating with TBH), ii) post-culture period (co-treating the cells with 100 nM hydromorphone and TBH), and iii) pre + post-culture (treating the cells with 100 nM hydromorphone for 1 hour followed by co-treatment with 100 nM hydromorphone and TBH). The remaining cells were pre-treated for 1 hour with 100 nM hydromorphone and each of the selective μ-, δ- and κ-opioid receptor antagonists (naltrindole, nor-binaltorphimine, or D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub> [CTOP]). The cells were treated with an opioid receptor antagonist followed by 100 nM hydromorphone and TBH and the intracellular ROS levels were measured by flow cytometry (Fig. 1).

**The measurement of the intracellular ROS production**

**Flow cytometry:** The control and TBH (50 μM) and/or hydromorphone (100 nM) treated astrocytes were incubated in a CO<sub>2</sub> incubator with DCF-DA (10 ug/ml), at a temperature of



**Fig. 1.** Experimental protocol. Experimental design. TBH: tert-butyl hydroperoxide, DCF-DA: 2',7'-dichlorofluorescein diacetate, OR antagonist: one of selective μ-, δ- and κ-opioid receptor antagonists (naltrindole, nor-binaltorphimine, and CTOP), respectively.



**Fig. 2.** Detection of intracellular ROS. Detection of intracellular ROS products. This graph shows the protective effect of hydromorphone on TBH induced toxicity in primary human glial cell cultures. The ROS level was measured with using FACS (DCF-DA) in the 100 nM hydromorphone i) pre-conditioned ii) post-conditioned iii) pre+post-conditioned primary human glial cells. \* < 0.05 versus control group. † < 0.05 versus pre-conditioned group. ‡ < 0.05 versus post-conditioned group.

37°C for a period of 20 minutes. The DCF-DA-treated astrocytes were washed with phosphate-buffered saline (PBS) and detached from the plate by using trypsin-EDTA (0.25%). The detached cells were diluted with PBS (300 ul) for flow cytometric analysis. At least 3 different sets of experiments, with the cells from different isolations, were performed.

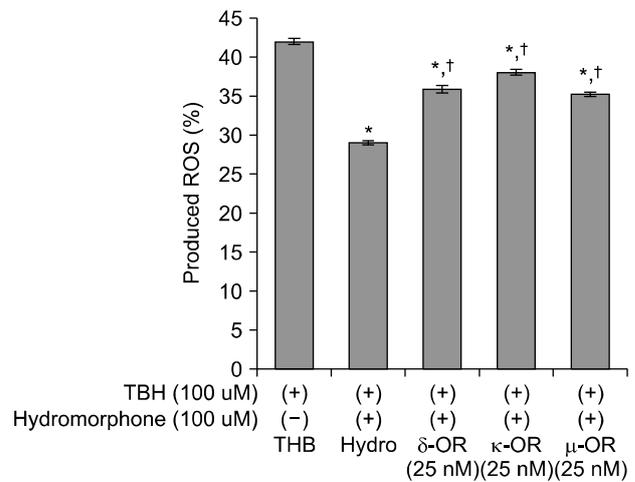
## RESULTS

### The effects of TBH and/or hydromorphone on ROS production

Production of ROS was lower in the hydromorphone-treated group as compared to the control, which was only TBH-treated, though the differences were not statistically significant. However, the ROS production was significantly lower in the pre + post-conditioned group compared to the preconditioned or the post-conditioned group (Fig. 2).

### The effects of opioid receptor antagonists on ROS production

Production of ROS was reduced in the hydromorphone-treated group. However, each of the selective  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptor antagonists partially negated the effects of hy-



**Fig. 3.** Detection of intracellular ROS. Effects of opioid receptor antagonists on ROS products. This graph shows that selective opioid receptor antagonists reverse the attenuation of the ROS induced by hydromorphone (pre + post-conditioned). The ROS level was measured with using FACS (DCF-DA) in the cells treated only TBH (control group), and the cells treated with TBH, hydromorphone and/or one of selective  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptor antagonists (naltrindole, nor-binaltorphimine, and CTOP), respectively. \* < 0.05 versus control group. † < 0.05 versus hydromorphone group.

dromorphone on the ROS production (Fig. 3).

## DISCUSSION

At present, TIVA is the preferred technique of general anesthesia in cerebral surgery for MEP monitoring [7]. In addition, the use of opioids is common in TIVA for intraoperative analgesia. In this study, we demonstrated the preconditioning- and post-conditioning effects of hydromorphone. Therefore, our findings provide theoretical evidence of the perioperative effectiveness of hydromorphone.

Recent studies show that the opioids, including morphine, have a protective preconditioning effect in several ischemia/ reperfusion models [9]. The anti-neuroinflammatory role and the prevention of memory deficit attributed to morphine, is due to its preconditioning effect, which is dependent on the opioid receptors [10]. However, the neuroprotective role of opioids in the case of astrocytes is unclear. Although, the glial cells play an important role in the antioxidant defense system in the brain [11], they are considered to be specialized functional units, in addition to providing physical support to the neurons. Astrocyte dysfunction is one of the causes of cognitive disorder or short-term memory loss [1]. In addition, neuroinflammation is highly related to ischemic stroke-induced cerebral dama-

ge and the progression of neurodegenerative diseases [12,13]. Astrocytes are important cells that are responsible for the neurophysiology of the brain and hence, represent potential therapeutic targets, as seen in this study.

Excessive oxidative stress is reported to be associated with a variety of diseases [14]. Although a certain ROS level is essential for cellular survival, cellular death may occur when oxidative stress caused by ROS production, exceeds the cellular antioxidant abilities [14,15]. An excessive ROS production has been reported to cause DNA damage, modify proteins and lipid functions, and activate the related signal pathways [15]. However, a moderate level of oxidative stress, referred to as positive oxidative stress, could be induced and modulated to produce an adaptive cellular response that is beneficial for cellular survival. Both, preconditioning and post-conditioning effects could be the result of positive oxidative stress [15,16]. Not only brief episodes of ischemia and reperfusion, but also a number of pharmacological agents can induce these phenomena [17]. TBH is commonly used to induce oxidative stress *in-vitro* and *in-vivo* [18]. In this study, we used TBH to induce oxidative stress-related injury to the murine-derived glial cells [19].

Certain studies demonstrated that morphine is involved in the functions of the immune system [20,21]. Morphine showed both, peripheral inflammatory and anti-inflammatory effects [22]. Although opioids induce glial activation and neuroinflammatory reactions [23], morphine-induced preconditioning showed an anti-neuroinflammatory effect [10].

Instead of morphine, we used hydromorphone, an opioid receptor agonist and a derivative of morphine [24], to prevent the TBH-induced ROS production. Hydromorphone, one of the first choice opioids as per the European guideline [25], is widely used in the management of acute or chronic cancer pain, in both, children and adults [25]. As compared to morphine, hydromorphone has certain advantages in clinical settings, such as lesser nausea and vomiting [26], and lower histamine release [27]. To demonstrate its protective effect, we used hydromorphone to reduce oxidative stress-related injury [28].

In this study, hydromorphone showed a protective effect towards TBH-induced oxidative stress-related injury, as assumed. Interestingly, the combined use of hydromorphone (preconditioning followed by co-administration) showed a superior effect when compared individually to preconditioning and co-administration treatments. It is known that the opioids not only suppress responses to various noxious stimuli (for example, short cyclic episodes of ischemia or ROS) but also show similar

preconditioning effects [10,17]. This cumulative effect of hydromorphone may form an important basis in clinical settings, such as the perioperative uses of hydromorphone in neurosurgery.

Although most studies have accepted the role of  $\delta$ -opioid receptors in ischemic models [3,9,29,30], the effects of  $\mu$ - and  $\kappa$ -opioid receptors are still unclear. However, some studies have discussed the role of  $\mu$ - and  $\kappa$ -opioid receptors in cardiac models [9,29]. It has been reported that the neuroprotective effects of hydromorphone, such as improved neurological score, reduced infarct volumes and tumor necrosis factor- $\alpha$  levels, are mediated through the  $\delta$ -opioid receptors and not through the  $\mu$ - and  $\kappa$ -opioid receptors, against transient focal cerebral ischemia in rats [30]. On the other hand, our results showed that each of the  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors are involved in the protective effects of hydromorphone in astrocytes. When using each of the  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptor antagonists, the production of ROS is similar to that in the TBH-treated group. Even though we did not evaluate the mechanism of the opioid receptors in knock-out models, it gives possibility to prove that each of the  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors contribute to the protective effects of hydromorphone. Unfortunately, we cannot conduct these experiments with other opioid receptors, including toll-like receptor 4 (TLR-4), which is involved in the modulation by the glial cells [23]. Despite these shortcomings, our data suggest that regardless of the timing of administration, hydromorphone has protective effects, which corresponds to each of the  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors. Hence, our findings support the therapeutic potential for hydromorphone in the prevention of ischemic cerebral injury.

In conclusion, this study provides evidence that hydromorphone has both, preconditioning and postconditioning effects on the TBH-induced, oxidative stress-related injuries. Furthermore, we proved that each of the  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors contributes to the mechanism of hydromorphone and its protective effect on the astrocytes under ischemic conditions. Further studies are required to evaluate the mechanism of opioid receptors, including TLR-4, using knock-out models.

## REFERENCES

1. Vicente E, Degerone D, Bohn L, Scornavaca F, Pimentel A, Leite MC, et al. Astroglial and cognitive effects of chronic cerebral hypoperfusion in the rat. *Brain Res* 2009; 1251: 204-12.
2. Bilotta F, Gelb AW, Stazi E, Titi L, Paoloni FP, Rosa G. Pharmacological perioperative brain neuroprotection: a qualitative review of randomized clinical trials. *Br J Anaesth* 2013; 110 Suppl

- 1: i113-20.
- Gross GJ, Baker JE, Hsu A, Wu HE, Falck JR, Nithipatikom K. Evidence for a role of opioids in epoxyeicosatrienoic acid-induced cardioprotection in rat hearts. *Am J Physiol Heart Circ Physiol* 2010; 298: H2201-7.
  - Goldsmith JR, Perez-Chanona E, Yadav PN, Whistler J, Roth B, Jobin C. Intestinal epithelial cell-derived mu-opioid signaling protects against ischemia reperfusion injury through PI3K signaling. *Am J Pathol* 2013; 182: 776-85.
  - Gwak MS, Li L, Zuo Z. Morphine preconditioning reduces lipopolysaccharide and interferon-gamma-induced mouse microglial cell injury via delta 1 opioid receptor activation. *Neuroscience* 2010; 167: 256-60.
  - Teo JD, Morris MJ, Jones NM. Hypoxic preconditioning reduces microglial activation, astrocyte and caspase activity, and inflammatory markers after hypoxia-ischemia in the neonatal rat brain. *Pediatr Res* 2015; 77: 757-64.
  - Scheufler KM, Zentner J. Total intravenous anesthesia for intraoperative monitoring of the motor pathways: an integral view combining clinical and experimental data. *J Neurosurg* 2002; 96: 571-9.
  - Kim EM, Park WY, Choi EM, Choi SH, Min KT. A retrospective analysis of prolonged propofol-remifentanyl anesthesia in the neurosurgical patients. *Anesth Pain Med* 2008; 3: 288-92.
  - Zhang Y, Irwin MG, Lu Y, Mei B, Zuo YM, Chen ZW, et al. Intracerebroventricular administration of morphine confers remote cardioprotection role of opioid receptors and calmodulin. *Eur J Pharmacol* 2011; 656: 74-80.
  - Rostami F, Oryan S, Ahmadiani A, Dargahi L. Morphine preconditioning protects against LPS-induced neuroinflammation and memory deficit. *J Mol Neurosci* 2012; 48: 22-34.
  - Iwata-Ichikawa E, Kondo Y, Miyazaki I, Asanuma M, Ogawa N. Glial cells protect neurons against oxidative stress via transcriptional up-regulation of the glutathione synthesis. *J Neurochem* 1999; 72: 2334-44.
  - Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 1996; 19: 312-8.
  - Brown GC, Bal-Price A. Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. *Mol Neurobiol* 2003; 27: 325-55.
  - Hu Y, Wang S, Wang A, Lin L, Chen M, Wang Y. Antioxidant and hepatoprotective effect of *Penthorum chinense* Pursh extract against t-BHP-induced liver damage in L02 cells. *Molecules* 2015; 20: 6443-53.
  - Yan LJ. Positive oxidative stress in aging and aging-related disease tolerance. *Redox Biol* 2014; 2C: 165-9.
  - Milisav I, Poljsak B, Suput D. Adaptive response, evidence of cross-resistance and its potential clinical use. *Int J Mol Sci* 2012; 13: 10771-806.
  - Das M, Das DK. Molecular mechanism of preconditioning. *IUBMB Life* 2008; 60: 199-203.
  - Rush GF, Gorski JR, Ripple MG, Sowinski J, Bugelski P, Hewitt WR. Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. *Toxicol Appl Pharmacol* 1985; 78: 473-83.
  - Holownia A, Mroz RM, Wielgat P, Skiepmo A, Sitko E, Jakubow P, et al. Propofol protects rat astroglial cells against tert-butyl hydroperoxide-induced cytotoxicity; the effect on histone and cAMP-response-element-binding protein (CREB) signalling. *J Physiol Pharmacol* 2009; 60: 63-9.
  - Hutchinson MR, Bland ST, Johnson KW, Rice KC, Maier SF, Watkins LR. Opioid-induced glial activation: mechanisms of activation and implications for opioid analgesia, dependence, and reward. *ScientificWorldJournal* 2007; 7: 98-111.
  - Madera-Salcedo IK, Cruz SL, Gonzalez-Espinosa C. Morphine decreases early peritoneal innate immunity responses in Swiss-Webster and C57BL/6J mice through the inhibition of mast cell TNF-alpha release. *J Neuroimmunol* 2011; 232: 101-7.
  - Askari N, Mahboudi F, Haeri-Rohani A, Kazemi B, Sarrami R, Edalat R, et al. Effects of single administration of morphine on G-protein mRNA level in the presence and absence of inflammation in the rat spinal cord. *Scand J Immunol* 2008; 67: 47-52.
  - Watkins LR, Hutchinson MR, Rice KC, Maier SF. The "toll" of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia. *Trends Pharmacol Sci* 2009; 30: 581-91.
  - Rickett A, Mateyoke G, Vallabh M, Owen C, Peppin J. A pilot evaluation of a hydromorphone dose substitution policy and the effects on patient safety and pain management. *J Pain Palliat Care Pharmacother* 2015; 29: 120-4.
  - Oosten AW, Oldenmenger WH, Mathijssen RH, van der Rijt CC. A systematic review of prospective studies reporting adverse events of commonly used opioids for cancer-related pain: a call for the use of standardized outcome measures. *J Pain* 2015; 16: 935-46.
  - Wirz S, Wartenberg HC, Nadstawek J. Less nausea, emesis, and constipation comparing hydromorphone and morphine? A prospective open-labeled investigation on cancer pain. *Support Care Cancer* 2008; 16: 999-1009.
  - Guedes AG, Papich MG, Rude EP, Rider MA. Comparison of plasma histamine levels after intravenous administration of hydromorphone and morphine in dogs. *J Vet Pharmacol Ther* 2007; 30: 516-22.
  - Wolf NB, Kuchler S, Radowski MR, Blaschke T, Kramer KD, Weindl G, et al. Influences of opioids and nanoparticles on in vitro wound healing models. *Eur J Pharm Biopharm* 2009; 73: 34-42.
  - Wu S, Wong MC, Chen M, Cho CH, Wong TM. Role of opioid receptors in cardioprotection of cold-restraint stress and morphine. *J Biomed Sci* 2004; 11: 726-31.
  - Jeong S, Kim SJ, Jeong C, Lee S, Jeong H, Lee J, et al. Neuroprotective effects of remifentanyl against transient focal cerebral ischemia in rats. *J Neurosurg Anesthesiol* 2012; 24: 51-7.