Iranian Journal of Basic Medical Sciences
Vol. 14, No. 1, Jan-Feb 2011, 1-8
Received: Apr 22, 2010; Accepted: June 22, 2010

IJBMS

Original article

Effect of Morphine Withdrawal Syndrome on Cerebral Ischemia Outcome in Rats

*[1,2]Mohammad Allahtavakoli, [1]Ruhollah Moloudi, [1]Mohammad Ebrahim Rezvani, [1,2]Ali Shamsizadeh

Abstract

Objective(s)
Opioid abuse is still remained a major mental health problem, a criminal legal issue and may cause ischemic brain changes including stroke and brain edema. In the present study, we investigated whether spontaneously withdrawal syndrome might affect stroke outcomes.

Materials and Methods
Addiction was induced by progressive incremental doses of morphine over 7 days. Behavioral signs of withdrawal were observed 24, 48 and 72 hr after morphine deprivation and total withdrawal score was determined. Cerebral ischemia was induced 18-22 hr after the last morphine injection by placing a natural clot into the middle cerebral artery (MCA). Neurological deficits were evaluated at 2, 24 and 48 hr after ischemia induction, and infarct size and brain edema were determined at 48 hr after stroke.

Results
Morphine withdrawal animals showed a significant increase in total withdrawal score and decrease of weight gain during the 72 hr after the last morphine injection. Compared to the addicted and control animals, infarct volume and brain edema were significantly increased in the morphine deprived animals ($P< 0.05$) at 48 hr after cerebral ischemia. Also, neurological deficits were higher in the morphine-withdrawn rats at 48 hr after stroke ($P< 0.05$).

Conclusion
Our data indicates that spontaneous withdrawal syndrome may worsen stroke outcomes. Further investigations are necessary to elucidate mechanisms of opiate withdrawal syndrome on stroke.

Keywords: Addiction, Cerebral ischemia, Embolic stroke, Morphine withdrawal syndrome, Opiates

1-Department of Physiology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
2-Physiology- Pharmacology Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
* Corresponding author: Tel: +98-391-5224003; Fax: +98-391-5225209; email: m_alahtavakoli@rums.ac.ir

Introduction

According to the report of world health organization, stroke is the second leading cause of death and the first cause of major adult disability in the world (1). Among the stroke patients, 85-90% of the cases are ischemic stroke with a predominant (75-80%) cause of cerebral arterial thrombosis and majorities of ischemic episodes occurring as a result of occlusion of the middle cerebral artery (MCA) or its branches (2, 3). Leading pathogenic mechanisms of cerebral ischemia include energy failure, elevation of intracellular Ca^{2+} level, excitotoxicity, spreading depression, generation of free radicals, blood brain barrier disruption, inflammation and apoptosis (2).

Recreational drug abuse is one of the most important risk factors for stroke in young adults (4). High rate of both ischemic and hemorrhagic stroke following opiate users have been described and there are several reports showing a broad range of neurologic complications in opiate users including embolic stroke and brain edema (5-7). Brain autopsy of patients that died from opiate heroin intoxication had presented cerebral edema (6).

Opiates are used in medicine as analgesic drugs and abused for their recreational effects (8). There are numerous studies which controversially indicate neuroprotective and neurotoxic properties of opioids (9-11). Chronic exposure to these compounds leads to tolerance and physical dependence which are characteristics of opiate addiction. Following chronic administration of morphine, termination of the drug leads to withdrawal syndrome which is characterized by severe pathophysiological and behavioral manifestations (8, 12). It has been reported that opiate withdrawal syndrome causes neurodegenerative alterations including increased neuronal excitability and apoptosis, blood brain barrier (BBB) dysfunction and oxidative stress (2, 13-16). To the best of our knowledge, whether withdrawal from morphine in the addicted humans or animals can affect stroke outcomes has not yet been reported.

Since opiate addiction is an illegal behavior in most countries, stroke addicted-patients or their relatives may prefer to keep it secret from health care providers or physicians and hence, they may go through opiate withdrawal syndrome during hospitalization. Therefore, studies in this area have clinical importance. In the present investigation we studied, for the first time, the effect of withdrawal from morphine on infarct size, brain edema and neurological deficits after stroke in rat.

Materials and Methods

Animals and treatments

Animals were handled in accordance with criteria outlined in the Guide for Care and Use of Laboratory Animals (NIH US publication 86-23 revised 1985; http://oacu.od.nih.gov/regs/guide/guidex.htm) and the experiments were approved by the University Research Council guidelines for conducting animal studies. Adequate measures were taken to minimize pain or discomfort of the animals. A total of 30 male Wistar rats weighting 250 to 300 g were maintained on a 12 hr light-dark cycle, with food and water available *ad libitum*. Eight animals were non-dependent and considered as control (vehicle) and 22 rats were subjected to morphine dependence. Dependence was induced by repeated subcutaneous injections of morphine sulfate (Darou-Pakhsh, Tehran, Iran) daily at 13 PM as previously reported (8). In brief, morphine doses were dissolved in a volume of 2 ml/kg saline and progressively increased for 7 days initiating with 6, then 16, 26, 36, 46, 56 and 66 mg/kg by a single dose. Control group was treated with saline following the same procedure. Both non-dependent (n= 8) and morphine dependent (n= 16) animals were gone under the surgery of embolic stroke at 18-22 hr after the last injection of saline or morphine (day 8), respectively. Then the dependent rats were randomly divided into two groups of spontaneously morphine withdrawal or morphine dependent animals by terminating or continuing the last dose of morphine injection (66 mg/kg), respectively. The remaining 6 rats were equally divided into 3 dependent and 3 withdrawal ones and used as sham-operated animals, accordingly. For sham-operated animals, the surgery was the

same except for injection of 5 µl saline into the MCA.

Measurement of withdrawal syndromes

Spontaneous withdrawal syndromes were induced by terminating morphine injection and observed for 30 min in circular Plexiglas boxes at 24 hr after the last morphine injection (day 8) and, 24 and 48 hr after stoke induction (days 9 and 10) as shown in Figure 1. Animals' reactions were observed individually by an observer who was unaware of the treatments animals received. Total withdrawal score (TWS) was determined using the method described in detail before (8). Briefly, 20 distinct withdrawal behaviors (16 scale): jumping, rearing, walk sniffing, sniffing, wet dog shakes, head shakes, body grooming, face wiping, penis licking, chewing, teeth chattering, swallowing, writhing, fore paw tremor, weight loss percentage and dysphoria time percentage; (2 ordinal): ptosis and diarrhea; and (2 checked) behaviors: irritability and eye twitch were scored. Body weight was measured daily during induction of morphine dependency and before each withdrawal observing periods.

Induction of embolic stroke

Rats were anesthetized with 1.5% halothane in a 21% O_2 and 79% N_2 mixture. Embolic stroke was induced by placing a preformed clot into the middle cerebral artery (MCA) as reported in detail before (17). Briefly, a longitudinal incision of 1.5 cm in length was made in the midline of the ventral cervical skin. The right common carotid artery, internal carotid artery, and external carotid artery were exposed. The distal portion of the external carotid artery was ligated and cut. A modified PE-50 tube with the 20 mm clot was connected to a 50-µl Hamilton lock syringe, and advanced 17-19 mm in the internal carotid artery until its tip was inside of MCA. The clot was then injected and the catheter was removed. The wound was closed, and the animal was returned to its cage. Rectal temperature was maintained at 36.5±0.5 ^{o}C with a heating pad throughout the surgical procedures. Blood gases and glucose and blood pressure were measured 5 min before and after embolization. For sham-operated animals, the surgery was the same except for injection of 5 µl saline into the MCA. The surgeon was blinded to the drug treatment regimen of animals he was working on.

Measurement of infarct volumes and brain edema

The quantification of infarct volume has been previously described in detail (17). Briefly, for these analyses rats were killed at 48 hr after MCA occlusion. The brains were removed from the skull, and using a brain matrix, the forebrain was sliced coronally at 2 mm intervals. A total of 6 coronal sections were collected, and these were incubated with a 2% 2, 3, 5- triphenyltetrazolum chloride solution (TTC; Sigma, UK) at 37 ºC, and fixed by immersion in a 10% phosphate-buffered formalin solution (Figure 3). The stained brain sections were scanned (Canon, CanoScan 9900f) and analyzed. The total volume of each hemisphere and infarction was determined by integration of the distance of the 6 sections. Infarctions were adjusted to the size of contralateral hemisphere by applying the following formula: Infarct volume= (volume of left hemisphere-(volume of right hemisphere-measured infarct volume))/ volume of left hemisphere. Brain edema was determined by using the following formula: Edema= (volume of right hemisphere-volume of left hemisphere)/volume of left hemisphere. The infarction volume and brain edema were expressed as percentages. For these analyses, the experimenter was blinded to the treatment assignations.

Neurological deficits evaluation

Neurological deficits were recorded at 2, 24 and 48 hr after embolic stroke and determined with a modified 6-point scoring system (17) as follows: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion plus decreased resistance to lateral push; 3, unidirectional circling; 4, unidirectional circling plus decreased level of consciousness; and 5, death.

Statistical analysis

Infarct volume and brain edema were presented as mean±SEM and were analyzed

with one-way ANOVA. Percentage of body weight changes (compared to the weight before the first injection) and total withdrawal score (TWS) were presented as mean±SEM and were analyzed by two-way repeated measure ANOVA or two-way ANOVA followed by Tukey's test, respectively. Neurological deficits are reported as medians and interquartile ranges (25th and 75th percentiles), and were analyzed with Mann-Whitney U test. A value of $P< 0.05$ was considered to be statistically significant.

Results

Weight changes

The weight gain was monitored daily throughout morphine treatment and withdrawal, as shown in Figure 1. An increase of 4.5% and 3.5% in weight gain was observed in the control and morphine treated rats during the 8 days of experiment, respectively. This difference was not statistically significant. At 24 hr (day 2 after the last morphine injection) after stroke induction, weight gain reduced in control, dependent and morphine withdrawal animals by 5.8±1.14%, 3.9±.81 and 11.04±1.15%, respectively, which was significantly different between the groups ($P< 0.01$). A maximum of significant difference in weight reduction was observed between morphine deprived animals and the other groups at 48 hr (day 3 after the last morphine injection) after stroke induction ($P< 0.001$). At this time point, weight reductions in the control, morphine dependent and morphine withdrawal animals were 7.6±1.3%, 7.97±.66 and 18.25±1.73%, respectively ($P< 0.001$).

Withdrawal syndrome

Morphine-withdrawn animals showed behavioral signs of opiate withdrawal and the total withdrawal score (TWS) was considered as an index of abstinence throughout our experiments (Figure 2). While at the day 8 (24 hr after the last morphine injection) there was no significant difference in TWS between control and morphine treated animals, withdrawal signs were significantly increased in morphine-deprived rats at 24 and 48 hr after stroke (days 2 and 3 after the last morphine injection; $P< 0.001$). Also, compared to 24 hr after stroke (day 2 after the last injection), TWS was increased in the morphine-deprived animals at 48 hr after cerebral ischemia onset ($P< 0.05$), as shown in Figure 2.

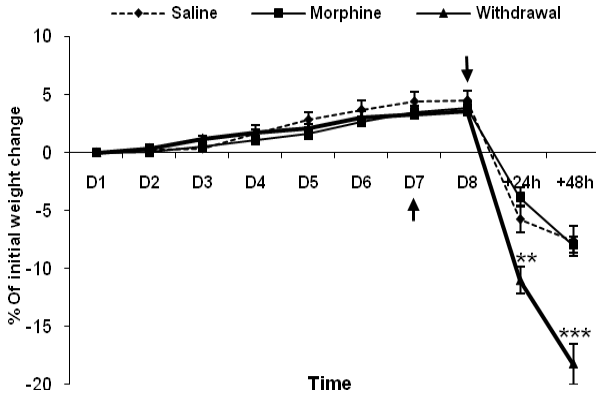

Figure 1. Evaluation of weight change compared to the weight before the first injection. Body weight of each rat was measured before the injection in each day, before stroke induction (24 hr after the last morphine injection, day 8) or at 24 and 48 hr after stroke. Results are expressed as mean±SEM. $**P< 0.01$; $***P< 0.001$ compared to the control (saline) or morphine dependent group at the same time point. D= day. The lower and the upper arrows show the day of last morphine injection and stroke induction, respectively.

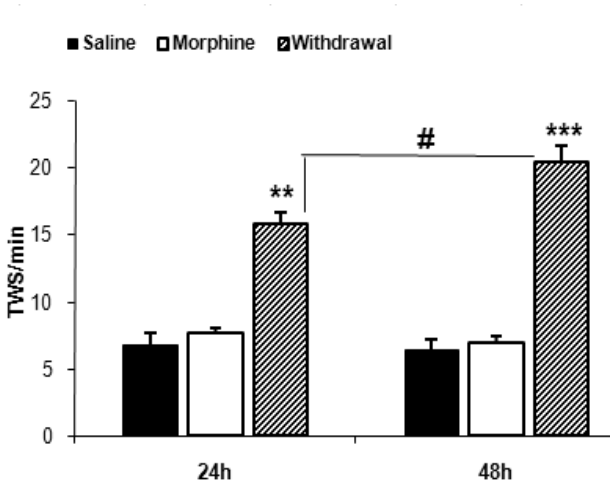

Figure 2. Evaluation of total withdrawal scores (TWS) in non-dependent (control), morphine dependent or withdrawal rats before stroke induction (24 hr after the last morphine injection= day 8) or at 24 and 48 hr after stroke. Data are expressed as Mean±SEM. $**P< 0.01$; $***P< 0.001$ compared to the control or dependent animals at the same time. $\#P<0.05$ compared to the same group at different time points.

Infarct volume and brain edema

We did not observe any cerebral infarction or brain edema in the morphine withdrawal or dependent sham-operated animals. The mean infarct volumes in control, morphine and morphine-deprived animals were 26.95±4.46%, 23.74±2.83 and 39.67±3.5%, respectively (Figure 3A). Compared to the control group, infarct volume in the morphine withdrawal rats was increased by 45% ($P< 0.05$). The mean brain edema in control, morphine-dependent and morphine withdrawal rats were 6.9±1.24, 5.45±1.3 and 10.44±1, respectively. Morphine-deprivation worsened brain edema compared to the control and morphine dependent groups when measured at 48 hr after embolic stroke ($P< 0.05$; Figure 3B). There were no significant differences in infarct volume or brain edema between control and morphine dependent animals.

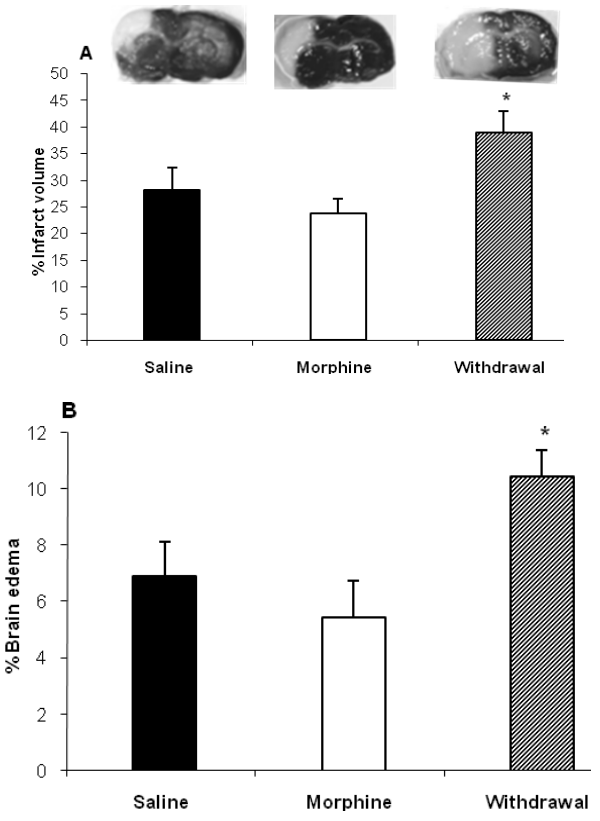

Figure 3. Morphine withdrawal worsens infarct volume (A) and brain edema (B) in the rats subjected to embolic cerebral ischemia. Infarct volume and brain edema were measured from TTC stained brain sections that were obtained 48 hr after embolic stroke (3 days after morphine deprivation). The data are presented as mean±SEM. *$P< 0.05$ compared to the control and morphine dependent rats.

Table 1. Morphine withdrawal worsens neurological deficits in the rats subjected to embolic cerebral ischemia.

Groups Hours	Control	Morphine	Withdrawal
2	4 (3-4.75)	3.5 (3-4.75)	4 (3-4.75)
24	4 (2.25-4)	2.5 (2-5)	4.5 (2.25-5)
48	3 (2.25-3.75)	2 (1-5)	5 (3.5-5)*

Neurological deficits were measured by a five-score scale at 2, 24 and 48 hr after embolic cerebral ischemia onset. The data are presented as median, 25th and 75th percentiles (percentiles in the parentheses). Non parametric Mann-Whitney U test showed a significant difference between morphine withdrawal animals and the other two groups at 48 hr after stroke. *$P< 0.05$ compared to the control group at the same time.

Neurological deficits evaluation

Neurological deficits at 2, 24 and 48 hr after stroke were evaluated. The data are shown in Table 1. Compared to the control and morphine dependent groups, morphine-deprived animals showed a higher score of neurological deficits at 48 hr after MCA embolization ($P< 0.05$). There were no significant difference between neurological scores of control, morphine dependent or withdrawal animals at 2 and 24 hr after stroke. No neurological deficit was observed in the sham operated rats.

Discussion

This is the first study to show that deprivation from morphine can deteriorate stroke outcomes in the addicted animals. To the best of our knowledge, there are no other publications discussing the effect of morphine withdrawal syndrome on cerebral ischemia outcomes. To mimic the human stroke and withdrawal syndrome, we chose the embolic model of stroke and spontaneously morphine withdrawal syndrome in our experiments.

Brain injury after an ischemic attack is an evolving process that can continue for days after injury (18-20). During the delayed phase of cerebral ischemia, which may last for several days or even weeks, secondary phenomena such as inflammation, apoptotic cell death and vasogenic edema may contribute to further progression of brain injury (18). In the present study, we found that morphine withdrawal worsened infarct size, brain edema and neurological function in the

dependent rats subjected to the embolic model of middle cerebral artery occlusion (MCAO). There was no significant difference in stroke outcome between addicted and non-addicted animals after focal embolic cerebral ischemia ($P> 0.05$).

Most studies implemented to date have used opioid receptor antagonist-precipitated withdrawal syndrome. This experimental method however differs from the clinical setting where the opiate withdrawal syndrome occurs as a result of drug intake cessation. Spontaneously morphine withdrawal syndrome however induces milder behavioral modulation than the naloxone-precipitated one (21), although it has been suggested that 40 hr after the last morphine administration, spontaneous morphine withdrawal symptoms showed no difference with the naloxone-precipitated one (22). Therefore, spontaneous withdrawal syndrome induced by cessation of morphine in the addicted animals more closely resembles the clinical conditions and may be better in future translation from bench to bedside. In the current study, we observed the classical signs in the spontaneous withdrawal model by depriving the dependent animals from morphine. Morphine-withdrawn animals showed behavioral signs of opiate withdrawal and the total withdrawal score was gradually increased as the interval of last morphine injection and testing time was increased. As expected, our data showed that weight loss of morphine withdrawal animals after stroke induction was significantly higher than the control and morphine dependent groups ($P< 0.05$).

The possible neuropathology and neurodegeneration mechanisms of worsening stroke outcomes by morphine withdrawal syndrome are still not well known. However, there are some similar mechanisms in the pathophysiology of stroke and opiate withdrawal syndrome which may have neurodegenerative or neurotoxic effects following stroke in the opiate withdrawal condition. First, excessive or prolonged stimulation of glutamate receptors, particularly N-methyl-D-aspartate (NMDA) receptors, which results in disruption of cellular ion homeostasis, oxidative stress, degeneration and death of neurons in a process called excitotoxicity, is one of the major pathophysiologies of stroke (2, 18). It has been reported that morphine withdrawal syndrome also augmented excitatory glutamate neurotransmitter release and subsequently increased neuronal excitability during morphine withdrawal (13, 22). Accordingly, NMDA receptor antagonists could disrupt opiate withdrawal signs (14, 23). Hence, it can be speculated that morphine withdrawal syndrome may have augmented excitotoxicity of ischemic neurons and then raised neural death after stroke. Second, both cerebral ischemia (18, 24) and morphine withdrawal syndrome (15) induce apoptotic cell death in the brain. Third, blood brain barrier (BBB) disruption is one of the leading mechanisms of stroke pathology (2, 18) and spontaneous withdrawal of morphine also leads to BBB dysfunction and brain edema (16). Finally, oxidative stress may play a role in both morphine induced withdrawal neurotoxicity and stroke induced neurodegeneration (2, 16, 18). Therefore, the above shared pathologic mechanisms in the cerebral ischemia and morphine withdrawal syndrome may have additive or even synergistic neurodegenerative deleterious effects on stroke outcome of morphine deprived animals.

In the present study, we did not observe any significant difference in stroke outcome between morphine-dependent or independent rats. In agreement with our results, it has been reported that there was no difference in stroke outcome between drug-associated and non-drug associated patients (4). Neuroprotective effects of δ and κ opioid receptor agonists (25) and also μ agonists (11) have been reported in animal models of cerebral ischemia. Paradoxically, neuroprotective effect of naloxone, a non-selective opioid receptor antagonist, in ischemic brain injury has been demonstrated (10). One reason that our findings are not parallel with the other investigators (11, 25), is that they have described neuroprotection of opiate receptor agonists in normal but not morphine-addicted animals. Therefore, down-

regulation of endogenous opioid peptides or opiate receptors which happens during morphine addiction, may affect neuroprotective properties of these compounds.

Conclusion

The present study indicates for the first time, that spontaneous withdrawal syndrome may worsen infarct size, brain edema and neurological deficits after stroke. However, further investigations are necessary to elucidate the exact effects and pathophysiologic mechanisms of opiate withdrawal syndrome on stroke.

Acknowledgment

This study was financially supported by the grant no 9/20/454 from the Research Vice Chancellor of Rafsanjan University of Medical Sciences, Iran.

References

1. Carmichael ST. Rodent models of focal stroke: size, mechanism, and purpose. NeuroRx 2005; 2: 396-409.
2. Durukan A, Tatlisumak T. Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav 2007; 87:179-197.
3. Mergenthaler P, Dirnagl U, Meisel A. Pathophysiology of stroke: lessons from animal models. Metab Brain Dis 2004; 19:151-167.
4. Sloan MA, Kittner SJ, Feeser BR, Gardner J, Epstein A, Wozniak MA, *et al*. Illicit drug-associated ischemic stroke in the Baltimore-Washington young stroke study. Neurology 1998; 50:1688-1693.
5. Somala RK. Rhabdomyolysis and brain ischemic stroke in a heroin-dependent male. Invited comment. Acta Psychiatr Scand 2009; 120:80-81.
6. Büttner A, Mall G, Penning R,Weis S. The neuropathology of heroin abuse. Forensic Sci Int 2000; 113:435-442.
7. Borne J, Riascos R, Cuellar H, Vargas D, Rojas R. Neuroimaging in drug and substance abuse part II: opioids and solvents. Top Magn Reson Imaging 2005; 16:239-245.
8. Mirzaii-Dizgah I, Ojaghi R, Sadeghipour-Roodsari HR, Karimian SM, Sohanaki H. Attenuation of morphine withdrawal signs by low level laser therapy in rats. Behav Brain Res 2009; 196:268-270.
9. Zhang Y, Chen Q, Yu LC. Morphine: a protective or destructive role in neurons? Neuroscientist 2008; 14:61-570.
10. Liao SL, Chen WY, Raung SL, Chen CJ. Neuroprotection of naloxone against ischemic injury in rats: role of mu receptor antagonism. Neurosci Lett 2003; 345:169-172.
11. Xiong LZ, Yang J, Wang Q, Lu ZH. Involvement of delta-and mu-opioid receptors in the delayed cerebral ischemic tolerance induced by repeated electroacupuncture preconditioning in rats. Chin Med J (Engl) 2007; 120:394-399.
12. Feng P, Meissler JR, Adler MW,Eisenstein TK. Morphine withdrawal sensitizes mice to lipopolysaccharide: elevated TNF-alpha and nitric oxide with decreased IL-12. J Neuroimmunol 2005; 164:57-65.
13. Dunbar SA, Karamian I, Yeatman A, Zhang J. Effects of recurrent withdrawal on spinal GABA release during chronic morphine infusion in the rat. Eur J Pharmacol 2006; 535:152-156.
14. Harris AC, Rothwell PE, Gewirtz JC. Effects of the NMDA receptor antagonist memantine on the expression and development of acute opiate dependence as assessed by withdrawal-potentiated startle and hyperalgesia. Psychopharmacology (Berl) 2008; 196: 649-660.
15. Emeterio EP, Tramullas M, Hurle MA. Modulation of apoptosis in the mouse brain after morphine treatments and morphine withdrawal. J Neurosci Res 2006; 83:1352-1361.
16. Sharma HS, Sjoquist PO, Ali SF. Drugs of abuse-induced hyperthermia, blood-brain barrier dysfunction and neurotoxicity: neuroprotective effects of a new antioxidant compound H-290/51. Curr Pharm Des 2007; 13:1903-1923.
17. Allahtavakoli M, Moloudi R, Arababadi MK, Shamsizadeh A, Javanmardi K. Delayed post ischemic treatment with rosiglitazone attenuates infarct volume, neurological deficits and neutrophilia after embolic stroke in rat. Brain Res 2009; 19:121-127.
18. Hossmann KA. Pathophysiology and therapy of experimental stroke. Cell Mol Neurobiol 2006; 26: 1057-1083.
19. Williams AJ, Berti R, Dave JR, Elliot PJ, Adams J, Tortella FC. Delayed treatment of ischemia/reperfusion brain injury: extended therapeutic window with the proteosome inhibitor MLN519. Stroke 2004; 35:1186-1191.
20. Alioglu B, Ozyurek E, Avci Z, Atalay B, Caner H, Ozbek N. Peripheral blood picture following mild head trauma in children. Pediatr Int 2008; 50:281-283.
21. Desjardins S, Belkai E, Crete D, Cordonnier L, Scherrmann JM, Noble F, *et al*. Effects of chronic morphine and morphine withdrawal on gene expression in rat peripheral blood mononuclear cells. Neuropharmacology 2008; 55:1347-1354.
22. Xiao H, Zhai DX, Yan BB, Wang JH, Xu WS, Wang GY, *et al*. A role for the parafascicular thalamic nucleus in the development of morphine dependence and withdrawal. Brain Res 2009; 28: 74-82.

23. Blokhina EA, Sukhotina IA, Bespalov AY. Pretreatment with morphine potentiates naloxone-conditioned place aversion in mice: effects of NMDA receptor antagonists. Eur J Pharmacol 2000; 406:227-232.
24. Giuliani D, Leone S, Mioni C, Bazzani C, Zaffe D, Botticelli AR, *et al*. Broad therapeutic treatment window of (Nle(4), D-Phe(7))alpha-melanocyte-stimulating hormone for long-lasting protection against ischemic stroke, in Mongolian gerbils. Eur J Pharmacol 2006; 538:48-56.
25. Kao TK, Ou YC, Liao SL, Chen WY, Wang CC, Chen SY, *et al*. Opioids modulate post-ischemic progression in a rat model of stroke. Neurochem Int 2008; 52:1256-1265.