Allopregnanolone suppresses diabetes-induced neuropathic pain and motor deficit through inhibition of GABAA receptor down-regulation in the spinal cord of diabetic rats

Samira Afrazi [1, 2], Saeed Esmaeili-Mahani [2]*

[1] Laboratory of Molecular Neuroscience, Kerman Neuroscience Research Center (KNRC), Kerman University of Medical Sciences, Kerman, Iran
[2] Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran

ARTICLE INFO	ABSTRACT
	Objective(s): Painful diabetic neuropathy is associated with hyperexcitability and hyperactivity of spinal cord neurons. However, its underlying pathophysiological mechanisms have not been fully clarified. Induction of excitatory/inhibitory neurotransmission imbalance at the spinal cord seems to account for the abnormal neuronal activity in diabetes. Protective properties of neurosteroids have been demonstrated in numerous cellular and animal models of neurodegeneration. *Materials and Methods:* Here, the protective effects of allopregnanolone, a neurosteroid were investigated in an *in vivo* model of diabetic neuropathy. The tail-flick test was used to assess the nociceptive threshold. Diabetes was induced by injection of 50 mg/kg (IP) streptozotocin. Seven weeks after the induction of diabetes, the dorsal half of the lumbar spinal cord was assayed for the expression of $\gamma 2$ subunit of GABAA receptor using semiquantitative RT-PCR. *Results:* The data shows that allopregnanolone (5 and 20 mg/kg) markedly ameliorated diabetes-induced thermal hyperalgesia and motor deficit. The weights of diabetic rats that received 5 and 20 mg/kg allopregnanolone did not significantly reduce during the time course of study. Furthermore, this neurosteroid could inhibit GABAA receptor down-regulation induced by diabetes in the rat spinal cord. *Conclusion:* The data revealed that allopregnanolone has preventive effects against hyperglycemic-induced neuropathic pain and motor deficit which are related to the inhibition of GABAA receptor down-regulation.

Introduction

Diabetes mellitus is a major health concern for human and according to the reports published by World Health Organization (WHO), the prevalence of diabetes for all age-groups worldwide is estimated to be 4.4% in 2030 (1). Diabetic neuropathies are heterogeneous groups of disorders which present a wide range of abnormalities. Such disorders are among the most common long-term complications of diabetes and are important sources of morbidity and mortality (2).

Almost 50% of the diabetic patients develop neuropathy with symptoms including spontaneous pain, hypoesthesia, allodynia and hyperalgesia (3). Despite significant progress in the treatment of pain, a variety of pathological pains, particularly diabetic neuropathic pain, cannot still be alleviated and they are refractory to the currently available analgesics. Therefore, biomedical investigations are performed to reveal cellular and molecular factors that are affected in the central nervous system during chronic pain to introduce potential targets for new drugs.

Although hyperglycemia is considered to be a major pathogenic factor in the development of diabetic neuropathy, the mechanisms associated with this disease have not yet been fully understood (4).

It has been demonstrated that changes in the extracellular GABA levels and impaired GABAA receptor-mediated inhibition are occurred in neuropathic situations (5, 6). Spinal delivery of bicuculline, a GABAA receptor antagonist reduced formalin-evoked flinching and tactile allodynia in diabetic rats (7). Furthermore, GABAA receptor-mediated depression of the spinal Hoffmann's reflex (a reflectory reaction of muscles after electrical stimulation of sensory fibers) was also absent in the spinal cord of diabetic rats (7). It has been reported

Corresponding author: Saeed Esmaeili Mahani. Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran. Tel/Fax: +98-341-3222032; email: semahani@yahoo.com

that GABA content as well as GABA receptor binding sites significantly decreased in the brain stem of pancreatectomised hyperglycemic rats (8).

Neurosteroids are synthesized in oligodendrocytes, astrocytes, schwann cells and some neurons, such as purkinje, retinal amacrine and ganglion cells and hippocampal neurons (9). Recent experimental findings bring new insights into the decline of neurosteroid synthesis in the CNS during aging and also in various neurodegenerative diseases (10).

Synthesis of neurosteroids declines during stressful conditions and in chronic inflammatory and neurodegenerative diseases. However, recent biochemical reports indicate that spinal neurosteroids are important factors for the control of pain (11) and also they show the potential applications of neurosteroids in the therapeutic management of painful neuropathic conditions (12, 13). It has been shown that some neurosteroids produce neuroprotective effects against streptozotocin (STZ)-induced diabetic neuropathy at the neurophysiological, neuropathological, biochemical and functional levels (14). Since neurosteroids had shown protective effects in numerous cellular models of neurodegenerative diseases (9, 10) and elicited antinociception in neuropathic situations (11), the present study was designed to evaluate the preventive effects of neurosteroid allopregnanolone in animal models of diabetic neuropathic pain by evaluating its possible effect on the predicted diabetes-induced spinal GABA$_A$ down-regulation.

Materials and Methods
Animals
The experiments were carried out on male Wistar rats that were housed in a room with 12 hr light/dark cycle with controlled temperature (22 ± 1°C). Food and water were available *ad libitum*. Animals (weighing 220-250 g) were handled daily (between 9:00-11:00 AM) 5 days before the experiment in order to adapt them to the experiment procedure and minimize nonspecific stress responses. Rats were randomly divided into several experimental groups, each containing 6-8 animals. The experiments followed the guidelines on ethics standard for investigation of experimental pain in animals (15) and approved by the Animal Experimentation Ethics Committee of Kerman Neuroscience Research Center (EC/KNRC/90).

In vivo experimental protocol
Diabetes was induced by a single intraperitoneal injection of 55 mg/kg (IP) streptozotocin (STZ) freshly dissolved in 0.1 mol/l citrate buffer. Rats receiving an injection of citrate buffer were used as control. One week later, diabetes was confirmed in STZ -injected rats by measuring serum glucose concentrations (16). The glucose concentration was assayed enzymatically using a glucose oxidase peroxidase (GOD-POD) kit (Pars Azmon Co, Iran). The rats with blood glucose ≥ 350 mg/dl were considered to have diabetes. One week after the STZ injection, the diabetic rats were given allopregnanolone (5 and 20 mg/kg/day for 6 weeks, orally). Seven weeks after the STZ administration, the animals were decapitated under CO_2 anesthesia. Control rats were killed in the same way 7 weeks after receiving vehicle injection.

Nociception assay
Nociceptive threshold was assessed by tail-flick test (17). The intensity of the beam was adjusted to produce a mean control reaction time between 4 and 6 sec. The cut-off time was fixed at 15 sec in order to avoid any damage to the tail. In this manner, we were able to reveal potential, subtle alternations that may occur in basal thermal nociception (18, 19). The tail-flick latency for each rat was determined three times and the mean was designated as the baseline latency value. Experimentally-induced decreases in control tail-flick latency provide an indication of hyperalgesia as a marker of neuropathy.

Tissue extraction and preparation
Rats were anesthetized (using CO_2) and decapitated. The spinal column was cut through the pelvic girdle and a hydraulic extrusion was performed by inserting a 16 gauge needle into the sacral vertebral canal and expelling with ice-cold saline. The tissues were placed on ice in a glass petri dish, and the dorsal half of the lumbar spinal cord was dissected. Tissue samples were weighed and immediately frozen in liquid nitrogen and stored at -70°C until assay.

Rota-rod treadmill
Rota-rod treadmill test was performed to evaluate motor coordination of the animals (20). Briefly, the rats were placed individually on the rotating rod apparatus for two trials; on day zero and in the fourth week of the test. The rats were initially trained to maintain themselves on the rotating rod for more than 3 min. The rats were scored for their latency to fall (in seconds) in each trial.

RT-PCR analysis of GABA$_A$ receptor
Total cellular RNAs was isolated from the dorsal half of the lumbar spinal cord by a modification of the guanidine isothiocyanate-phenol-chloroform method using RNX+ reagent and a semiquantitative RT-PCR method was used (21). Briefly, the RT-PCR reaction was performed using Oligo-dT primer and M-MuLV reverse transcriptase, based on the manufacturer protocol (Fermentas GMBH, Germany). The reactions were incubated at 42°C for 60 min and then inactivated at 70°C for 10 min. Three separate PCR reactions were used for studying the gene expression in the samples obtained from each rat.

Table 1. Effect of allopregnanolone (ALLO) on serum glucose levels and body weight of diabetic animals. Values represent mean ± SEM (n=6-8)

Groups	Serum glucose (mg/dl)		Body weight	
	Start of study	End of study	Start of study	End of study
Control	100.62±4.41	98.53±3.71	213.57±6.70	269.11±4.01***
Diabetic	427.86±14.93	498.33±17.41	220.29±6.46	155.83±4.41*
Diabetic+ Veh	482.83±41.53	530.75±45.85	215.30±3.75	150.20±8.80*
Diabetic+ALLO5	393.75±24.43	443.17±27.66	216.50±6.19	207.17±8.79++
Diabetic+ ALLO20	430.75±13.11	502.33±51.93	220.75±4.57	198.33±15.75++

Each PCR reaction was carried out using selective forward and reverse primers for β-actin (as an internal standard) and γ2 subunit of GABA$_A$ receptor. The primers of γ2 subunit were designed using online NCBI's facility and cDNA sequences of the gene taken from Gene Bank.

The sequence of primers used was:

γ2 forward: 5'- AGC CCG GAA GTC TCT GCC CAA - 3', γ2 reverse: 5'- CCT CCC GTG TCT CCA GGC TCC -3', β-actin forward: 5'-CCC AGA GCA AGA GAG GCA TC- 3', β-actin reverse: 5'-CTC AGG AGG AGC AAT GAT CT-3'.

Taq DNA polymerase (Roche, Germany) was used for DNA amplification and reactions were set up according to the manufacturer protocol. The PCR reactions were incubated for 5 min at 94°C, followed by 25 cycles of thermal cycling (45 sec at 94°C, 45 sec at 55°C and 45 sec at 72°C). The final cycle was followed by a 5 min extension step at 72°C. The reaction parameters were adjusted to obtain a condition with a linear relation between the number of PCR cycles and PCR products and with linear relation between the initial amount of cDNA template and PCR product. According to the results obtained from these tests, 25 cycles of amplification were used for analyzing all samples. Then, PCR products were subsequently analyzed on 1.5% agarose LMMP (Roche, Germany) gel and bands were quantified by densitometry using Lab Works analyzing software (UVP, UK). The possibility of the presence of contaminating genomic DNA was ruled out by using the yield of reverse transcriptase-minus (RT⁻) reaction, instead of cDNA template which caused no DNA amplification (data not shown). Finally, a semiquantitative PCR technique was used to estimate the levels of γ2 subunit of GABA$_A$ receptor mRNA in tissue samples, normalized to an internal standard (β-actin).

Statistical analysis

The results are expressed as mean ± SEM. The differences in the mean of data between groups were determined by one-way ANOVA, followed by the Newman–Keuls test. *P*<0.05 was considered significant.

Results
The effect of allopregnanolone (ALLO) on serum glucose levels and body weight in diabetic animals

Diabetic rats developed hyperglycemia within one week after streptozotocin injection and serum glucose concentrations were significantly increased in diabetic animals compared with control animals (*P*<0.001). The levels of glucose remained at high levels 7 week after STZ injection. The data showed a significant change (increase) in the weight of control rats during the time curse of the study. At the end of the study (week 7), the weights of diabetic group were significantly lower than those at the start of study (Table 1). Treatment with ALLO (5 and 20 mg/kg/day for 7 weeks) had no attenuating effect on the serum glucose of the diabetic animals (Table 1). The weights of diabetic rats that received 5 and 20 mg/kg ALLO did not significantly decrease during the time course of the study and were higher than those in diabetic non-treated and vehicle-treated animals.

The effect of ALLO on nociceptive threshold in diabetic animals

As shown in Figure 1A, diabetic animals showed a significant decrease in the nociceptive threshold (hyperalgesic response). The hyperalgesia appeared 2 weeks after STZ injection and persisted almost unchanged for up to the end of study (week 7). However, 5 and 20 mg/kg ALLO attenuated diabetes-induced hyperalgesia (Figure 1A).

At the end of the study, the tail flick latency was significantly (*P*<0.001) lower in diabetic rats (5.54±0.13) as compared to the control animals (2.69±0.19). However, administration of ALLO at doses of 5 and 20 mg/kg significantly prevented the induction of diabetes-induced thermal hyperalgesia in the 7th week of the test (Figure 1B).

The effect of ALLO on diabetes induced motor deficit

Rota-rod test has been conducted to assess motor coordination of rats (20). Diabetic rats showed significant reduction in their motor coordination and their ability to maintain themselves over the rota rod apparatus was significantly decreased (*P*<0.001) as compared to the control non-diabetic animals. Treatment of diabetic animals with 5 and 20 mg/kg of ALLO increased retention time to 85.5 and 76.3 % of the control value, respectively (Figure 2).

The effect of ALLO on γ2 mRNA in diabetic animals

Ethidium bromide staining of PCR products showed a single band of the expected size for β-actin (830 bp) and for γ2 subunit (337 bp). Diabetic animals showed a significant decrease in γ2 mRNA
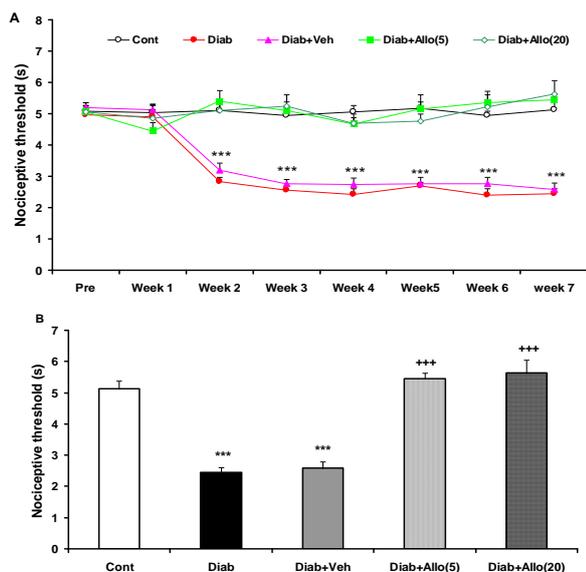
Figure 1. Effect of allopregnanolone (ALLO, 5 and 20 mg/kg) on the development of diabetes-induced thermal hyperalgesia during the time course of the study (A) and comparison of nociceptive threshold at the end (week 7) of the study (B) in rats. Values are expressed as the mean ± SEM (n=6–8). ***P<0.001 versus control (Cont) group. +++P<0.001 versus the diabetic group
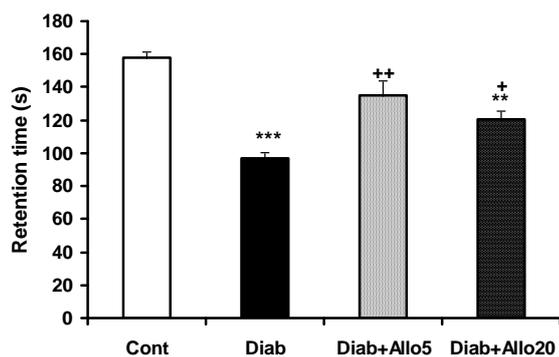

Figure 2. Effect of allopregnanolone (ALLO) on the Rotarod treadmill performance of diabetic rats. Values are expressed as the mean ± SEM (n=6–8). ** P<0.01, *** P<0.001 compared to control. +P<0.05, ++P<0.01 compared to diabetic group

level. Allopregnanolone at the dose of 5 mg/kg could prevent γ2 down-regulation in diabetic animals. Furthermore, treatment with 20 mg/kg ALLO significantly attenuated diabetes-induced down-regulation of γ2 subunit (Figure 3).

Discussion
Diabetic neuropathy is one of the most common complications affecting more than 50-60% of diabetic patients. The pathology of diabetic neuropathy involves oxidative stress, advanced glycation end, polyol pathway flux and protein kinase C activation (3). Here, the STZ-induced diabetic rats were used as an *in vivo* model for the study of diabetic neuropathic pain. This study shows that the neurosteroid allopregnanolone has a protective effect against diabetes-induced hyperalgesia.
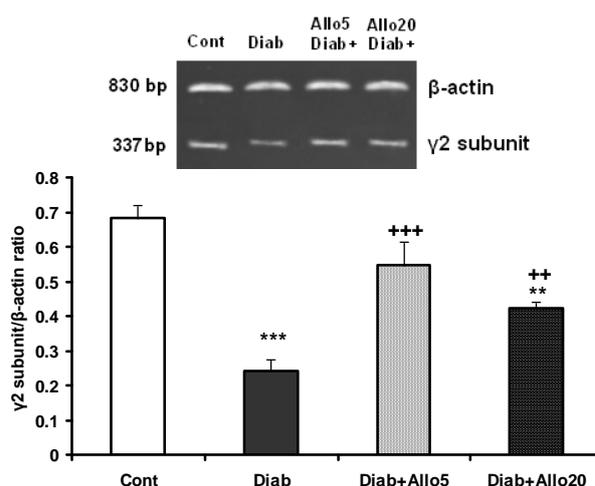

Figure 3. The level of γ2 mRNA in dorsal portion of lumbar spinal cord in control, diabetic (Diab) and allopregnanolone-treated diabetic rats (Diab+ALLO). Each value in the graph represents the mean ± SEM band density ratio for each group (n=5). β-actin was used as an internal control. **P<0.01, ***P <0.001 versus control animals. ++P < 0.01, +++P<0.001 versus diabetic group

Hyperglycemia induces oxidative stress in diabetic neurons and then mitochondrial damage occurs due to excess formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (22). Therefore, it seems that compounds with antioxidant property might exert neuroprotective effects and may be promising in practice. It has been reported that ALLO markedly induces a protective effect on sleep deprivation-induced anxiety-like behavior and oxidative damage in mice (23). Furthermore, ALLO has a beneficial effect in the treatment of Niemann-Pick C disease due to its ability to restore the intracellular antioxidant defense (24). The protective effects of neurosteroids against oxidative stress could be through both genomic and non-genomic pathways (25). Not surprisingly, the mentioned neurosteroid with antioxidant activity could be protective in this *in vivo* model of diabetic neuropathy.

An impaired spinal GABAergic inhibitory function is known to have a critical role in neuropathic pain conditions (5, 6). However, it is unknown whether the severity of neuropathic pain is determined by the degree of these GABAergic impairments. Our data suggest that dysfunctional GABA$_A$ receptor production is coincident with the induction of pain behavior in STZ-induced diabetic rats. ALLO could significantly prevent diabetes-induced spinal GABA$_A$ receptor down-regulation.

Impairment of GABAergic transmission is developed in different models of neuropathic pain (5, 6) as well as in diabetic rats (7). It has been reported that the expression of the genes encoding the α1, α2, β2 and β3 GABA$_A$ channel subunits is down-regulated in pancreatic islets of type 2 diabetic individuals (26). In addition, GABA content as well as

GABA receptor binding sites was significantly decreased in the brain stem of diabetic rats (8).

It seems that impairment in GABA signaling is involved in the induction of painful diabetic neuropathy. Surprisingly, the use of neural cell lines that are able to deliver GABA, as inhibitory neurotransmitters, in a model of chronic pain offers a novel approach to pain management. Transplants of neuronal cells bioengineered to synthesize GABA near the spinal dorsal horn, can reverse the development of chronic neuropathic pain and inhibit allodynia, tactile and thermal hyperalgesia following peripheral nerve injury (27). In addition, intra-spinal transplantation of GABAergic neural progenitor cells reduces neuropathic pain and suppresses the exaggerated dorsal horn neuronal firing by restoring dorsal horn inhibition in rats with unilateral chronic constriction injury of the sciatic nerve (28).

It has been documented that spinal GABA$_A$ agonists may provide a specific therapy for neuropathic pain. It was reported that isoguvacine, a selective GABA$_A$ receptor agonist prevents tactile allodynia and thermal hyperalgesia produced by spinal nerve ligation model of neuropathic pain (29). HZ166, a novel GABA$_A$ receptor subtype-selective benzodiazepine site ligand has antihyperalgesic property in mouse models of inflammatory and neuropathic pain (30).

Therefore, based on our results it seems that the inhibition of diabetes-induced spinal GABA$_A$ receptor down-regulation by neurosteroid ALLO could be accompanied in anti-hyperalgesic effect of this drug. Hormones play an important role in modulating the overall excitability of neurons. GABA$_A$ receptors contain γ subunits that mediate tonic inhibition in the central nervous system. The most potent endogenous modulators of GABA$_A$ receptors are neurosteroids that act as positive allosteric modulators (31). However, the exact mechanism(s) underlying the regulation of GABA subunit gene expression by neurosteroids has not been fully clarified and needs to be studied in future investigation.

Conclusion

Taken together, the results suggest that ALLO prevents diabetic neuropathic pain. The mechanisms underlying such effects may be, at least in part, due to resduction of diabetes-induced GABA receptor down-regulation. Our findings propose therapeutic potential of neurosteroids in the attenuation of diabetes consequences such as neuropathy.

Acknowledgment

References

1. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27:1047-1053.

2. Melmed S, Polonsky KS, Reed P, Kronenberg L, Kronenberg H. Williams Textbook of Endocrinology. 12th ed. New York: Elsevier Mosby/Saunders; 2012.

3. Ramos KM, Jiang Y, Svensson CI, Calcutt NA. Pathogenesis of spinally mediated hyperalgesia in diabetes. Diabetes 2007; 56:1569-1576.

4. Pacher P, Szabó C. Role of poly (ADP-ribose) polymerase-1 activation in the pathogenesis of diabetic complications: endothelial dysfunction, as a common underlying theme. Antioxid Redox Signal 2005; 7:1568-1580.

5. Janssen SP, Truin M, Van Kleef M, Joosten EA. Differential GABAergic disinhibition during the development of painful peripheral neuropathy. Neuroscience 2011; 184:183-194.

6. Janssen SP, Gerard S, Raijmakers ME, Truin M, Van Kleef M, Joosten EA. Decreased intracellular GABA levels contribute to spinal cord stimulation-induced analgesia in rats suffering from painful peripheral neuropathy: the role of KCC2 and GABA (A) receptor-mediated inhibition. Neurochem Int 2012; 60:21-30.

7. Jolivalt CG, Lee CA, Ramos KM, Calcutt NA. Allodynia and hyperalgesia in diabetic rats are mediated by GABA and depletion of spinal potassium-chloride co-transporters. Pain 2008; 140:48-57.

8. Balarama Kaimal S, Gireesh G, Paulose CS. Decreased GABAA receptor function in the brain stem during pancreatic regeneration in rats. Neurochem Res 2007; 32:1813-1822.

9. Benarroch EE. Neurosteroids: endogenous modulators of neuronal excitability and plasticity. Neurology 2007; 68:945-957.

10. Charalampopoulos I, Alexaki VI, Tsatsanis C, Minas V, Dermitzaki E, Lasaridis I, *et al.* Neurosteroids as endogenous inhibitors of neuronal cell apoptosis in aging. Ann N Y Acad Sci 2006; 1088:139-152.

11. Kibaly C, Meyer L, Patte-Mensah C, Mensah-Nyagan AG. Biochemical and functional evidence for the control of pain mechanisms by dehydroepiandrosterone endogenously synthesized in the spinal cord. FASEB J 2008; 22:93-104.

12. Meyer L, Patte-Mensah C, Taleb O, Mensah-Nyagan AG. Allopregnanolone prevents and suppresses oxaliplatin-evoked painful neuropathy: multi-parametric assessment and direct evidence. Pain 2011; 152:170-181.

13. Patte-Mensah C, Meyer L, Kibaly C, Mensah-Nyagan AG. Regulatory effect of dehydro-epiandrosterone on spinal cord nociceptive function. Front Biosci 2010; 2:1528-1537.

14. Leonelli E, Bianchi R, Cavaletti G, Caruso D, Crippa D, Garcia-Segura LM, *et al*. Progesterone and its derivatives are neuroprotective agents in experimental diabetic neuropathy: a multimodal analysis. Neuroscience 2007; 144:1293-12304.

15. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16:109-110.

16. Hajializadeh Z, Esmaeili-Mahani S, Sheibani V, Kaeidi A, Atapour M, Abbasnejad M. Changes in the gene expression of specific G-protein subunits correlate with morphine insensitivity in streptozotocin-induced diabetic rats. Neuropeptides 2010; 44:299-304.

17. DʼAmour FE, Smith DL. A method of determining loss of pain sensation. J Pharmacol Exp Ther 1941; 27:74-79.

18. Esmaeili-Mahani S, Fereidoni M, Javan M, Maghsoudi N, Motamedi F, Ahmadiani A. Nifedipine suppresses morphine-induced thermal hyperalgesia: evidence for the role of corticosterone. Eur J Pharmacol 2007; 567:95-101.

19. Kaeidi A, Esmaeili-Mahani S, Sheibani V, Abbasnejad M, Rasoulian B, Hajializadeh Z, Afrazi S. Olive (Olea europaea L.) leaf extract attenuates early diabetic neuropathic pain through prevention of high glucose-induced apoptosis: *in vitro* and *in vivo* studies. J Ethnopharmacol 2011; 136:188-196.

20. Cartmell SM, Gelgor L, Mitchell D. A revised rotarod procedure for measuring the effect of antinociceptive drugs on motor function in the rat. J Pharmacol Methods 1991; 26:149-159.

21. Esmaeili-Mahani S, Javan M, Motamedi F, Ahmadiani A. Post-adrenalectomy changes in the gene expression of specific G-protein subunits involved in morphine sensitization. Neuropeptides 2008; 42:169-175.

22. Pacher P, Obrosova IG, Mabley JG, Szabo C. Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. Curr Med Chem 2005; 12:267-275.

23. Singh A, Kumar A. Possible GABAergic modulation in the protective effect of allopregnanolone on sleep deprivation-induced anxiety-like behavior and oxidative damage in mice. Methods Find Exp Clin Pharmacol 2008; 30:681-689.

24. Zampieri S, Mellon SH, Butters TD, Nevyjel M, Covey DF, Bembi B, Dardis A. Oxidative stress in NPC1 deficient cells: protective effect of allopregnanolone. J Cell Mol Med 2009; 13:3786-3796.

25. Simoncini T, Genazzani AT. Non-genomic action of sex steroids hormons. Eur J Endocrinal 2003; 148:281-292.

26. Taneera J, Jin Z, Jin Y, Muhammed SJ, Zhang E, Lang S, *et al*. γ-Aminobutyric acid (GABA) signalling in human pancreatic islets is altered in type 2 diabetes. Diabetologia 2012; 55:1985-2994.

27. Eaton MJ, Plunkett JA, Martinez MA, Lopez T, Karmally S, Cejas P, *et al*. Transplants of neuronal cells bioengineered to synthesize GABA alleviate chronic neuropathic pain. Cell Transplant 1999; 8:87-101.

28. Jergova S, Hentall ID, Gajavelli S, Varghese MS, Sagen J. Intraspinal transplantation of GABAergic neural progenitors attenuates neuropathic pain in rats: a pharmacologic and neurophysiological evaluation. Exp Neurol 2012; 234:39-49.

29. Malan TP, Mata HP, Porreca F. Spinal GABA (A) and GABA(B) receptor pharmacology in a rat model of neuropathic pain. Anesthesiology 2002; 96:1161-1167.

30. Di Lio A, Benke D, Besson M, Desmeules J, Daali Y, Wang ZJ, *et al*. HZ166, a novel GABAA receptor subtype-selective benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain. Neuropharmacology 2011; 60:626-632.

31. Ferando I, Mody I. GABAA receptor modulation by neurosteroids in models of temporal lobe epilepsies. Epilepsia 2012; 53:89-101.