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Original Article

Metformin enhances analgesia of low-dose fluoxetine in models of acute and persistent pain in mice: A role of astrocytic activation

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A B S T R A C T

Abstract: This work was designed to investigate the antinociceptive effects of combinations of pioglitazone or metformin with fluoxetine in acute and persistent pain models in Swiss mice. Besides control groups, treatment groups (n=8) were given intraperitoneally (mg/kg) the following: fluoxetine (10, 20 and 40), pioglitazone (20), metformin (50), fluoxetine (20) + pioglitazone (20) and fluoxetine (20) + metformin (50). Sixty min post-injection, mice were subjected to three sets of experiments: 1) hot plate test (acute nociception), 2) formalin test (persistent pain) followed by measurement of paw edema, serum cytokines and immunoreactivity of glial fibrillary acidic protein (GFAP, a marker for astrocytic activation), and 3) rotarod test. In hot plate test, pretreatments with fluoxetine (20), fluoxetine (40), metformin, and the combinations significantly increased the latency time. Moreover, pretreatments with fluoxetine (40), pioglitazone, metformin and the combinations significantly decreased licking time in the second phase of formalin test, formalin-induced paw edema, and formalin-induced GFAP overexpression. All treatments significantly decreased serum levels of tumor necrosis factor-α, interleukin-6 and monocyte chemoattactant protein-1 while increased level of interleukin-10. In rotarod test, treatments did not affect motor function. In conclusion, combination of metformin with low-dose fluoxetine effectively inhibits nociceptive behavior in acute and persistent pain models in mice, suggesting its potential clinical benefit in treatment of pain comorbidities.

Key Words: GFAP; fluoxetine, formalin test; hot plate test; metformin; pain; pioglitazone**.**

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1. INTRODUCTION

Pain is a common cause of seeking medical advice and it causes a heavy load on health and community resources (Breivik *et al.,* 2006). Chronic pain (such as inflammatory and neuropathic pain) differs from acute pain in onset, duration and underlying mechanisms (Nicotra *et al.,* 2012). It is caused by abnormal neuronal responses along the pain transmission pathway, however it was shown that persistent activation of spinal glial cells (astrocytes and microglia) have an important role in initiation and maintenance of chronic pain (Milligan and Watkins, 2009, and Xu and Yaksh, 2011). A key component in glial activation is stimulation of the nuclear factor kappa B (NF-κB) signaling pathway which causes a release of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), monocyte chemoattractant protein-1(MCP-1), and prostaglandins in the spinal cord (Okun *et al.,* 2011). On the other hand, interleukin-10 (IL-10), being an anti-inflammatory cytokine, reduces inflammatory pain through modulation of membrane TNF-α in spinal cord microglia (Zhou *et al.,* 2008).

A role for adenosine monophosphate-activated protein kinase (AMPK) activation in relieving pain (both acute and chronic) was detected. It occurs through inhibition of the mammalian target of rapamycin (mTOR) and the extracellular signal-regulated kinase (ERK) pathways which are involved in the sensitization of peripheral nociceptors and blocking of sodium channel-mediated stimulation of sensory neurons (Tillu, 2012). Being treated through activation rather than inhibition, AMPK represents a good chance for treatment of chronic pain (Price and Dussor, 2013). The activation of AMPK increases glucose uptake and improves hyperglycemia. Pioglitazone and metformin, oral antidiabetic drugs, stimulate AMPK through different signaling pathways

suggesting that AMPK may play a wider role in the cellular stress response (Fryer *et al.,* 2002). Pioglitazone, an agonist on the nuclear peroxisome proliferator-activated receptor gamma (PPAR-γ), is a thiazolidinedione acting as insulin sensitizer in type 2 diabetes (Larsen *et al.,* 2008). It has important neuroprotective, antioxidative, and anti-inflammatory effects that could be mediated through PPAR-γ receptor activation and mostly independent of its insulin-sensitizing effect (Garcia-Bueno *et al.,* 2010) Metformin reduces hepatic glucose production and insulin resistance (Viollet *et al.,* 2012). It showed anti-inflammatory and antinociceptive effects in two different models of inflammatory nociception (Russe *et al.,* 2013). Fluoxetine, the widely-used selective serotonin reuptake inhibitor (SSRI) antidepressant, relieved the nociceptive behavior in models of persistent and neuropathic pain in animals through 5-HT_{2A} receptor stimulation (Anjaneyulu and Chopra 2004). Moreover, a daily administration of fluoxetine for seven days revealed an antiinflammatory and antiarthritic effect against murine and human arthritis which could result of inhibition of signaling of toll-like receptors suppressing the inflammatory cytokine production including TNF-α and IL-6 (Sacre *et al.,* 2010).

The formalin test (injection of formalin into the hind-paw) is a commonly used model for tonic (persistent) pain in rodents (Ellis *et al.,* 2008). Generally, persistent pain is difficult to be treated with the typical analgesics and it is usually associated with other disorders such as diabetes mellitus and depression. Moreover, diabetic patients experience pain due to multiple causes (Ortiz, 2013) and they are more prone to depression (Siddiqui, 2014). Consequently, use of therapies that target, as much as possible, these comorbidities are encouraged (Nicholson and Verma, 2004).

Taken together, the present work was designed to investigate the potential antinociceptive effects of combination of pioglitazone or metformin with fluoxetine in acute and persistent pain models in mice and investigating the underlying mechanisms. Mice were subjected for tests for acute nociception (hot plate test) and persistent pain (formalin test). Following the formalin test, serum levels of cytokines (TNF-α, IL-6, MCP-1 and IL-10) and immunoreactivity of glial fibrillary acidic protein (GFAP, a marker for activation of astrocytes) were measured. The rotarod test was conducted to exclude any possible effects of the tested drugs on the motor function which might affect the nociceptive behavioral results.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

The study protocol was approved by King Abdulaziz University Research Ethics Committee (KAU-REC) and adhered to the National Institute of Health (NIH) guidelines for the Care and Use of Laboratory Animals. Swiss (SWR) male mice (25-30 g) were obtained from King Fahd Research Center and housed in cages at 20-22°C room temperature in a 12 h light-dark cycle. Food and water were available *ad libitum*. All drugs and chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless mentioned otherwise. As far as possible, the same procedure was done by the same personnel to minimize mistakes. After one week of acclimatization, mice were randomly divided by a blinded assistant into twenty five groups (n=8) which were subjected to three sets of experiments: 1) the hot plate test, 2) the formalin test (followed by measurement of paw edema and serum cytokines, and immunohistochemistry), and 3) the rotarod test.

2.2. The first set: Hot plate test (acute thermal pain model)

Eight groups of mice (n=8) were given by intraperitoneal (i.p.) administration the following: 0.9% saline solution (0.1 ml/10 g, the vehicle of the tested drugs) and served as the control (C) group, fluoxetine 10, 20 and 40 mg/kg (Singh *et al.,* 2003), pioglitazone 20 mg/kg (Oliveira *et al.,* 2007), metformin 50 mg/kg (Montes *et al.,* 2012), fluoxetine 20+pioglitazone, and fluoxetine 20+metformin. Then after 60 min. mice were tested using the hot-plate apparatus (UgoBasile, Varese, Italy). Mice were habituated to a plate temperature at 45°C for 2 min. On testing, mice were placed inside a 10-cm diameter Plexiglas cylinder on a hot plate whose temperature set at 55°C. The thermal withdrawal latency was taken as the time in seconds the mouse spent on the hot plate before showing signs of nociception (e.g. licking its paw or jumping). To avoid tissue damage, a cut-off time was set at 30 sec (Zhao *et al.,* 2007 and AlSharari *et al.,* 2012).

2.3. The Second set:

Nine groups (n=8) were used. A group was used as a negative control (NC) where mice were injected 20 µl of 0.9% saline solution (the vehicle of formalin) into the plantar surface of the left hind paw. Another group was used as a positive control (PC) where mice were given by the i.p. route 0.1 ml/10 g of 0.9% saline solution (the vehicle of the tested drugs). The treatment groups were exactly the same as in the hot plate test. Sixty min post-injection all groups except the NC group were subjected to the formalin test. Two hours after the formalin injection, paw

edema was measured, blood was collected from the retroorbital plexus for biochemical measurements and then mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and sacrificed for harvesting the spinal cord for immunohistochemical examination. The 2 h post-injection is considered the most suitable time for measurement of formalin-induced changes in the GFAP expression in the spinal cord (Kang *et al.*, 2011).

2.3.1. Formalin test (persistent pain model)

Mice were allowed 30 min for habituation in an open Plexiglas cage. Twenty µl of 1% formalin solution in 0.9% saline were administered subcutaneously (s.c.) into the plantar surface of mouse's left hind paw using a 30-gauge needle. Each mouse was then immediately placed in the Plexiglas cage. A mirror was placed at a 45° angle behind the cage for clear observation and the licking time (time spent licking the formalin-injected hind paw) in seconds was recorded by a blinded observer using a digital stopwatch from 0 to 5 min (phase 1) and from 20 to 25 min (phase 2) post- injection (Hunskaar and Hole, 1987).

2.3.2. Paw edema

A micrometer caliper was used to measure the alteration of the paw diameter (Zhao *et al.*, 2007).

2.3.3. Serum measurements

The levels of serum cytokines were measured spectrophotometrically at 450 nm wavelength using commercially available ELISA kits for IL-6, IL-10, MCP-1 (RayBiotech,Inc, GA, USA) and TNF-alpha (R&D Systems, MN, USA).

2.3.4. Immunohistochemistry

The activity and distribution of astrocytes was examined by measuring the immune expression of GFAP in the lumbar region of spinal cord (L4–L6) (Martin and O'Callaghan, 1995). The spinal cord at lumbar segments level was formalin-fixed and paraffin-embedded. Paraffin sections (3-5 µm thick) were cut (using a microtome), deparaffinized and rehydrated then processed as described by Makhlouf et al. (2014). Anti GFAP (goat polyclonal Ig G, anti-rat produced by Dako Cytomation, USA) antibody was used at a 1:1000 dilution. The secondary antibody biotinylated rabbit antimouse immunoglobulin (1:600 for 30 min (37ºC), Dako, USA) was used. A computerized image analyzer system software (Pro Plus image analysis software version 6.0) connected to an Olympus Microscope BX-51 with a digital camera connected to a computer at the microscope center, KFMRC was used for photographing and morphometric study. The mean intensity (MI) of GFAP immune reaction in the spinal cord sections were measured using an objective lens of ×20 at the magnification x100. Ten readings from five non-overlapping sections from each mouse of all groups were examined.

2.4. The third set: Rotarod test

It was done to exclude any potential effects of the tested drugs on the motor function which might affect the nociceptive behavioral results. Eight groups (n=8) of naïve rats (ones without previous exposure to this test) were used exactly in the same way as in the hot plate test. Sixty min post-injection, mice were placed on a RotaRod Treadmill (Ugo Basile, Comerio, Italy) at a constant speed of rotation at 32 rpm. The fall-off latency (time spent until the mouse fell off from the rotarod) was calculated from five tests. The upper cut-off time was 90 seconds (Russe *et al.*, 2013)

2.5. Statistical analysis

Data were given as mean values ± SD. Comparisons for two groups were made using Student's T-test. One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used for comparison of variance between groups. All the analysis was done by SPSS version 18.0. A *p* value of <0.05 was considered statistically significant.

3. RESULTS

3.1. Hot plate test

Pretreatments with fluoxetine (20), fluoxetine (40), metformin, and the combinations significantly increased the withdrawal latency time compared to the control group while pretreatments with fluoxetine (10) and pioglitazone failed to affect it. The fluoxetine (20)+metformin showed the most significant increase *versus* other groups. The fluoxetine (20)+pioglitazone showed significant increases *versus* fluoxetine (10) and pioglitazone, non-significant differences from fluoxetine (20) and metformin, and a significant decrease *versus* fluoxetine (40) (table 1).

3.2. Formalin test

The NC mice receiving the s.c. injection of saline into the hind paw did not display any nociceptive behavior. The s.c. injection of formalin resulted in significant increases in the paw licking time in both phases compared to the NC group. All pretreatments failed to affect the nociceptive behavior in the first phase (Fig. 1A). Pretreatments with fluoxetine (40), pioglitazone, metformin and the combinations significantly decreased the licking time in the second phase compared with the PC group. Non-significant differences were found between monotherapy groups (fluoxetine (40), pioglitazone, metformin) while the combinations showed significant decreases compared to monotherapy groups (Fig. 1B).

3.3. Paw edema

The s.c. injection of formalin in mice resulted in a significant increase in the paw volume compared to the NC group. The formalin-induced paw edema was significantly inhibited by pretreatments with fluoxetine (40), pioglitazone, metformin, and the combinations compared with the PC group. There were non-significant differences among all treatment groups (Fig. 1C).

3.4. Immunohistochemistry

The lumbar spinal cord of the NC group showed some GFAP positive astrocytes that exhibited many processes while that of the PC group showed many astrocytes with increased GFAP activity and significant increase in the mean intensity of the GFAP immunoexpression compared to the NC group. The formalin-induced increases in GFAP expression were significantly inhibited by pretreatments with fluoxetine (40), pioglitazone, metformin and the combinations compared with the PC group. The combination groups completely reversed the changes and showed non-significant differences from the NC group. Also both combinations showed more significant decreases compared with fluoxetine (40). The fluoxetine (20)+metformin group showed more significant decreases compared with pioglitazone and metformin (Fig. 1D and 2).

3.5. Serum cytokines measurements

The s.c. injection of formalin in mice resulted in significant increases in the serum levels of TNF-α, IL-6 and MCP-1 and a significant decrease in level of IL-10 compared to the NC group. All treatments significantly reversed the formalin-induced changes and fluoxetine showed a dose-dependent effect. Fluoxetine (10, 20, 40), pioglitazone, and metformin showed significant differences *versus* each other. The combination groups showed significant differences *versus* monotherapy groups while showed a non-significant difference in-between (Fig. 3).

3.6. Rotarod test

The fall-off latency time ranged from 77.93±3.85 to 84.54±6.16 second in all groups with non-significant differences among each other.

Table 1. Effect of various treatment regimens on the withdrawal latency time in the hot plate test done 60 min post-injection

Group	Withdrawal latency time (sec)	Percentage change
Control	**11.90 ± 0.80**	
Fluoxetine10 mg/kg	**10.93 ± 0.57**	**8.15**
Fluoxetine 20 mg/kg	**14.59 ± 2.24** [a]	**22.61**
Fluoxetine 40 mg/kg	**20.66 ± 1.08** [a]	**73.61**
Pioglitazone 20 mg/kg	**12.06 ± 0.98**	**1.34**
Metformin 50 mg/kg	**18.49 ± 0.7** [a]	**55.38**
Fluoxetine 20+Pioglitazone	**15.44 ± 1.73** [a,b]	**29.75**
Fluoxetine 20+Metformin	**26.15 ± 2.06** [a,c]	**119.75**

Withdrawal latency time is the time the mouse spent on the hot plate before licking its paw or jumping. All drugs were given intraperitoneally, n=8. Data are expressed as mean ± SD. Comparisons were made using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. [a] $p < 0.05$ vs. control (C), [b] $p < 0.05$: F20+P vs. F10, F40, and P, [c] $p < 0.05$: F20+M vs. all other therapy.

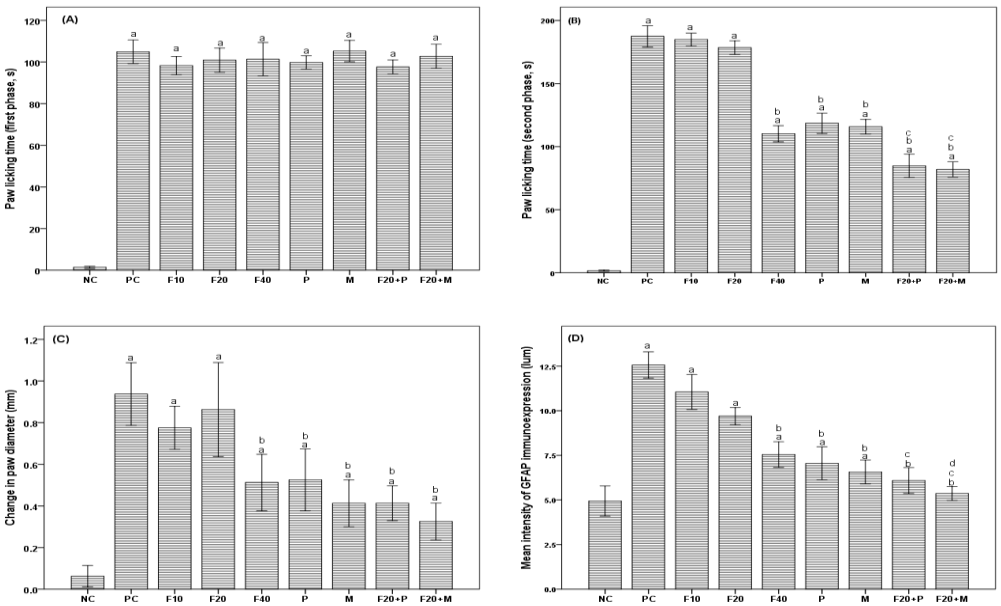

Figure 1. Effect of various treatment regimens on the licking time in seconds in the first (0-5 min) and second (20-25 min) phases of formalin test (A and B respectively), on the change in paw diameter (C), and on GFAP immunoexpression in mice (D)

The treated groups were given fluoxetine (F 10, 20 and 40), pioglitazone (P, 20), metformin (M, 50), fluoxetine (20)+pioglitazone (F20+P) and fluoxetine (20)+metformin (F20+M) (mg/kg, n=8), sixty min post-treatment , 20 µl of 1% formalin solution were injected subcutaneously into the plantar surface of left hind paw while the positive control (PC) was injected with saline. Data are expressed as mean ± SD. Comparison was made using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. [a] $p < 0.05$ vs. normal control (NC), [b] $p < 0.05$ vs. positive control (PC), [c] $p < 0.05$: F20+P & F20+M vs. F40, P and M, [d] $p < 0.05$: F20+M vs. F40.

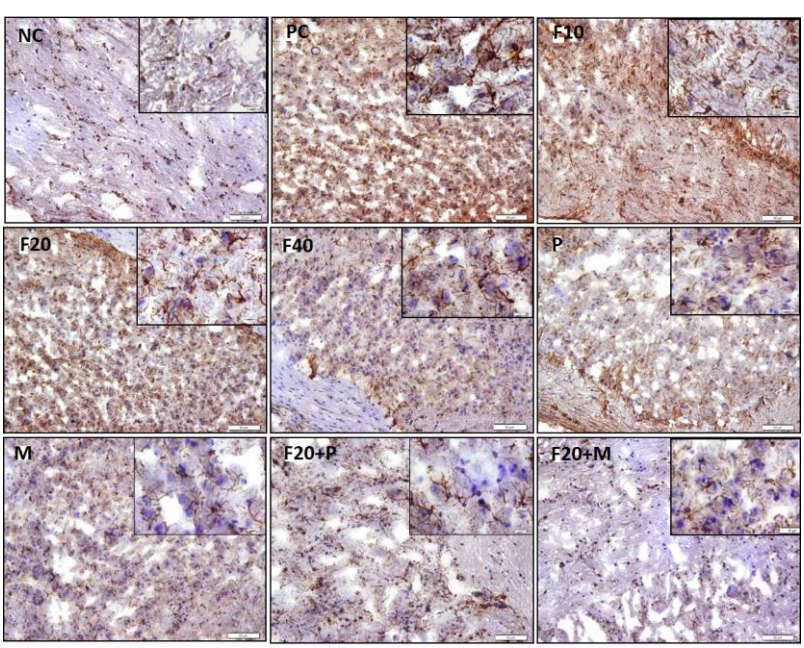

Figure 2. Photomicrographs of GFAP immunoexpression in the lumbar spinal cord two-hours after formalin test in mice (using an objective lens of ×20 at magnification GFAP X200, insert X600

Normal control shows some GFAP positive astrocytes with many processes. Positive control (formalin-injected) shows many astrocytes with increased GFAP immunoexpression. The treated groups (fluoxetine (10, 20, 40), pioglitazone, metformin, fluoxetine(20)+pioglitazone, and fluoxetine(20)+metformin) show inhibition of the formalin-induced increases in GFAP expression with nearly normal picture in the combination groups.

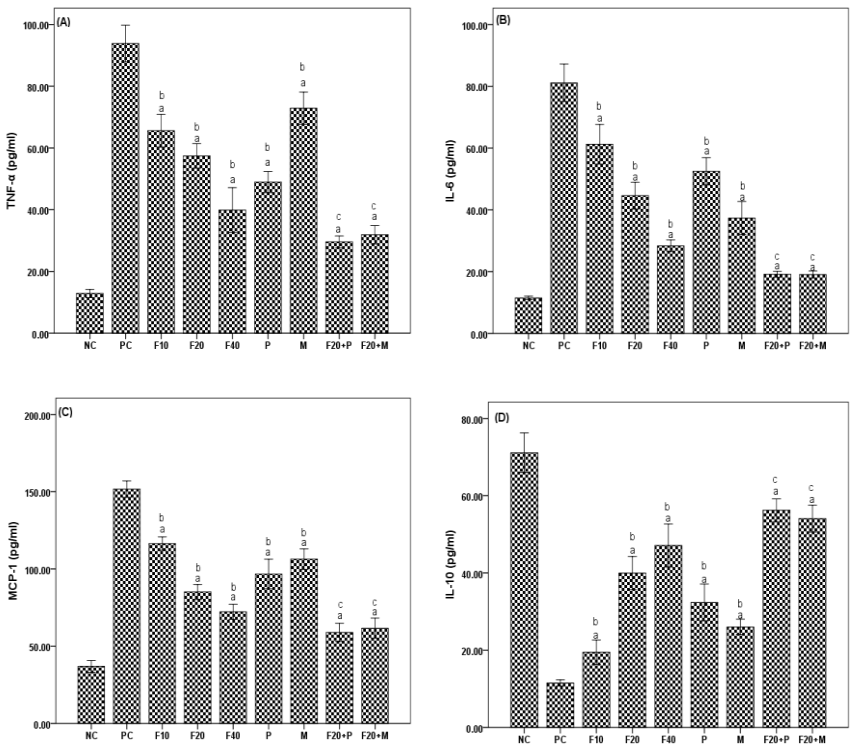

Figure 3. Effect of various treatment regimens on serum cytokines (pg/ml): (A) TNF-α, (B) IL-6, (C) MCP-1, and (D) IL-10

Drugs given were fluoxetine (F10, 20, 40), pioglitazone (P, 20), metformin (M, 50), fluoxetine (20)+pioglitazone (F20+P) and fluoxetine (20)+metformin (F20+M) (mg/kg, n=8). Data are expressed as mean ± SD. Comparison was made using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. [a] $p < 0.05$ vs. normal control (NC) & positive control (PC), [b] $p < 0.05$ monotherapy vs. each other, and [c] $p < 0.05$: F20+P & F20+M vs. monotherapy.

4. DISCUSSION

The current study showed that pretreatment with fluoxetine(20), fluoxetine(40), metformin, and the combinations significantly increased the latency time in the hot plate test (a model for acute pain). In addition, pretreatment with fluoxetine(40), pioglitazone, metformin and the combinations significantly decreased the licking time in the second phase of formalin test (a model for persistent pain). They also significantly decreased the formalin-induced paw edema and GFAP overexpression. All treatments significantly decreased the serum levels of tumor necrosis factor-α, interleukin-6 and monocyte chemoattactant protein-1 while increased the level of interleukin-10. In the rotarod test, all treatments did not affect motor function. These results are in accordance with the findings of previous studies. The injection of formalin into the hind paw of mice produces an immediate peripheral inflammation and a biphasic nociceptive behavior shown by lifting, licking, flinching, and even biting of the injected hind paw. The first (early) phase results from acute direct stimulation of peripheral nociceptors causing neurogenic non-inflammatory pain, while the second (late) phase results from inflammation and central sensitization causing an inflammatory or tonic pain (AlSharari *et al.,* 2012). Prostaglandins do not have an important role in the early phase while prostaglandins and inflammatory cytokines mediate the inflammatory response and pain in the late phase (Hunskaar and Hole, 1987). Formalin injection also increases the paw volume and modulates the levels of the pro- and anti-inflammatory mediators (Lin *et al.,* 2007). Moreover, it activates astrocytes in the spinal (ipsilateral) lumbar dorsal horn manifested by GFAP overexpression (Tanga *et al.,* 2004) and release of cytokines and prostaglandins (Romero-Sandoval *et al.,* 2008). The MCP-1 recruits macrophages and microglia contributing to the inflammatory and neuropathic pain conditions (Abbadie *et al.,* 2003). Release of cytokines and prostaglandins from the activated astrocytes occurs within 10 min post-formalin injection (Shi *et al.,* 2006) while astrocytic GFAP up-regulation occurs at 60 min or longer post-injection (Sun *et al.,* 2009). The GFAP overexpression at 2 h

post-formalin injection was significantly greater than that at 30 min post-injection and thus the 2 h post-injection time point is the most suitable time for measuring the changes in formalin-induced GFAP expression in the spinal cord (Fu *et al.,* 2007 and Kang *et al.,* 2011). The antinociceptive treatments reduce spinal GFAP expression in animal models of inflammatory or neuropathic pain (Sweitzer *et al.,* 1999 and Tsuda *et al.,* 2011).

The antinociceptive effects of the antidepressants are influenced by the type of the nociceptive stimulus whether chemical, mechanical, or thermal (Eschaller *et al.,* 1992). In the hot plate test in mice, fluoxetine (10 and 20 mg/kg) produced time-dependent antinociceptive effect up to three hours post-administration. The hot plate test is mediated through supraspinal centers (Abbott *et al.,* 1982). Loss of fluoxetine's antinociceptive effect in serotonin-depleted animals (Singh *et al.,* 2001) confirms involvement of serotoninergic pathways. In the formalin test i.p. fluoxetine (3-30 mg/kg given an hour before formalin injection) attenuated flinching in both phases of the test, however this attenuation was significant only with the highest dose in the second phase. The antinociceptive doses of fluoxetine did not affect the motor function up to 2 h post-administration (Pedersen *et al.,* 2005). Pretreatment with an intrathecal serotonergic neurotoxin in rats abolished the fluoxetine's modest antinociception in the second phase of formalin test indicating a role of central 5-HT level (Nayebi *et al.,* 2001). The 5-HT concentration in the dorsal horn of the rat spinal cord increases in the analgesic state (Nayebi and Ahmadiani, 1999). Central 5-HT mediates both the pronociceptive and antinociceptive actions at the spinal level through descending pathways from the rostroventral medulla (Suzuki *et al.,* 2004). Fluoxetine (20 mg/kg, i.p.) exerted a significant analgesic effect in the second phase of formalin test in mice; however, this effect was lower than its analgesic effect in the acute thermal pain model indicating that central 5-HT does not play the same role in the different pain conditions. The formalin-induced paw edema in mutant mice (lacking the central 5-HT system) did not significantly differ from that in control mice suggesting no role of central 5-HT in this respect (Zhao *et al.,* 2007). Fluoxetine therapy for seven days suppressed inflammation and decreased the production of TNF-α and IL-6 in arthritis (Sacre *et al.,* 2010). Peripheral 5-HT is mainly pronociceptive and thus has no role in the analgesic action of antidepressants (Sommer, 2004). In addition, mechanisms other than changes in 5-HT level may mediate effects of the antidepressants on inflammation. Fluoxetine decreased PGE_2 and substance P levels in inflammatory exudates denoting a possible role of local mediators in its anti-inflammatory effect (Bianchi *et al.,* 1994). A dose dependent anti-inflammatory effect of fluoxetine was manifested by reducing carrageenan-induced paw edema while sertraline dose-dependently enhanced edema denoting escalation of inflammation (Abdel-Salam *et al.,* 2003). A role of the opioid system is suggested to mediate the fluoxetine's peripheral anti-inflammatory effect (Abdel-Salam *et al.,* 2004). Further, in animal models of pain inhibition of reuptake of both 5-HT and noradrenaline causes more antinociceptive effect than selective inhibition of 5-HT (Pedersen *et al.,* 2005).

Pioglitazone (1-50 mg/kg, i.p.) suppressed the second phase of formaldehyde test and carrageenan-induced paw edema in mice, but it did not inhibit the nociceptive response in the hot plate test and the first phase of formaldehyde test (Oliveira *et al.,* 2007). The anti-inflammatory and antinociceptive activity of pioglitazone may be due to inhibition of production of inflammatory mediators such as prostaglandins and nitric oxide, TNF-α, and IL-6 (Chichorro *et al.,* 2004). A role of central PPAR-γ receptors is also suggested (Shibata *et al.,* 2008). Pioglitazone decreased expression of TNF-α suggesting an inhibitory effect on glial cell proinflammatory activities (Duvanel *et al.,* 2003). Pioglitazone (1-10 mg/kg/day for 7 days) reduced the increased expression of GFAP in the spinal dorsal horn in the nerve injury model and thus reduced the neuropathic pain. Block of this effect by a PPARγ antagonist confirms PPARγ dependence. Pioglitazone neither affect acute thermal nociception nor motor function (Morgenweck *et al.,* 2013). AMPK activation suppresses inflammation through decreasing cytokines such as IL-6 and TNF-α in primary astrocytes, microglia, and macrophages (Giri *et al.,* 2004). It was reported that the spinal dorsal horn mTOR pathway has an important role in preclinical pain models (Xu *et al.,* 2011). The antinociceptive effect of metformin may be attributed to a central mechanism (Labuzek *et al.,* 2010) where it activates AMPK in the spinal cord and inhibit central mTOR pathways (Melemedjian *et al.,* 2011). Metformin (100 mg/kg) significantly inhibited the second phase of the formalin test and zymosan-evoked paw edema without affecting the motor function in the rotarod test (Russe *et al.,* 2013). In rats, metformin (100-300 mg/kg) given orally 30 min before formalin injection did not affect the nociceptive response in the first phase, but affected it in the second phase (Montes *et al.,* 2012). In addition, metformin showed a significant antinociceptive effect in the hot plate test in rats with diabetic neuropathy (Kumar *et al.,* 2012 and Nagilla and Reddy, 2014). Moreover, metformin activated AMPK, suppressed the inflammatory responses and decreased serum TNF-α and IL-6 (Soraya *et al.,* 2014).

In conclusion, metformin significantly inhibited the nociceptive behavior in the hot plate test while pioglitazone failed to affect it. Moreover, combination of metformin with low-dose fluoxetine most effectively decreased the formalin-induced inflammatory and nociceptive changes. Metformin is a safe widely-used drug, thus its combination with low dose-fluoxetine might represent a potential treatment option for patients having acute or persistent pain and diabetes and/or depression.

5. ACKNOWLEDGEMENT

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