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Experimental Trial

Co-Administration of Pioglitazone Improves Fluoxetine's Antinociceptive, Neuroprotective, and Antidepressant Effects in Chronic Constriction Injury in Rats

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Background: Chronic pain may be associated with diabetes mellitus and/or depression. Use of therapies that target both comorbidities is encouraged.

Objective: This study was designed to investigate the potential antinociceptive, neuroprotective, and antidepressant effects of combinations of pioglitazone or metformin with fluoxetine in chronic constriction injury (CCI) in rats.

Study Design: Experimental trial in rats.

Setting: University lab in Saudia Arabia.

Methods: Two sets of experiments were performed. In each one, 9 groups of rats (n = 8) were used: sham, CCI, and 7 CCI-treated groups. Treatments were given orally starting on day 7 post-surgery as follows (mg/kg/day): fluoxetine (10, 20, and 40), pioglitazone (20), metformin (50), fluoxetine (20) + pioglitazone, and fluoxetine (20) + metformin. In the first set, on day 14 post-surgery mechanical allodynia, thermal hyperalgesia, and serum cytokines were measured. Moreover, immunoreactivity of glial fibrillary acidic protein (GFAP, a marker for astrocytic activation) in the spinal cord was assessed and histopathological changes in the ipsilateral sciatic nerve were examined. In the second set, on days 14 and 21 post-surgery the forced swimming test was done.

Results: In the first set, all treatments significantly decreased mechanical allodynia while all treatments except F10 and F20 significantly decreased thermal hyperalgesia compared to the CCI group. The F20+M group showed the highest decreases, however still significantly lower than those of the sham group. The treatments didn't impair motor function in the rotarod test. All treatments significantly decreased serum levels of tumor necrosis factor-α, interleukin-6, and monocyte chemoattractant protein-1 while increasing the level of interleukin-10. The CCI-induced marked increase of GFAP immunoexpression has been reduced to moderate with fluoxetine (40) and pioglitazone, and to mild with metformin and the combination groups. The CCI-induced changes in sciatic nerve were less in fluoxetine (40), pioglitazone, and metformin groups, and least in the combination groups. In the second set, the immobility duration was significantly reduced by F20, F40, P, F20+P, and F20+M compared to the CCI group. The F20+P group showed the highest decrease, however still significantly lower than that of the sham group. The treatments didn't affect locomotor activity in the open field test.

Limitations: Measuring the cytokines levels only in blood and not in the spinal cord and sciatic nerve and measuring the outcome measures in the first set of experiments at only one time-point.

Conclusions: Co-administration of pioglitazone or metformin with low-dose fluoxetine improved mechanical allodynia, thermal hyperalgesia, and neurohistopathological changes while co-administration of pioglitazone, but not metformin, improved the depressive-like behavior in the peripheral nerve injury model of neuropathic pain in rats. Extrapolation of the current results to clinical reality could be beneficial for pain patients with diabetes and/or depression, however this needs further confirmatory studies.

Key words: Antidepressant, antinociceptive, chronic constriction injury, fluoxetine, GFAP, metformin, neuroprotective, pain, pioglitazone, sciatic

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Chronic neuropathic pain due to peripheral nerve injury, manifested as hyperalgesia and allodynia, occurs through increased excitability in nociceptive pathways, constant stimulation of spinal glial cells causing central sensitization (1,2), and inflammatory cell infiltration into the nerve (3). Activation of nuclear factor kappa B (NF-κB) pathway (a chief element in glial activation) and involvement of different immune cells result in release of proinflammatory cytokines which trigger release of pain-producing substances such as prostaglandins and bradykinins (4). Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1(MCP-1) act as proinflammatory cytokines, while interleukin-10 (IL-10) acts as an anti-inflammatory cytokine (5).

Activation of adenosine monophosphate-activated protein kinase (AMPK) has been involved in relieving pain mainly through suppression of mammalian target of rapamycin (mTOR) pathway (6). Being managed by activation rather than inhibition, AMPK is a good target for treatment of chronic pain (7). AMPK activation also increases glucose uptake and improves hyperglycemia. Pioglitazone and metformin, widely-used oral antidiabetics, activate AMPK through different signaling pathways (8). Pioglitazone has important neuroprotective, antioxidant, and anti-inflammatory properties independent of its insulin-sensitizing effect (9). Metformin has anti-inflammatory and antinociceptive effects in models of inflammatory nociception (10). Pain is a common complaint in diabetic patients (11) and they are more liable to depression (12). Moreover, chronic pain is usually associated with depression (13). Fluoxetine, a selective serotonin reuptake inhibitor antidepressant, has antinociceptive effects in persistent and neuropathic pain in animals through 5-HT_{2A} receptor stimulation (14). Research investigating use of therapies that target both pain and its comorbidities is encouraged (15).

The chronic constriction injury (CCI) is a commonly used model of chronic neuropathic pain in rats. It has both inflammatory and peripheral nerve injury components. It mimics neuropathic pain in humans and thus it is a useful model for study of neuropathic pain and its treatment (16,17).

Methods

Animals and Experimental Design

This work was designed to investigate the antinociceptive, neuroprotective, and antidepressant effects of combinations of pioglitazone or metformin with fluoxetine in a CCI model in rats. The protocol was approved by the Institutional Research Ethics Committee and adhered to the National Institute of Health guidelines for the Care and Use of Laboratory Animals. All drugs and chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless mentioned otherwise.

In the first set of experiments, 72 male Sprague-Dawley rats (300 – 350 g) were used, 64 rats were subjected to CCI operation while 8 rats were subjected to the same procedure except for application of ligatures (sham, negative control group, received saline). On day 7 post-CCI, mechanical allodynia and thermal hyperalgesia were tested to confirm successful model creation. Thereafter, CCI rats were randomly divided into 8 groups (n = 8) treated by oral gavage as follows (mg/kg/day): CCI (saline, positive control), F10, F20, F40 (fluoxetine 10, 20, 40) (18,19), P (pioglitazone 20) (20), M (metformin 50) (21), F20+P (fluoxetine 20 + pioglitazone), and F20+M (fluoxetine 20 + metformin) groups. On day 14 post-CCI, mechanical allodynia and thermal hyperalgesia were reassessed. Later, blood was collected for measurements of serum levels TNF-α, IL-6, MCP-1, and IL-10. Finally, rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and sacrificed. The spinal cord was harvested for immunohistochemical study of glial fibrillary acidic protein (GFAP, a marker for activation of astrocytes) and the ipsilateral sciatic nerve was excised for histopathological examination. In order to exclude any possible effect of the treatments on the motor function, which might affect the nociceptive behavioral results, the rotarod test was done on naïve rats (ones without surgery or previous exposure to the rotarod test).

In the second set of experiments, another 9 groups of rats were used (exactly as mentioned before) to examine the effects of the tested drugs on the depressant behavior in a CCI model using the forced swimming test (FST). In order to exclude any possible effect of surgery and/or treatments on the locomotor activity, which might affect performance, the open field test (OFT) was done immediately before FST. The FST was done on day 7 post-CCI (to confirm development of depression), and on days 14 and 21 post-CCI (after one- and 2-week therapy) (22).

The Chronic Constriction Injury Model

Under anesthesia with pentobarbital sodium, a skin incision was made 3 mm below and parallel to

the left femur. Three loose ligatures (using chromic gut 50) were applied around the sciatic nerve. Within one week, rats developed mechanical allodynia and thermal hyperalgesia on the ipsilateral hindpaw (16,17,23).

Mechanical Allodynia

Rats were positioned on a mesh floor covered by a transparent Plexiglas chamber. After 30 minutes of habituation, 9 von Frey filaments (Stoelting, Kiel, USA) equivalent to 0.6, 1, 1.4, 2, 4, 6, 8, 10, and 15 g bending forces were introduced in an ascending way to the mid plantar skin surface of the ipsilateral hindpaw. Each filament was applied with sufficient force to bend for 5 seconds (repeated at 5-minute intervals). The paw mechanical withdrawal threshold (MWT, g) was defined as the least filament size that caused at least 3 withdrawal responses in 5 measurements. A cutoff value was defined as 15 g because more force could cause paw lifting (24).

Thermal Hyperalgesia

Rats were placed in a Plexiglas chamber over an elevated transparent glass surface. After 30 minutes of habituation, the mid plantar surface of ipsilateral hindpaw was exposed to a beam of a light source beneath the floor at least 3 times at 5-minute intervals. The thermal withdrawal latency (TWL) was recorded as the time for paw withdrawal. A cutoff time of 40 seconds was used to avoid tissue damage (25).

Rotarod Test

A control (C, untreated, saline) group and 7 groups (treated as previously mentioned) (n = 8) were utilized. Fall-off latency (time spent until the rat fell off from the rotarod) was calculated (26).

Serum Measurements

Commercially available ELISA kits for TNF-α (Koma Biotech Inc., Korea), IL-6 (Anogen, Canada), MCP-1 (Thermo Scientific, USA), and IL-10 (DRG International Inc., USA) were used.

Immunohistochemistry of the Spinal Cord

The activity and distribution of astrocytes were examined by measuring the immunoexpression of GFAP in the lumbar region of the spinal cord (27). On day 14 post-CCI the spinal cord at the lumbar segments level was removed, formalin-fixed, and paraffin-embedded (28). Sections were processed as previously described (29). The primary anti-GFAP antibody (anti-rat goat polyclonal IgG, Dako Cytomation, USA) was used at a dilution of 1:1000. Sections were counterstained with hematoxylin. Negative controls were performed by substituting the primary antibody with non-specific IgG. Cells containing GFAP (e.g., astrocytes) appeared brown and the nuclei appeared blue. The scale set by Colburn et al (30) was used: "Score (0); resting state; unperturbed astrocytes showing extensive fine projections, well-spaced, and neatly arranged. Score (+); mild response; astrocytes exhibit numerous long but thickening projections, less area between individual astrocytes, and more apparent GFAP immunoreactivity. Score (++); moderate response; astrocytes are less ramified/exhibit bold projections, increased density of cells/occasionally overlapping, and prominent GFAP immunoreactivity. Score (+++); intense response; astrocytes are rounded with few projections, densely arranged/overlapping, and intense GFAP immunoreactivity." A computerized image analyzer system (Pro Plus image analysis software version 6.0) connected to an Olympus microscope BX-51 with a digital camera connected to a computer was used for photographing and the morphometric study. Mean intensity (MI) of GFAP immune reaction was measured using an objective lens of x20 at magnification x100. Ten readings from 5 non-overlapping sections from each rat were examined.

Histologic Examination of the Sciatic Nerve

The left sciatic nerve was resected. Samples were fixed with 4% paraformaldehyde, and then a piece of the nerve was post-fixed with osmium tetroxide to examine the myelin sheath while the other piece was left without post-fixation. Both pieces were dehydrated and embedded in paraffin. Cross and longitudinal sections were stained with hematoxylin and eosin (HE) while those post-fixed in osmic acid was left unstained. Samples were examined using light microscopy regarding abundance, size, and arrangement of nerve fibers. The state and thickness of epi-, peri- and endo-neurium, and integrity of the myelin sheath were examined (28).

Forced Swimming Test

The FST is a useful test for studying the antidepressant effects. Rats were placed in a glass cylinder (20 cm diameter and 45 cm high) filled with 30 cm deep water (at 25°C). A 15-minute training session was done 24 hours before the test. During FST, rats were placed in water for 6 minutes and the duration of immobility

(described as making "only those movements essential to keep head above water") during the last 4 minutes was recorded (31,32).

Open Field Test

The locomotor activity was recorded in an Opto-Varimex apparatus (Columbus Instruments, USA). The travelled distance and the number of vertical movements were measured during 30 minutes (33).

Statistical Analysis

Data were given as mean values ± SD. Student's T-test and ANOVA with Bonferroni post-hoc test were used for 2 groups' and more than 2 groups' comparison, respectively. SPSS 18.0 was used. *P* values of < 0.05, < 0.01, and < 0.001 indicated grades of significance.

Results

Mechanical Allodynia and Thermal Hyperalgesia

The ipsilateral hind paws in all CCI groups on day 7 post-surgery showed significantly reduced MWT (from 2.46 ± 0.34 to 3.18 ± 0.41) and TWL (from 13.25 ± 2.07 to 16.03 ± 1.60) compared to sham rats (12.91 ± 0.78 and 31.38 ± 2.58, respectively) indicating successful model induction. On day 14 post-CCI all treatments significantly increased MWT compared to the CCI group, and fluoxetine showed dose-dependent effects. All treatments (except F10 and F20) significantly increased TWL compared to the CCI group, and F40 and M showed significant differences versus F20. The F20+M group showed the highest increases, however still significantly lower than those of sham group (Table 1).

Rotarod Test

There were non-significant differences among all groups indicating that treatments didn't impair motor function (fall-off latencies ranged from 119.84 ± 2.92 to 126.71 ± 2.12).

Serum Cytokines

The CCI rats showed significant increases in serum levels of TNF-α, IL-6, and MCP-1 and a significant decrease in the level of IL-10 compared to sham group. All treatments significantly reversed these changes mainly with combination groups, and fluoxetine showed dose-dependent effects (Table 2).

GFAP Immunoexpression in the Spinal Cord

The sham group showed GFAP immunoexpression in astrocytes having many fine processes (score 0). The astrocytic activity increased in the CCI group (score 3) and MI of GFAP immunoexpression significantly increased. The extent of CCI-induced changes were nearly the same in the F10 and F20 groups, significantly reduced to moderate activity in the F40 and P groups, and significantly reduced to mild activity in the M, F20+P, and F20+M groups. P and M caused more significant reductions than F40, and the combinations showed the most significant reductions (Table 3 and Fig. 1).

Histopathological Changes in the Sciatic Nerve

The cross and longitudinal sections of the sham group showed abundant nerve fibers with large axons and a thick myelin sheath, and the epi-, peri- and endoneurium appeared intact. The CCI group showed fewer nerve fibers, smaller axons, some degenerated myelin

Table 1. *Effects of treatments (mg/kg/day) on mechanical allodynia (MWT, g) and thermal hyperalgesia (TWL, s) in rats on day 14 post-CCI.*

Group	Sham	CCI	F10	F20	F40	P	M	F20+P	F20+M
MWT	13.06 ± 0.70	2.44 ± 0.25***	3.91 ± 0.35$^{***,!!}$	5.18 ± 0.59$^{***,!!!,\dagger}$	6.99 ± 1.02$^{***,!!!,\dagger\dagger\dagger}$	7.05 ± 0.93$^{***,!!!,\dagger\dagger\dagger}$	7.50 ± 0.65$^{***,!!!,\dagger\dagger\dagger}$	9.79 ± 0.67$^{***,!!!,\wedge\wedge\wedge}$	10.25 ± 0.76$^{***,!!!,\wedge\wedge\wedge}$
TWL	33.08 ± 1.92	13.25 ± 2.07$^{\#\#\#}$	14.55 ± 2.23$^{\#\#\#}$	15.71 ± 1.28$^{\#\#\#}$	20.19 ± 2.38$^{\#\#\#,+++,\ddagger\ddagger,\ddagger}$	19.91 ± 1.58$^{\#\#\#,+++,\ddagger\ddagger}$	21.60 ± 2.77$^{\#\#\#,+++,\ddagger\ddagger\ddagger,\ddagger\ddagger}$	25.83 ± 4.12$^{\#\#\#,+++,xxx,xx}$	25.99 ± 3.35$^{\#\#\#,+++,xxx,xx,x}$

CCI (chronic constriction injury), F (fluoxetine 10, 20, 40), P (pioglitazone 20), M (metformin, 50), F20+P (fluoxetine (20) + pioglitazone), F20+M (fluoxetine (20) + metformin) (n = 8). Data are expressed as mean ± SD.

$^{***}P < 0.001$: all groups vs. sham, $^{!!!}P < 0.001$: all groups (except F10) vs. CCI, $^{!!}P < 0.01$: F10 vs. CCI ($P = 0.003$), $^{\dagger\dagger\dagger}P < 0.001$: (F40, P & M) vs. (F10 & F20), $^{\dagger}P < 0.05$: F20 vs. F10 ($P = 0.021$), $^{\wedge\wedge\wedge}P < 0.001$: combination groups vs. (F10, F20, F40, P & M).

$^{\#\#\#}P < 0.001$: all groups vs. sham, $^{+++}P < 0.001$: all groups (except F10 & F20) vs. CCI, $^{\ddagger\ddagger\ddagger}P < 0.001$: M vs. F10, $^{\ddagger\ddagger}P < 0.01$: F40 & P vs. F10 ($P = 0.001$ & 0.003), and M vs. F20 ($P = 0.001$), $^{\ddagger}P < 0.05$: F40 vs. F20 ($P = 0.03$), $^{xxx}P < 0.001$: F20+P vs. (F10 & F20), and F20+M vs. (F10, F20 &P), $^{xx}P < 0.01$: F20+P vs. (F40 & P) ($P = 0.001$ & 0.001), and F20+M vs. F40 ($P = 0.001$), $^{x}P < 0.05$: F20+M vs. M ($P = 0.037$).

Table 2. *Effects of treatments (mg/kg/day) on serum cytokines (pg/ml): tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1(MCP-1), and interleukin-10 (IL-10) in rats on day 14 post-CCI.*

Group	TNF-α	IL-6	MCP-1	IL-10
Sham	39.86 ± 5.92	19.31 ± 1.47	53.73 ± 3.01	81.45 ± 4.63
CCI	139.68 ± 7.50	98.88 ± 3.48^{fff}	179.25 ± 5.66	16.71 ± 1.48^{ooo}
F10	113.94 ± 6.03^{aaa}	87.10 ± 7.67^{fff}	143.74 ± 12.30	25.10 ± 2.68 ^{ooo,pp}
F20	102.90 ± 6.14^{aaa,bb}	75.05 ± 6.23^{fff,ggg}	112.96 ± 6.97^{kkk}	33.64 ± 4.93 ^{ooo,ppp,qq}
F40	91.46 ± 3.49^{aaa,bbb,cc}	64.71 ± 6.75^{fff,ggg,hh}	102.26 ± 4.87 ^{kkk,l}	41.29 ± 3.75 ^{ooo,ppp,qqq,rr}
P	80.03 ± 6.41^{aaa,bbb,ccc,dd}	53.73 ± 3.01^{fff,ggg,hhh,ii}	91.71 ± 3.42 ^{kkk,lll,m}	47.60 ± 4.72 ^{ooo,ppp,qqq,rrr}
M	69.76 ± 3.35^{aaa,bbb,ccc,ddd,d}	45.65 ± 5.10^{fff,ggg,hhh,iii}	80.50 ± 2.75 ^{kkk,lll,mmm,mm}	56.36 ± 4.53 ^{ooo,ppp,qqq,rrr,rr}
F20+P	57.65 ± 6.26^{aaa,dd,eee}	33.64 ± 4.93^{fff,ggg,jjj}	70.50 ± 2.31 ^{kkk,nnn,n}	67.51 ± 3.56 ^{ooo,ppp,sss}
F20+M	54.31 ± 2.74^{aaa,eee}	29.58 ± 1.93^{ff,ggg,jjj}	68.59 ± 3.48 ^{kkk,nnn,nn}	70.60 ± 2.41 ^{ooo,ppp,sss}

CCI (chronic constriction injury), F (fluoxetine 10, 20 and 40), P (pioglitazone 20), M (metformin 50), F20+P (fluoxetine (20) + pioglitazone), F20+M (fluoxetine (20) + metformin) (n = 8). Data are expressed as mean ± SD.
$^{aaa}P < 0.001$: all groups vs. sham & CCI, $^{bbb}P < 0.001$: (F40,P &M) vs. F10, $^{bb}P < 0.01$: F20 vs. F10 ($P = 0.006$), $^{ccc}P < 0.001$: (P &M) vs. F20, $^{cc}P < 0.01$: F40 vs. F20 ($P = 0.004$), $^{ddd}P < 0.001$: M vs. F40, $^{dd}P < 0.01$: P vs. F40 ($P = 0.004$), and F20+P vs. M ($P = 0.002$), $^{d}P < 0.05$: M vs. P ($P = 0.016$), $^{eee}P < 0.001$: F20+P vs. (F10, F20, F40 & P) and F20+M vs. (F10, F20, F40, P & M).
$^{fff}P < 0.001$: all groups (except F20+M) vs. sham, $^{ff}P < 0.01$: F20+M vs. sham ($P = 0.004$), $^{ggg}P < 0.001$: all groups vs. (CCI & F10), $^{hhh}P < 0.001$: (P & M) vs. F20, $^{hh}P < 0.01$: F40 vs. F20 ($P = 0.003$), $^{iii}P < 0.001$: M vs. F40, $^{ii}P < 0.01$: P vs. F40 ($P = 0.001$), $^{jjj}P < 0.001$: (F20+P & F20+M) vs. (F10, F20, F40, P & M).
$^{kkk}P < 0.001$: all groups vs. (sham, CCI, F10), $^{lll}P < 0.001$: (P & M) vs. F20, $^{l}P < 0.05$: F40 vs. F20 ($P = 0.016$), $^{mmm}P < 0.001$: M vs. F40, $^{m}P < 0.05$: P vs. F40 ($P = 0.019$), $^{mm}P < 0.01$: M vs. P ($P = 0.009$), $^{nnn}P < 0.001$: (F20+P & F20+M) vs. (F20, F40 & P), $^{n}P < 0.05$: F20+P vs. M ($P = 0.036$), $^{nn}P < 0.01$: F20+M vs. M ($P = 0.004$).
$^{ooo}P < 0.001$: all groups vs. sham, $^{ppp}P < 0.001$: all groups (except F10) vs. CCI, $^{pp}P < 0.01$: F10 vs. CCI ($P = 0.002$), $^{qqq}P < 0.001$: (F40, P & M) vs. F10, $^{qq}P < 0.01$: F20 vs. F10 ($P = 0.001$), $^{rrr}P < 0.001$: (P & M) vs. (F20), and M vs. (F40), $^{rr}P < 0.01$: F40 vs. F20 ($P = 0.006$), and M vs. P ($P = 0.001$), $^{sss}P < 0.001$: (F20+P & F20+M) vs. (F10, F20, F40, P & M).

Table 3. *Effects of treatments (mg/kg/day) on mean intensity of GFAP immunoexpression (lum) in the lumbar spinal cord in rats on day 14 post-CCI.*

Group	Sham	CCI	F10	F20	F40	P	M	F20+P	F20+M
GFAP	5.34 ± 0.27	14.05 ± 0.55$^{!!!}$	13.50 ± 0.37$^{!!!}$	13.33 ± 0.76$^{!!!}$	12.93 ± 0.84$^{!!!,\#}$	12.66 ± 0.71$^{!!!,\#\#}$	9.08 ± 0.62$^{!!!,\#\#\#,\wedge\wedge\wedge}$	6.05 ± 0.6$^{6\#\#\#,***}$	5.85 ± 0.24$^{\#\#\#,***}$

CCI (chronic constriction injury), GFAP (glial fibrillary acidic protein) F (fluoxetine 10, 20 and 40), P (pioglitazone 20), M (metformin 50), F20+P (fluoxetine (20) + pioglitazone), F20+M (fluoxetine (20) + metformin) (n = 8). Data are expressed as mean ± SD.
$^{!!!}P < 0.001$: all groups (except the combinations) vs. sham, $^{\#\#\#}P < 0.001$: M and combination groups vs. CCI, $^{\#\#}P < 0.01$: P vs. CCI ($P = 0.001$), $^{\#}P < 0.05$: F40 vs. CCI ($P = 0.013$), $^{\wedge\wedge\wedge}P < 0.001$: M vs. (F10, F20, F40 & P), $^{***}P < 0.001$: combination groups vs. (F10, F20, F40, P & M).

sheaths, many Schwann-cell nuclei, inflammatory cell infiltrate, and increased thickness of the peri- and endo-neurium. The extent of CCI-induced changes were nearly the same in F10 and F20, less in F40, pioglitazone, and metformin, and least in combination groups which showed small nerve fibers suggesting regeneration of myelin sheaths (Figs. 2 and 3).

FST and OFT

On day 7 post-surgery, all CCI groups showed significantly increased immobility duration (from 185.88 ± 8.48 to 197.38 ± 2.62) compared to sham rats (122.13 ± 9.01), indicating development of depression-like behavior. On day 14 post-CCI, there were insignificant changes in the immobility duration indicating failure of one week-therapy to reverse depression. On day 21 post-CCI (Table 4) the immobility duration was significantly reduced by F20, F40, P, F20+P, and F20+M compared to the CCI group. The F20+P group showed the highest decrease, however still significantly lower than that of sham group. F10 and M failed to exert any effect while F20 and F40 showed non-significant differences in-between. In OFT on days 7, 14, and 21 days post-CCI, there were non-significant differences in locomotor activity among all groups. The distance travelled ranged from 2147.00 ± 455.10 to 2618.75 ± 252.1 cm, and the

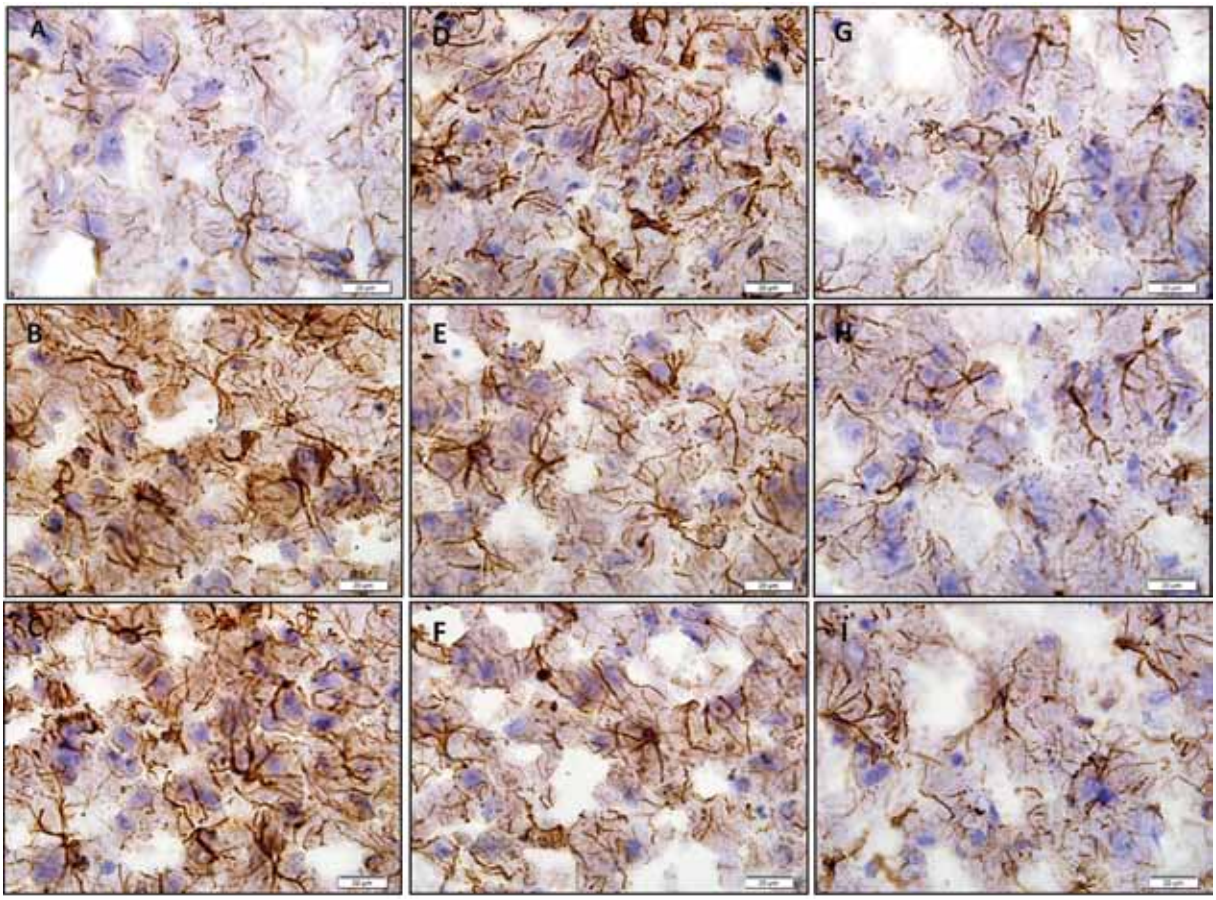

Fig. 1. *Immunoexpression of glial fibrillary acidic protein (GFAP) in the lumbar spinal cord in CCI rats. (A) Negative control showing score 0. (B, C & D) Positive control, fluoxetine (10), and fluoxetine (20) groups showing score (+++). (E & F) Fluoxetine (40) and pioglitazone groups showing score (++). (G, H & I) Metformin, fluoxetine (20) + pioglitazone, and fluoxetine (20) + metformin groups showing mild score (+) (GFAP immnuostaining x1000).*

number of vertical movements ranged from 144.00 ± 12.24 to 168.25 ± 7.31.

Discussion

Pain hypersensitivity to mechanical and thermal stimuli could persist for at least 3 weeks post-CCI (17,34,35). Following sciatic nerve injury, IL-6 mRNA in spinal cord neurons and IL-6 levels in plasma and the sciatic nerve increased (36). The immune cells producing IL-6 are sensitive to low doses of analgesics and the decreased production of IL-6 may partially mediate analgesics' effects in CCI animals (37). The spinal TNF-α protein level increased in CCI rats, however serum levels of TNF-α and IL-6 remained unchanged. Rapamycin reversed the increased spinal TNF-α level and exerted antihyperalgesic and antiallodynic effects (38). AMPK activation reduced proinflammatory cytokines in primary astrocytes, microglia, and macrophages (39). After sham operation, IL-10 protein increased in the lumbar dorsal root ganglia (40). In addition, CCI rats showed prolonged immobility duration in the FST and normal unimpaired locomotor activity (41-43). CCI mice developed thermal hyperalgesia and prolonged immobility time in the FST (44).

The GFAP expression in astrocytes has been associated with chronic pain behaviors (45). In the current study, the CCI markedly increased GFAP immunoexpression in the lumber spinal cord grey matter in agreement with the findings from previous studies (28,30,46,47). This increase was attributed to hypertrophy of astrocytes rather than proliferation or migration (30,47). Examination of the sciatic nerve in CCI rats showed re-

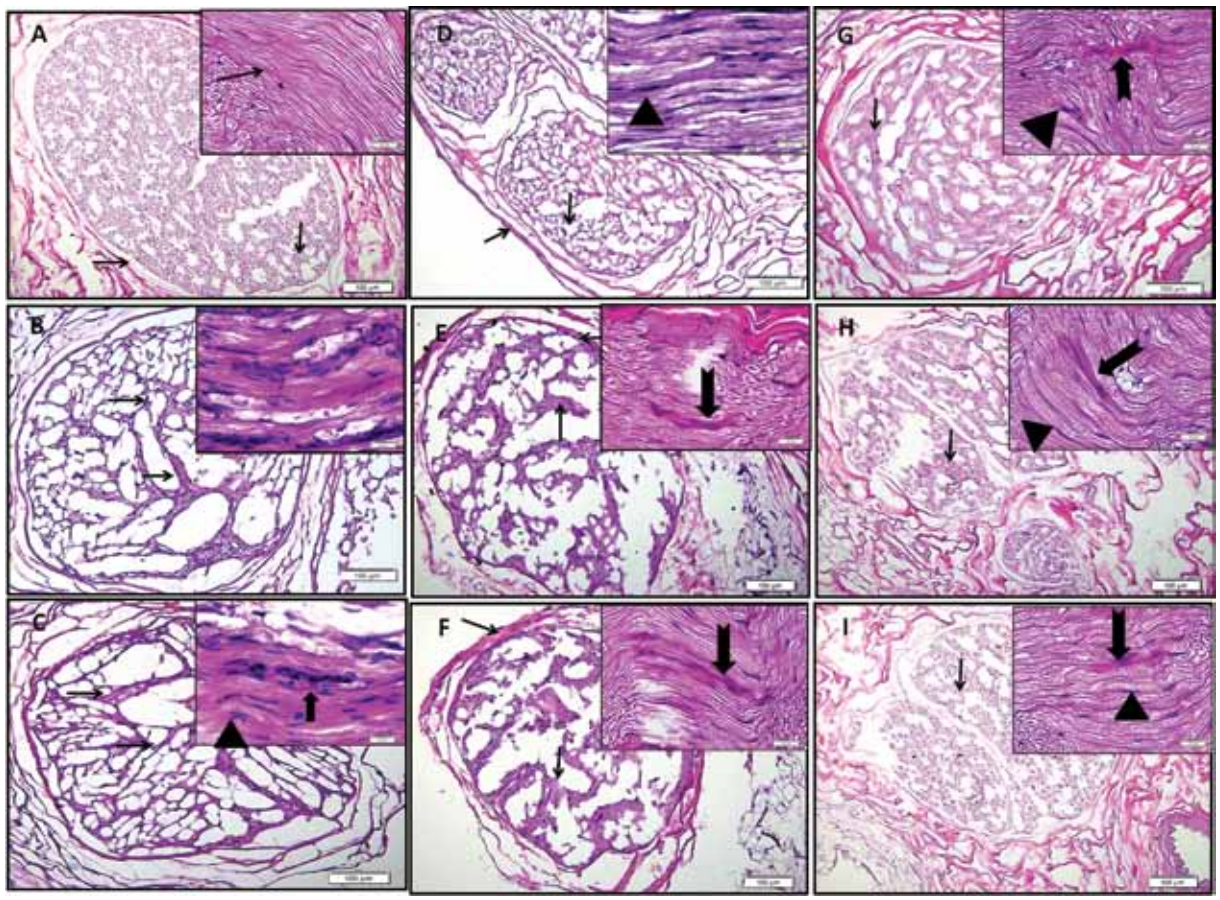

Fig. 2. *Cross sections and longitudinal inserts of the ipsilateral sciatic nerve in CCI rats. (A) Negative control showing abundant nerve fibers with tidy arrangement, and intact epi-, peri-, and endo-neurium (thin arrow). (B, C, D & E) Positive control, fluoxetine (10), fluoxetine (20), and fluoxetine (40) groups showing fewer nerves, increased thickness of epi- and endo-neurium (thin arrow), inflammatory cell infiltrate (thick arrow), many Schwann-cell nuclei (arrow head), and scar tissue (bifid arrow). (F, G, H & I) pioglitazone, metformin, fluoxetine (20) + pioglitazone, and fluoxetine (20) + metformin groups showing slightly increased number of nerve fibers (HE, x 400, insert x1000).*

duced number of nerve fibers, nerve degeneration, and demyelination in agreement with the findings from Murakami et al (28). There were inflammation-induced edema between nerve fibers and infiltration of inflammatory cells (48). CCI-treated animals showed more epi- and endo-neurial integrity, more tidy-arranged fibers, less inflammation, and less degeneration of myelinated fibers (49).

Oral fluoxetine produced antiallodynic effects without altering locomotor activity in CCI mice (22). The increased 5-HT level in rat spinal dorsal horn and loss of fluoxetine's antinociceptive effect in 5-HT-depleted animals confirm involvement of serotoninergic pathways (50). Fluoxetine for 7 days suppressed inflammation and decreased levels of TNF-α and IL-6 in murine and human arthritis (51). Fluoxetine attenuated mechanical allodynia and thermal hyperalgesia without affecting motor function in CCI rats. However, antidepressants inhibiting reuptake of both 5-HT and noradrenaline were more effective (19). Central 5-HT mediates both pronociceptive and antinociceptive actions at the spinal level through descending pathways (52). Peripheral 5-HT is mainly pronociceptive and thus not involved in the antidepressants' analgesia (53). In CCI rats, chronic fluoxetine treatment (10 and 30 mg/kg for 14 days) reduced the immobile behavior in FST (43). The locomotor activity didn't vary between sham, CCI, and CCI-fluoxetine-treated mice (22). In CCI mice, acute oral

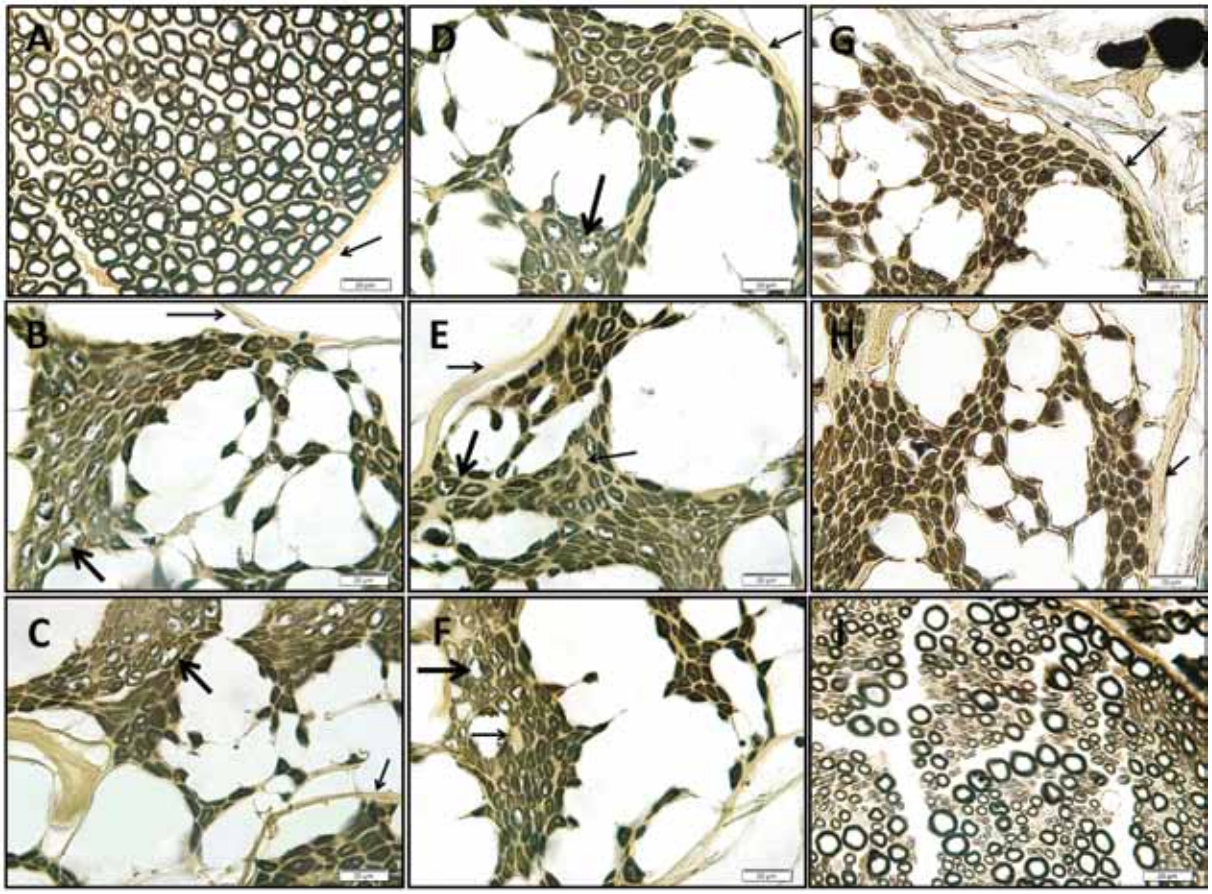

Fig. 3. *Cross sections of the ipsilateral sciatic nerve post-fixed in osmic acid in CCI rats. (A) Negative control showing abundant nerve fibers with large axons and thick myelin (black rings). (B, C & D) Positive control, fluoxetine (10), and fluoxetine (20) groups showing fewer nerves with smaller axons, degeneration in some myelin sheaths (thick arrow), and increased epi- and endo-neurium connective tissue (thin arrow). (E, F, G & H) Fluoxetine (40), pioglitazone, metformin, fluoxetine (20) + pioglitazone groups showing the same changes but to less extent. (I) fluoxetine (20) + metformin group showing large number of intact nerve fibers as well as many smaller ones (osmic acid x1000).*

fluoxetine exerted an antiallodynic effect but failed to decrease immobility time in FST indicating dissociation between the antiallodynic and antidepressant effects. In contrast, antidepressants inhibiting reuptake of both 5-HT and noradrenaline succeeded to suppress immobility (54), indicating that selective block of 5-HT reuptake is not enough to reverse depression in CCI animals. Mechanisms other than changes in 5-HT level may mediate fluoxetine's effects. The fluoxetine's anti-inflammatory effect may be mediated with decreasing levels of local inflammatory mediators (55) and affecting the opioid system (56) while its antidepressant and analgesic effects in CCI rats may be explained by decreasing nitric oxide (NO) levels through inhibiting NO synthase (NOS) overexpression (57). In CCI rats, levels of nitrate and nitrite increased in the injured sciatic nerve. Drugs that increase NO level potentiated hyperalgesia and allodynia while NOS inhibitors alleviated pain (58). Moreover, NOS inhibitors potentiated fluoxetine's effects in FST in mice (59).

Oral pioglitazone attenuated mechanical hyperalgesia and reduced central TNF-α and NF-κB levels after spinal nerve transaction in rats (60). Pioglitazone also decreased levels of NF-κB, IL-6, MCP-1, and TNF-α in mice suggesting inhibition of glial cell activities (61). Pioglitazone in 2 sets of experiments (intraperitoneally for 7 days and orally for 7 weeks) dose-dependently reduced mechanical hyperalgesia and allodynia resulting from

Table 4. *Effects of treatments (mg/kg/day) on the immobility duration (s) in the FST in rats on day 21 post-CCI.*

Group	Sham	CCI	F10	F20	F40	P	M	F20+P	F20+M
Immobility duration	125.63 ± 7.84	193.38 ± 6.37$^{!!!}$	189.38 ± 7.33$^{!!!}$	179.38 ± 7.07$^{!!!,\#}$	178.13 ± 5.46$^{!!!,\#\#}$	166.50 ± 8.94$^{!!!,\#\#\#,\wedge}$	185.88 ± 8.48$^{!!!}$	153.13 ± 7.68$^{!!!,\#\#\#,**,*}$	176.25 ± 7.85$^{!!!,\#\#,\wedge}$

CCI (chronic constriction injury), FST (forced swimming test) F (fluoxetine 10, 20 and 40), P (pioglitazone 20), M (metformin 50), F20+P (fluoxetine (20) + pioglitazone), F20+M (fluoxetine (20) + metformin) (n = 8). Data are expressed as mean ± SD.
$^{!!!}P < 0.001$: all groups vs. sham. $^{\#\#\#}P < 0.001$: P & F20+P vs. CCI. $^{\#\#}P < 0.01$: F40 & F20+M vs. CCI ($P = 0.005$ & 0.001). $^{\#}P < 0.05$: F20 vs. CCI ($P = 0.015$). $^{\wedge}P < 0.05$: F20+M vs. F10 ($P = 0.032$), and P vs. F20 ($P = 0.039$). $^{***}P < 0.001$: F20+P vs. (F10, F20, F40, M & F20+M). $^{*}P < 0.05$: F20+P vs. P ($P = 0.026$).

nerve injury. It also reduced the increased GFAP expression in the spinal dorsal horn without affecting motor function. Pioglitazone's rapid effects, occurring after 7 days, are mediated by PPARγ-dependent transcription-independent mechanisms while its long-lasting effects occur through PPARγ-dependent transcription-dependent mechanisms. Moreover, pioglitazone didn't show analgesic tolerance (62). In mice, sciatic nerve ligation induced nerve inflammation, thermal hyperalgesia, tactile allodynia, upregulation of TNF-α and IL-6, and increased the number of activated microglia in the ipsilateral spinal dorsal horn. Pioglitazone given during the first 2 weeks post-ligation reversed these changes through stimulation of PPARγ receptors in neurons of the spinal dorsal horn, adipocytes at the epineurium of the sciatic nerve, and macrophages (3,63). Moreover, oral pioglitazone decreased immobility duration in mouse FST without affecting locomotor activity through PPARγ activation, in brain areas involved in pathogenesis of depression, and NOS inhibition (64).

Oral metformin activated AMPK, reversed tactile allodynia, and increased apolipoprotein E (a protein linked to nerve regeneration) after sciatic nerve injury in rats (65). Metformin also activated AMPK, suppressed inflammatory responses, and decreased serum TNF-α and IL-6 in rats with myocardial infarction (66). In animals with peripheral nerve injury, metformin for 7 days, possibly through activating AMPK and inhibiting mTOR pathway in the spinal cord, alleviated tactile allodynia and decreased sensory excitability without affecting motor function (67) and thus a central mechanism may be involved (68). Further, oral metformin didn't decrease immobility duration in FST and didn't affect locomotor activity in diabetic rats (69). In contrast, oral metformin significantly reduced immobility duration in mouse FST (70). The reported antidepressant effect of chronic metformin therapy in depressed diabetic patients (71) may be attributed to cognitive improvement induced by loss of weight and control of diabetes (72).

Conclusion

In conclusion, co-administration of pioglitazone or metformin with low-dose fluoxetine effectively improved mechanical allodynia, thermal hyperalgesia, and neurohistopathological changes while co-administration of pioglitazone, but not metformin, effectively improved the depressive-like behavior in a peripheral nerve injury model of neuropathic pain in rats.

Limitations and Generalizability

Limitations of this study included measuring levels of cytokines only in blood and not in the spinal cord and sciatic nerve and measuring the outcome measures in the first set of experiments at only one time-point. Absence of randomization, lack of power analysis, and incomplete blinding are common flaws in animal studies (73). If the present findings can be translated to clinical reality, it could be valuable in treatment of pain patients with comorbidities such as diabetes and/or depression, however this translation needs further confirmatory studies.

Disclosure of Conflicts of Interest

Author Contributions: Dr. Hussam Murad and Dr. Nasra Ayuob had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Dr. Hussam Murad designed the study protocol, managed the literature searches and summaries of previous related work, conducted the pharmacological work, and wrote the manuscript. Dr. Nasra Ayuob conducted the histopathological work and related writing.

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