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Fluoxetine attenuates UPR-activation, neuroinflammation, and oxidative stress in a nitroglycerin-induced rat model of migraine

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ABSTRACT

Objective(s): Migraine is a debilitating neurological disorder characterized by oxidative stress, neuroinflammation, and dysregulation of the unfolded protein response (UPR) in the brain. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), has demonstrated neuroprotective properties, but its effects on UPR activation, neuroinflammation, and oxidative stress in migraine are not well defined. This study evaluates the effects of fluoxetine on UPR-related gene expression, inflammatory cytokines, and oxidative stress markers in a nitroglycerin (NTG)-induced chronic migraine rat model.

Materials and Methods: Rats were divided into control, NTG-induced chronic migraine, and fluoxetine-treated groups. Behavioral light aversion was assessed. Trigeminal ganglion tissues were analyzed for expression of UPR-associated genes (Bip, XBP-1, CHOP) and inflammatory markers (IL-6, IL-1β, IL-10, NF-κB, TNF-α) using molecular techniques. Serum levels of oxidative stress indicators, including catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), and total antioxidant capacity (TAC), were measured.

Results: Our results demonstrate that fluoxetine treatment effectively tempered the maladaptive UPR by modulating the expression of Bip, XBP-1, and CHOP. It also suppressed neuroinflammation by reducing pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and enhancing the anti-inflammatory cytokine IL-10. Fluoxetine also counteracted oxidative stress by restoring the activity of antioxidant enzymes (SOD, CAT), reducing lipid peroxidation (MDA), and enhancing overall antioxidant capacity (TAC). Importantly, these positive biochemical changes were correlated with a significant reduction in light aversion behavior.

Conclusion: Fluoxetine exerts protective effects in an NTG-induced migraine model by modulating UPR pathways, reducing neuroinflammation, and alleviating oxidative stress. These findings highlight its potential as a therapeutic agent targeting multiple pathways in migraine pathophysiology.

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Introduction

Migraine is a prevalent chronic neurological disorder characterized by recurrent, often unilateral and pulsatile headaches accompanied by sensory hypersensitivities such as photophobia (1, 2). Chronic migraine is characterized by headaches occurring on 15 or more days per month, with at least 8 of those days fulfilling migraine diagnostic criteria (3-5). These frequent headaches significantly impair daily functioning and quality of life. The pathophysiology of migraine involves intricate interactions among genetic, environmental, and neurobiological factors (6), including cortical spreading depression and sensitization of neurons within the trigeminal system. This activation triggers the release of pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α) and transcription factors like NF-κB, while reducing anti-inflammatory mediators such as IL-10 (7-10). The serotonergic system, originating from the brainstem raphe nuclei, is also strongly implicated in migraine pathophysiology (11), with alterations in serotonin metabolism observed during migraine attacks (12). A low serotonergic state may facilitate activation of the trigeminovascular nociceptive pathway (13). Moreover, serotonin receptor dysfunction is implicated in various neuropsychiatric conditions. Various triggers, such as neuroendocrine imbalances, sleep disturbances, inflammation, and oxidative stress (14), converge on cellular stress pathways (14). These pathways include disruptions in protein folding within the endoplasmic reticulum (ER). The accumulation of unfolded or misfolded proteins in the ER lumen induces ER stress, activating the UPR, a cellular mechanism that restores proteostasis by enhancing protein-folding capacity and eliminating misfolded proteins (15-17). While initially protective, prolonged or severe ER stress can overwhelm the UPR, potentially triggering cell

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death. Dysregulation of UPR has been linked to chronic inflammation and diseases such as neurodegeneration, cancer, and metabolic disorders (18, 19). Furthermore, maladaptive UPR signaling may alter neuronal excitability and neurotransmitter release, thus influencing pain pathways. Although oxidative stress's role in migraine is recognized, connections between oxidative stress, ER stress, and UPR in migraine remain poorly understood. The potential of targeting UPR pathways for migraine prevention or treatment remains unexplored. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), has shown promise in reducing migraine frequency and possesses antioxidant properties that could confer neuroprotection through regulation of cellular stress pathways (20-22); however, its influence on UPR pathways had not been previously investigated. Therefore, this study used a nitroglycerin-induced rat model of chronic migraine (23) to examine UPR target gene expression and to evaluate the effects of fluoxetine on UPR regulation, antioxidant enzyme activity, and the ER stress-inflammation axis. Our findings may uncover new molecular targets for migraine treatment.

Materials and Methods

Chemicals

Nitroglycerine (NTG) injection was obtained from Caspian Company. (LOT: CT113, Rasht, Iran). Fluoxetine was purchased from Razak Company (Tehran, Iran) and dissolved in saline. All chemicals were obtained from Sigma (Gillingham, UK) unless otherwise stated.

Animals

Adult male Sprague-Dawley rats weighing 230 ± 20 g were obtained from the Laboratory Animal Centre of Shiraz University of Medical Sciences, Shiraz, Iran. Rats were housed 4 to a cage in plastic cages (59 × 38 × 20 cm) at 25 °C and received water and food *ad libitum* under a 12 hr light/dark cycle. All experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Medical and Research Ethics Committee of the Shiraz University of Medical Sciences, Shiraz, Iran. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.AEC. 1403. 055).

Chronic migraine model establishment and drug administration

The chronic migraine (CM) model was established as described by Pradhan and Smith (24). The concentration of the NTG stock solution was 5 mg/ml. Before injection, NTG was diluted to 1 mg/ml with normal saline. Animals in the CM model were administered nitroglycerin (NTG) intraperitoneally (IP) at 10 mg/kg of NTG every second day for 9 days (1st, 3rd, 5th, 7th, and 9th day). The vehicle control solution consisted of 0.9% saline, 6% propylene glycol, and 6% alcohol. Thirty-six rats were randomly divided into six groups (six for each group): A) Control group, rats received five saline injections over the course of 9 days, B) Vehicle control group, rats received five intraperitoneal injections of the vehicle solution every second day for 9 days. C) Chronic migraine model group, rats received five NTG (10 mg /kg) injections intraperitoneal on alternate days (1st, 3rd, 5th, 7th, and 9th day). D) Fluoxetine alone groups, rats received five fluoxetine (5 mg/kg IP) injections every other day (Days 1-9). To examine the ability of fluoxetine to reverse chronic migraine, E) prophylactic group, rats received NTG (10 mg/kg IP) every other day (Days 1-9) followed 15 min later by fluoxetine (5 mg/kg, IP) dissolved in saline. F) Therapeutic group, rats received NTG over the course of 9 days, followed by the administration of fluoxetine daily (5 mg/kg, i.e.) for ten consecutive days (Days 10-19). All experimental rats underwent the light/dark box behavioral test before and 2 hr after NTG injection.

Behavioral assessment

A hallmark symptom of migraine is photophobia or light aversion, which is reflected in the NTG-induced rat migraine model through increased light sensitivity. The dark/light box test is a validated behavioral assessment used to evaluate light aversion and anxiety-like behaviors in rodents. This method offers a measurable way to assess migraine-related light sensitivity (25, 26).

Light/dark box test

Behavioral assessments were performed between 9:00 AM and 3:00 PM in a quiet environment. Photophobia was assessed using a light/dark box consisting of two equal-sized chambers: one illuminated and the other dark. Rats were acclimated to the box for 10 min on Day 0. Baseline behavior was recorded for 10 min on Day 1 (pre–NTG injection). Following chronic NTG administration (10 mg/kg, IP, every other day for 9 days), light avoidance was recorded for 10 min, 2 hr post final NTG injection (Day 10). Time spent in the light chamber, latency to enter the light zone, and total number of transitions between chambers were quantified.

Biochemical determination

After behavioral observation, animals were decapitated after CO_2 inhalation. Blood samples were collected via cardiac puncture, allowed to clot, and centrifuged (3000 rpm, 15 min, 4 °C) to obtain serum, which we stored at -80 °C for oxidation stress marker analysis. The trigeminal ganglia were isolated from animals immediately. The tissues were wrapped in aluminum foil, frozen in liquid nitrogen, and stored at − 80 °C until RNA extraction.

Determination of oxidative stress and anti-oxidant markers in serum

Determination of catalase (CAT) activity

Catalase (CAT) activity was assessed by monitoring hydrogen peroxide (H_2O_2) decomposition at 240 nm spectrophotometrically (27). One unit of CAT activity was defined as the amount of enzyme required to degrade 1 µmol of H_2O_2 per minute at pH 7.0, and results were expressed as IU/ml.

Determination of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was determined using a spectrophotometric method based on its ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals (O_2^-), generated via the xanthin/ xanthin oxidase system (28). The reduction of NBT produces a purple formazan, measurable at 570 nm. One SOD unit was defined as the amount of enzyme that causes 50% inhibition of NBT reduction, and activity was expressed as IU/ml.

Measurement of malondialdehyde (MDA)

Malondialdehyde (MDA) or lipid peroxidation level was measured using the thiobarbituric acid reactive substances

(TBARS) assay (29). Briefly, serum samples reacted with thiobarbituric acid (TBA) under acidic conditions at 95 °C, forming a pink MDA- TBA complex. Absorbance was measured at 532 nm, and MDA concentration was calculated using a standard curve and expressed as µmol/ml.

Evaluation of total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) of serum samples was measured using the ferric reducing antioxidant power (FRAP) assay (30). Briefly, antioxidants in the sample reduce Fe^{+3} to $Fe^{+2,}$ forming a colored complex with 2,4,6-tripyridyl-S-triazine (TPTZ) at low pH. Serum was mixed with FRAP reagent (acetate buffer + TPTZ + $FeCl_3$), and the absorbance was read at 593 nm after 4 min incubation at 37 °C. TAC was calculated against a Trolox standard curve and expressed as µmol Trolox equivalents/ml serum.

Real-time quantitative PCR

We used qRT-PCR to assess transcript levels of the unfolded protein response genes *Xbp-1, CHOP*, and *Bip,* and proinflammatory cytokine genes *TNF-α, IL*-1β, and IL-6 during the development of migraine and reversal of effect by fluoxetine treatment. Total RNA was extracted from the trigeminal ganglion using the Biozol reagent (Bioflux, Japan), according to the manufacturer's instructions, and quantified by Nano-drop (Thermo Fisher Scientific, USA) to determine its concentration and purity. Denaturing gel electrophoresis was used to assess RNA integrity (Fleige and Pfaffl, 2006). The RNA extracted for cDNA synthesis was treated with DNase I (EN0521, Fermentas, Germany) to remove any DNA contamination. cDNAs were synthesized using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Eu). All procedures were based on the manufacturer's instructions. q RT-PCR was performed, using a 7500 real-time PCR system (Applied Biosystem, Foster City, CA, USA). All genes were amplified under the following conditions: initial denaturing at 95 °C for 30 sec, 40 cycles of 95 °C for 5 sec, and annealing and extension at 60 °C for 30 sec. Each PCR reaction was run in duplicate. The ΔΔCT method was used to calculate the relative expression level of UPR and proinflammatory genes of the animals. Amplification efficiencies were calculated from a relative standard curve derived from tenfold serial dilutions of pooled cDNA. The quality and accuracy of the PCR products were checked using electrophoresis on 2% agarose gels.

The following primer pairs were used for the qRT-PCR:
BIP F, 5' CAGCCCACCGTAACAATCA AG 3' and R, 5' TCCTGTCCCTTTGTCTTCAGC3'; *CHOP* F, 5' TACACCACCACACCTGAAAGC 3' and R, 5' GCAGGGTCAAGAGTAGTGAAG 3'; *XBP-1* F, 5' GGACACGCTTGGGGATGAATG 3' and R, 5' CTGCACCTGCTGCGGACT 3';
TNF-a F, 5' ATGGGCTCCCTCTCATCAGT 3' and R, 5' TGGTTTGCTACGACGTGGG 3';
β actin F, 5' TAGAGCGAACACGAA CCATCC 3' and R, 5' GAAGGAAGGCTGGAAGAG 3';
IL-1b F, 5' AACATAAGCCAACAAGTG 3' and R, 5' ACAGGACAGGTATAGATTC3';
IL-6 F, 5' GAAAGTCAACTCCATCTGCC3' and R, 5' TACTGGTCTGTTGTGGGTGGT3';
IL-10, 5' CCCAGAAATCAAGGAGCA3' and R, 5' CTGCTCCACTGCCTTGCTT 3';
NF-KB, 5'CCAGCACCAAGACCGAAGCAA3' and R, 5' TTCACATCTCCCGTAACCGC3';

Data analysis

GraphPad Prism (Version 9; GraphPad Software, San Diego, CA, USA) was used for statistical analysis of data presented as mean ± SEM, which were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. A *P*-value less than 0.05 is set as the level of significance.

Results

Light /dark box behavioral analysis

The light/dark box test was used to assess photophobia and anxiety-like behaviors in an NTG-induced chronic migraine model (Figure 1). NTG administration significantly increased anxiety-like responses (Table 1). The time spent in the light chamber was reduced in the NTG group (1.73 ± 0.16 seconds) compared to the control group (2.74 ± 0.72 seconds; $P<0.05$). The frequency of transitions also decreased (1.82 ± 0.13 in NTG vs 4.71 ± 0.15 in controls; $P<0.001$). Furthermore, latency to re-enter the light chamber was longer in the NTG group (66.04 ± 5.26 seconds) than in the control group (32.10 ± 1.90 seconds; $P<0.05$). No significant difference was observed between vehicle- and saline-treated rats ($P= 0.998$), confirming that the effects were NTG-specific. Fluoxetine treatment exerted differential effects depending on the administration regimen (Figure 1, Table 1). Prophylactic fluoxetine did not significantly alter the

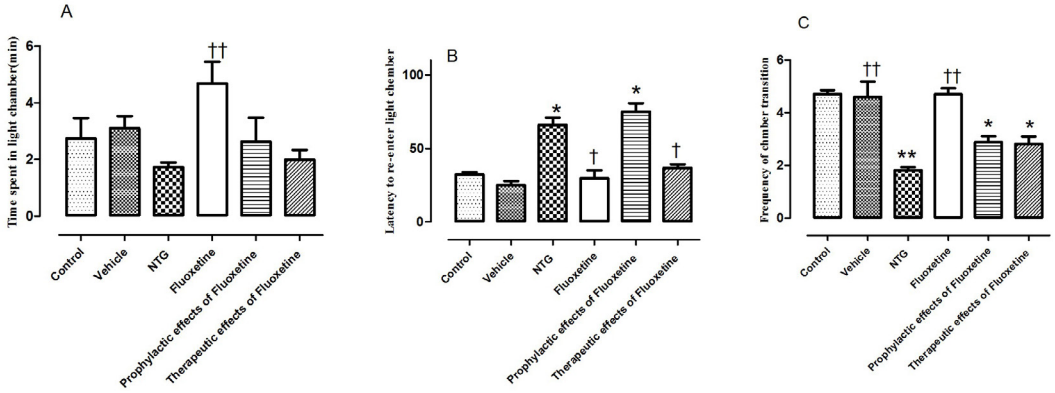

Figure 1. Assessment of rat light avoidance behavior in the light/dark box test following NTG-induced migraine and fluoxetine treatment
(A) Time spent in the light chamber (seconds). (B) Latency to first enter the light chamber (seconds). (C) Number of transitions between the dark and light chambers. Experimental groups (n=6/group): 1. Control (saline, 10 days); 2. NTG (10 mg/kg IP, every other day, days 1–9); 3. Prophylactic fluoxetine (5 mg/kg, 15 min pre-NTG); 4. Therapeutic fluoxetine (5 mg/kg, initiated 24 hr post-first NTG). Groups treated with vehicle or fluoxetine alone showed no significant differences relative to control and are therefore omitted for clarity. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.001 vs Control; †*P*<0.05, ††*P*<0.001 vs NTG group (one-way ANOVA followed by Tukey's *post hoc* test).

Table 1. Effects of fluoxetine on nitroglycerin (NTG)-induced photophobia in migraine rats

Groups (Intra-peritoneal injection)	Time spent in the light chamber	Latency to re-enter the light chamber	Frequency of chamber transitions
Control (n = 6)	2.74 ± 0.722	32.10 ± 1.90	4.71 ± 0.146
Vehicle (n = 6)	3.10 ± 0.430	25.23 ± 2.49	4.60 ± 0.575
NTG (n = 6)	1.725 ± 0.164	66.037 ± 5.255*	1.82 ± 0.1253*
Fluoxetine (n=6)	4.68 ± 0.766	29.71 ± 5.51†	4.70 ± 0.229†
Prophylactic effects of Fluoxetine (pre-NTG) (n = 6)	2.63 ± 0.853	75.19 ± 5.64*	2.88 ± 0.224*
Therapeutic effects of Fluoxetine (post NTG) (n =6)	1.99 ± 0.346	35.77 ± 2.50†	2.81 ± 0.269*

Light-avoidance behaviors in the light/dark box test in control, NTG-treated, and fluoxetine-treated rats.Values are expressed as mean ± SEM (n=6 per group). *P<0.05, **P<0.01 vs Control; † P<0.05, †† P<0.01 vs NTG group (one-way ANOVA followed by Tukey's *post-hoc* test).

time spent in the light chamber (2.63 ± 0.85 seconds) or the number of transitions (2.88 ± 0.22) compared to the NTG group (P> 0.05 for both). It further increased the latency to re-enter the light chamber (75.19 ± 5.64 seconds), although this difference was not statistically significant compared with the NTG group (P= 0.641). In contrast, fluoxetine alone significantly increased the time spent in the light chamber (4.68 ± 0.77 seconds) compared to both the control and NTG groups (P<0.05 for both), and normalized latency (29.71 ± 5.51 seconds) and transitions (4.70 ± 0.23) to control levels. Therapeutic fluoxetine significantly reduced the re-entry latency to 35.77 ± 2.50 seconds compared to the NTG group (P<0.05), restoring it to a level not significantly different from controls. However, the number of transitions (2.81 ± 0.27), although improved relative to the NTG group (P<0.05), remained significantly lower than in the control group (P<0.01).

Effects of fluoxetine on serum oxidative stress markers in the nitroglycerin- induced migraine model

Administration of NTG significantly increased oxidative stress (Figure 2, Table 2). This was evidenced by elevated MDA levels (2.47 ± 0.14 µmol/l in the NTG group vs 1.65 ± 0.19 µmol/l in controls; P<0.05) and a concurrent reduction in TAC (3.87 ± 0.25 µmol/ml in the NTG group vs 5.28 ± 0.28 µmol/ml in controls; P<0.05). We observed non-significant numerical increases in SOD (48.39 ± 5.67 IU/ml vs control 33.32 ± 3.17 IU/ml, P= 0.064) and CAT

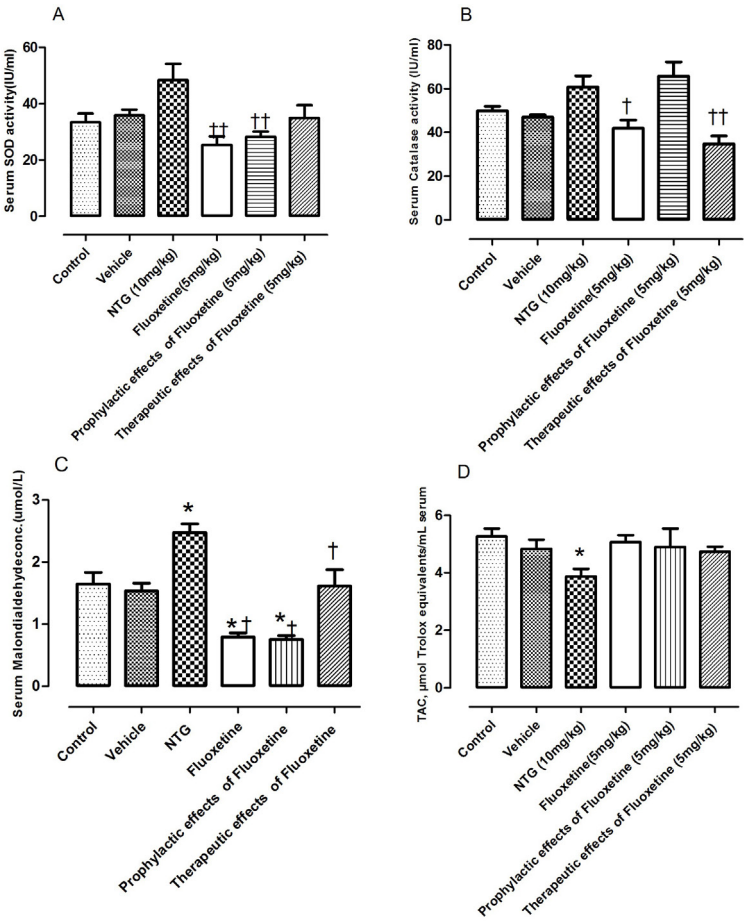

Figure 2. Effects of fluoxetine on serum oxidative stress parameters in a nitroglycerin (NTG)-induced migraine model in rats
(A-D) Serum levels of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) malondialdehyde (MDA), and (D) total antioxidant capacity (TAC) were measured 2 hr after the final NTG injection on day 9. Experimental groups (n=6/group): 1. Control; 2. NTG (10 mg/kg, every other day); 3. Prophylactic fluoxetine (5 mg/kg, 15 min pre-NTG); 4. Therapeutic fluoxetine (5 mg/kg, initiated 24 hr post-first NTG). Groups treated with vehicle or fluoxetine alone showed no significant differences relative to control and are therefore omitted for clarity. Values are mean ± SEM. *P<0.05, **P<0.001 vs Control; †P<0.05, ††P<0.001 vs NTG group (one-way ANOVA, Tukey's test).

Table 2. Serum levels of oxidative stress markers in control, nitroglycerin (NTG)-treated, and fluoxetine-treated rats

Groups (IP Injection)	Superoxide Dismutase (Iu/ml)	Catalase (Iu/ml)	Malondialdehyde (µmol/l)	Total antioxidant capacity (TAC) µmol/ml serum
Control (n = 6)	33.32 ± 3.17	49.82 ± 2.18	1.65 ± 0.187	5.28 ± 0.281
Vehicle (n = 6)	35.73 ± 2.17	46.94 ± 1.06	1.53 ± 0.126	4.84 ± 0.31
NTG (n = 6)	48.39 ± 5.67	60.74 ± 5.18	2.47 ± 0.144**	3.87 ± 0.254*
Fluoxetine (n = 6)	32.00 ± 8.76	41.67 ± 3.76†	0.794 ± 0.067 †*	5.07 ± 0.243
Prophylactic effects of Fluoxetine (pre-NTG) (n = 6)	28.25 ± 1.86	65.75 ± 6.42	0.753 ± 0.062 †*	4.89 ± 0.648
Therapeutic effects of Fluoxetine (post–NTG) (n = 6)	34.88 ± 4.58	34.79 ± 3.57 † †	1.61 ± 0.266† †	4.74 ± 0.173

Data are expressed as mean ± SEM (n=6 per group). *P<0.05, **P<0.001 vs Control; †P<0.05, ††P<0.001 vs NTG group (one-way ANOVA followed by Tukey's *post-hoc* test).

CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; TAC: total antioxidant capacity

(60.74 ± 5.18 IU/ml vs control 49.82 ± 2.18 IU/ml, P= 0.391) activity in NTG-treated rats. Fluoxetine treatment exerted differential effects on these markers. Prophylactic fluoxetine significantly reduced MDA levels to 0.75 ± 0.06 µmol/l compared to the NTG group (P<0.01) and increased CAT activity to 65.75 ± 6.42 IU/ml (P<0.05 vs NTG). Therapeutic fluoxetine significantly reduced MDA to 1.61 ± 0.27 µmol/l and SOD to 34.88 ± 4.58 IU/ml compared to the NTG group (P<0.05 for both), restoring them to levels that were not significantly different from the control group (P>0.05). Both fluoxetine regimens counteracted the NTG-induced depletion of TAC, maintaining levels (Prophylactic: 4.89 ± 0.65 µmol/ml; Therapeutic: 4.74 ± 0.17 µmol/ml) that were significantly higher than in the NTG group (3.87 ± 0.25 µmol/ml, P<0.05) and statistically similar to controls. Fluoxetine alone also demonstrated significant antioxidant effects, reducing MDA to 0.79 ± 0.07 µmol/l (P<0.01 vs NTG) and maintaining TAC at 5.07 ± 0.24 µmol/ml. The most pronounced reduction in lipid peroxidation was observed with prophylactic fluoxetine (0.75 ± 0.06 µmol/l), which was also the only regimen to significantly enhance CAT activity above all other groups.

Fluoxetine modulates UPR transcriptional responses in an NTG-induced chronic migraine model

In our NTG-induced chronic migraine model, we observed significant alterations in the mRNA expression levels of key UPR markers in the trigeminal ganglion. Quantitative RT-PCR analysis revealed that, compared to the control group (1.00 ± 0.018), NTG-treated rats exhibited a significant reduction in XBP-1 mRNA expression (0.173 ± 0.043) conversely, NTG administration significantly elevated the mRNA levels of Bip (GRP78) (2.26 ± 0.11 vs control: 1.11 ± 0.09) and CHOP (3.90 ± 0.07vs control: 0.97 ± 0.02). These findings show NTG– induced ER-stress and activation of pro-apoptotic UPR signaling in chronic migraine pathogenesis (Figure 3 A-C).

Notably, fluoxetine treatment modulated these UPR alterations. A trend toward increased XBP-1 mRNA expression was observed in the prophylactic (0.57 ± 0.01) and therapeutic (0.48 ± 0.08) fluoxetine groups compared to the NTG group (0.86 ± 0.03); however, these changes were not statistically significant (Figure 3A). In contrast, fluoxetine administration significantly normalized the NTG-induced elevations in both Bip and CHOP mRNA levels. Both prophylactic and therapeutic fluoxetine regimens reduced Bip expression to 0.26 ± 0.09 and 1.06 ± 0.13, respectively (Figure 3B), and CHOP expression to 1.06 ± 0.10 and 0.32 ± 0.06, respectively (Figure 3C). This demonstrates a robust modulatory effect of fluoxetine on these specific UPR pathways, suggesting it mitigates NTG-induced ER stress with particular efficacy in regulating the Bip and CHOP branches.

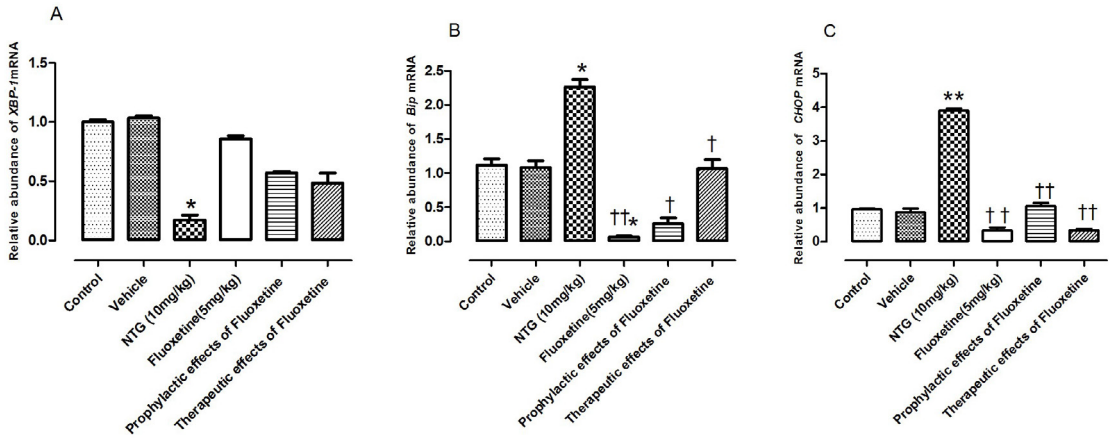

Figure 3. Effects of nitroglycerin (NTG) and fluoxetine on the expression of unfolded protein response (UPR) markers in the rat trigeminal ganglion
(A-C) Relative mRNA expression levels of (A) XBP-1, (B) Bip (Hspa5), and (C) CHOP (Ddit3) determined by qRT-PCR. Experimental groups (n=6/group): 1. Control (saline, 10 days); 2. NTG (10 mg/kg IP, every other day, days 1–9); 3. Prophylactic fluoxetine (5 mg/kg, 15 min pre-NTG); 4. Therapeutic fluoxetine (5 mg/kg, initiated 24h post-first NTG). The trigeminal ganglion was harvested 2 hr after the final injection on day 10. Gene expression was normalized to β-actin/GAPDH and calculated using the 2^(-ΔΔCt) method. Data are expressed as mean ± SEM fold change versus the Control group. *P<0.05, **P<0.001 vs Control; †P<0.05, ††P<0.001 vs NTG group (one-way ANOVA followed by Tukey's *post hoc* test).

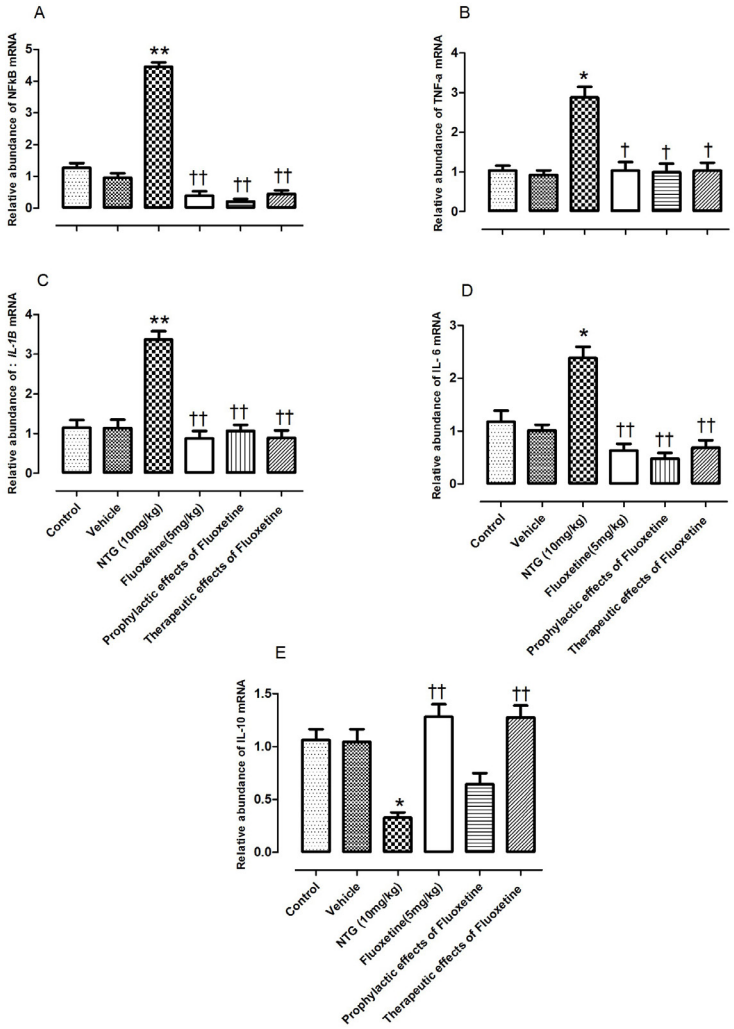

Figure 4. Effects of nitroglycerin (NTG) and fluoxetine on the expression of inflammatory genes in the rat trigeminal ganglion
(A-E) Relative mRNA expression levels of pro-inflammatory cytokines (A) IL-1β, (B) IL-6, (C) TNF-α, (D) NF-κB, and the anti-inflammatory cytokine (E) IL-10. Experimental groups (n=6/group): 1. Control (saline); 2. NTG (10 mg/kg IP, every other day); 3. Prophylactic fluoxetine (5 mg/kg, 15 min pre-NTG); 4. Therapeutic fluoxetine (5 mg/kg, initiated 24 hr post-first NTG). Tissue was collected 2 hr after the final injection. Gene expression was normalized to β-actin/GAPDH and calculated using the ΔΔCt method. Data are expressed as mean ± SEM fold change versus the Control group. **P*<0.05, ***P*<0.001 vs Control; †*P*<0.05, ††*P*<0.001 vs NTG group (one-way ANOVA followed by Tukey's *post hoc* test).

Fluoxetine attenuates NTG-induced pro-inflammatory signaling and restores IL-10 expression

Further qRT-PCR analysis revealed significant dysregulation of inflammatory mediators in the trigeminal ganglion. NTG administration significantly elevated the mRNA expression of pro-inflammatory genes, including NF-κB (4.45 ± 0.15 vs control: 1.27 ± 0.15, *P*<0.01), TNF-α (2.88 ± 0.27 vs control: 1.04 ± 0.12, *P*<0.05), IL-1β (3.37 ± 0.21 vs control: 1.15 ± 0.19, *P*<0.01), and IL-6 (2.38 ± 0.21 vs control: 1.18 ± 0.21, *P*<0.001). Fluoxetine treatment (both therapeutic and prophylactic regimens) significantly reversed these effects, reducing mRNA expression of all four pro-inflammatory markers compared to the NTG group (*P*<0.05 for all) (Figure 4A–D). In contrast, NTG administration suppressed the anti-inflammatory cytokine *IL-10* (0.45 ± 0.09 vs control: 1.00 ± 0.10, *P*<0.01). Fluoxetine treatment restored IL-10 expression in both the therapeutic group (1.27± 0.11 vs NTG, *P*<0.05) and fluoxetine-only group (1.28 ± 0.12 vs NTG, *P*<0.01). However, the prophylactic fluoxetine regimen (0.64 ± 0.11) did not significantly alter *IL-10* levels compared to the NTG group (*P*>0.05), suggesting timing-dependent effects on anti-inflammatory modulation (Figure 4E). Collectively, fluoxetine normalized NTG-induced inflammatory imbalances by suppressing pro-inflammatory mediators and enhancing *IL-10* expression, with therapeutic administration showing broader efficacy than prophylaxis.

Discussion

Our study demonstrates that NTG induces photophobia-like behavior, manifested as light avoidance and increased latency to enter the light chamber (31). The effects of fluoxetine on this behavior were complex and dependent on the treatment regimen. While therapeutic administration after NTG onset showed partial rescue, prophylactic administration unexpectedly worsened light-avoidance behavior, a finding that warrants critical discussion (32). A notable and unexpected finding was that prophylactic fluoxetine co-administered with NTG showed a trend toward exacerbating light-avoidance behavior, specifically by increasing the latency to enter the light chamber. This contrasts with the beneficial effects of therapeutic fluoxetine and fluoxetine alone (33). We must critically consider why a drug with known anxiolytic properties might produce

this effect in a prophylactic setting (34). One plausible explanation is serotonergic overstimulation. In the highly sensitized state induced by chronic (35) NTG, the rapid increase in synaptic serotonin caused by fluoxetine might initially exacerbate anxiety-like responses in certain individuals, a phenomenon sometimes observed in the early stages of SSRI treatment in clinically anxious populations (36). Alternatively, the specific neurobiological state of the 'pre-migraine' brain in our model may be uniquely vulnerable to modulation of the serotonin system(37), leading to a paradoxical anxiogenic effect (38). When the drug is administered concurrently with the migraine trigger. This suggests that the timing of fluoxetine administration is critical and that its prophylactic effect on pain pathways may be dissociable from its acute effects on affective behaviors (39). This intriguing dissociation merits further investigation to optimize the use of SSRIs in migraine prophylaxis.

NTG-treated rats exhibited a paradoxical elevation of antioxidant enzymes SOD and CAT. Alongside the significant increase in lipid peroxidation (MDA) and reduction in TAC, we noted a non-significant numerical trend toward higher SOD and CAT levels in NTG-treated rats. While this observation did not reach statistical significance in our study, it contrasts with some reports of antioxidant depletion in migraine and could be explored in future work as a potential adaptive response to oxidative challenge (40). The significant elevation in MDA confirms NTG's role in promoting lipid peroxidation, consistent with clinical studies reporting increased MDA in migraine patients (41). Furthermore, a concomitant 33% reduction in TAC parallels findings in individuals with chronic migraine, indicating systemic antioxidant depletion (14). Interestingly, fluoxetine treatments were associated with decreased SOD levels (-24% in fluoxetine-alone and -15%, prophylactic, respectively), likely reflecting down-regulation of compensatory antioxidant mechanisms as oxidative stress resolves. Fluoxetine also directly inhibits superoxide production by reducing NADPH oxidase activity in neural tissues (42). For catalase, a greater reduction was observed in therapeutic (-30%) compared to prophylactic fluoxetine groups, suggesting time-dependent regulation of hydrogen peroxide metabolism. This may be influenced by serotonin-mediated modulation of CAT transcription (43).

All fluoxetine regimens significantly normalized MDA levels ($P<0.01$), demonstrating robust protection against lipid peroxidation, and restored TAC to baseline, supporting its role in enhancing endogenous antioxidant capacity. Fluoxetine's multimodal antioxidant properties in NTG-induced migraine appear to operate via: 1) direct free radical scavenging, 2) modulation of oxidative stress markers, and 3) recovery of global antioxidant capacity (TAC restoration). These findings position fluoxetine as a promising therapeutic candidate for migraine patients exhibiting oxidative stress biomarkers (44). Future studies should determine whether these biochemical improvements correlate directly with behavioral symptom relief.

Our results demonstrate significant neuroinflammation in the trigeminal ganglion of NTG-treated rats, evidenced by up-regulated pro-inflammatory genes (*NF-kB*, *TNF-α*, *IL-1β*, and *IL-6*) and suppression of the anti-inflammatory cytokine *IL-10*. This finding aligns with both clinical and preclinical evidence linking migraine to neuroinflammatory cascades (45, 46). *NF-κB*, a master regulator of inflammation, induces TNF-α and IL-1β transcription, which sensitize trigeminal nociceptors and perpetuate central sensitization (47). Elevated *IL-6* levels further support its contribution to migraine chronification, paralleling clinical data correlating serum IL-6 with migraine attack frequency (48, 46).

Both therapeutic and prophylactic fluoxetine administration effectively suppressed pro-inflammatory genes, indicating a direct anti-neuroinflammatory action independent of its antidepressant effects. Notably, fluoxetine's reduction of *NF-κB* is significant, as *NF-κB* inhibition mitigates pain behaviors in rodent migraine models (49). This effect may involve fluoxetine's inhibition of microglial activation or modulation of serotonergic signaling, which suppresses cytokine release. The prophylactic regimen produced a weaker impact on *IL-10* compared to therapeutic treatment, suggesting that timing influences anti-inflammatory outcomes, potentially via differential serotonin receptor engagement or downstream transcriptional regulator activity (50, 51).

The reduction of *IL-10* in NTG-treated rats and its rescue by fluoxetine (notably in the therapeutic groups) underscores the critical role of *IL-10* as an anti-inflammatory brake in migraine pathogenesis. Low *IL-10* levels are associated with prolonged migraine attacks, and fluoxetine may help mitigate this deficit. The lack of significant restoration in prophylactic groups indicates the need for further investigation into dose and duration dependency.

Chronic migraine involves a dysregulation of cellular stress responses, including the UPR. Our study assessed fluoxetine's impact on UPR-related genes (*XBP-1, Bip, and CHOP)* in the NTG-induced model. NTG treatment suppressed *XBP-1* while up-regulating *Bip* and *CHOP*, indicating sustained ER stress and a shift toward pro-apoptotic UPR signaling. Fluoxetine administration significantly counteracted the NTG-induced up-regulation of *Bip* and *CHOP*. (52, 53). This specific action suggests that fluoxetine mitigates ER stress by enhancing protein-folding capacity (via *Bip* reduction) and reducing ER stress-induced apoptotic signaling (via *CHOP* reduction) (54, 55). This finding aligns with previous reports that antidepressants, including fluoxetine, modulate ER stress in neurological disorders (56). In contrast, the effect of fluoxetine on *XBP-1* expression was a non-significant trend, indicating that its primary UPR-related mechanism does not robustly involve this specific adaptive branch**.** The differential effects of fluoxetine on *XBP*-1 versus *Bip/CHOP* suggest that its therapeutic benefit derives from attenuating excessive, maladaptive ER stress rather than completely restoring the full spectrum of UPR signaling. The differential effects of fluoxetine on *XBP-1* versus *Bip/CHOP* suggest that its therapeutic benefit derives from attenuating excessive, maladaptive ER stress rather than completely restoring the full spectrum of UPR signaling. Taken together, our findings indicate that fluoxetine selectively mitigates the maladaptive *(Bip/CHOP*) arm of the UPR in response to NTG-induced ER stress (55, 56)**.** Given chronic migraine's features of sustained neuronal hyperexcitability and oxidative stress, this targeted mitigation of pro-apoptotic ER stress may significantly contribute to its prophylactic efficacy. Our data reveal that NTG-induced chronic migraine involves a complex cascade of oxidative stress, ER stress, and neuroinflammation. Elevated antioxidant enzymes (SOD, CAT) alongside increased MDA represent a failed compensatory response, triggering UPR

activation characterized by *XBP-1* suppression and *Bip/CHOP* up-regulation (57, 58). This ER stress amplifies neuroinflammation via *NF-κB* activation, increasing pro-inflammatory cytokines (*TNF-α, IL-1β, IL-6*) and suppressing *IL-10*, thereby weakening anti-inflammatory regulation. A self-perpetuating cycle emerges, where oxidative stress induces ER dysfunction, fueling inflammation and neuronal hyperexcitability, further exacerbating oxidative damage. Fluoxetine interrupts this pathological feedback loop through complementary mechanisms: potent antioxidant effects reduce MDA and restore TAC; normalization of antioxidant enzymes reinstates redox balance; ER stress modulation via Bip and *CHOP* down-regulation promotes protein homeostasis; and anti-inflammatory actions inhibit *NF-κB* and pro-inflammatory cytokines while up-regulating *IL-10*. Differences between the prophylactic and therapeutic regimens—such as weaker modulation of *IL-10* and CAT in prophylaxis—suggest temporal aspects in targeting oxidative/ER stress versus immune regulation (59, 60). Compared to single-target therapies, fluoxetine's broad-spectrum modulation of mitochondrial dysfunction, protein misfolding, and neurogenic inflammation underscores its potential as an effective, comprehensive agent in migraine management, consistent with clinical biomarker profiles in individuals with migraine (60, 61). Our study highlights important avenues for further research. First, a limitation of this work is the absence of a standard positive control drug, such as a triptan (for acute effects) or a known prophylactic agent like topiramate. Furthermore, measuring protein-level expression of inflammatory markers (e.g., by western blotting for NF-κB or by ELISA for *IL-10*) would clarify translational regulation and temporal dynamics beyond mRNA assessment. It is also crucial to validate whether serum biomarker changes reflect alterations in the central nervous system, particularly in migraine-relevant regions such as the trigeminal ganglion and brainstem. Comparative studies exploring whether other SSRIs, such as sertraline, exhibit similar multimodal effects would advance the understanding of fluoxetine's unique or shared mechanisms.

Conclusion

In summary, our study demonstrates that fluoxetine counters chronic migraine pathology through a coordinated mechanism: it alleviates oxidative stress, restores protein homeostasis by modulating the UPR, and suppresses neuroinflammation. By targeting these interconnected pathways, fluoxetine effectively disrupts the self-perpetuating cycle that drives migraine chronification. The differential outcomes of prophylactic versus therapeutic regimens further suggest that treatment timing is a key factor in its efficacy. These findings establish a strong mechanistic foundation for fluoxetine's potential repurposing as a multi-target therapy for migraine and justify further clinical investigation.

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Competing Interests and Funding

The authors declare they have no financial interests.

Ethical Approval

We have complied with all ethical guidelines concerning animal research.

Authors' Contributions

MS M and AH YN designed the experiments. MS M performed the experiments and collected data; Z KH and MR N proposed and developed the idea and designed the study. SM S performed the analysis and interpretation of results. F Z and Z KH wrote the manuscript.

Conflicts of Interest

The authors have no competing interests to declare that are relevant to the content of this article.

Declaration

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