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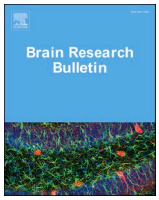

Research Report

The impact of chronic fentanyl administration on the cerebral cortex in mice: Molecular and histological effects

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ABSTRACT

Purpose: Fentanyl, a fully synthetic opioid, is widely used for severe pain management and has a huge abuse potential for its psychostimulant effects. Unlike other opioids, the neurotoxic effects of chronic fentanyl administration are still unclear. In particular, little is known about its effect on the cerebral cortex. The current study aims to test the chronic toxicity of fentanyl in the mice model.

Methods: Adult male Balb/c mice were chronically treated with low (0.05 mg/kg, i.p) and high (0.1 mg/kg, i.p) doses of fentanyl for 5 consecutive weeks, and various neurotoxic parameters, including apoptosis, oxidative stress, and neuroinflammatory response were assessed in the cortex. Potential histological as well as neurochemical changes were also evaluated.

Results: The results of this study show that chronic fentanyl administration induced intense levels of apoptosis, oxidative stress, and neuroinflammation in the cerebral cortex. These findings were found to be correlated with histopathological characteristics of neural degeneration and white matter injury. Moreover, fentanyl administration was found to reduce the expression of both NMDA receptor subunits and dopamine receptors and elevate the level of epidermal growth factor (EGF).

Conclusion: Fentanyl administration induced neurotoxic effects in the mouse cerebral cortex that could be primarily mediated by the evoked oxidative-inflammatory response. The altered expression of NMDA receptors, dopamine receptors, and EGF suggests the pernicious effects of fentanyl addiction that may end in the development of toxic psychosis.

1. Introduction

Fentanyl is a fully synthetic opioid that is widely utilized for both anesthesia and severe pain management, but also with huge abuse potential (Liu et al., 2016; Wilde et al., 2019). Like most opioids, it produces its analgesic and rewarding effects preferentially via activation of

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mu (μ) opioid receptors (Wilde et al., 2019; Comer and Cahill, 2019). However, the unique pharmacological properties of fentanyl, including high lipid solubility and strong binding affinity at μ opioid receptors result in a rapid onset, short duration of action, and greater analgesic potency compared to other opioids like morphine (Peng and Sandler, 1999; Rajan et al., 1998).

Despite its medically beneficial use, the widespread recreational use of illicit fentanyl for its powerful rewarding effects has markedly increased in recent years (Di Trana and Del Rio, 2020; Volkow, 2021). Synthesis of fentanyl is relatively simple and cheap compared to other synthetic or semisynthetic opioids such as heroin, and because it is so potent that it can produce strong rewarding and reinforcing effects, fentanyl is considered among the most commonly abused drugs found in the illicit drug market (Volkow, 2021; Han et al., 2022). In addition, fentanyl is commonly mixed with heroin and other illicit drugs to increase their potency at a low cost (Kuczynska et al., 2018).

It is now well documented that acute and chronic use of fentanyl is associated with numerous adverse health effects. Adverse effects linked with the acute use of fentanyl include, but are not limited, to respiratory depression, bradycardia, nausea and vomiting, and muscle rigidity (Mystakidou et al., 2006). Whereas its chronic use can be additionally associated with the emergence of neuropsychiatric symptoms such as cognitive impairment, severe sedation, hallucinations, and delirium. Moreover, the long-term use of fentanyl can result in tolerance and physical dependence which ultimately may lead to an increase in the minimal effective dose and raise the risk of overdose death (Colak et al., 2015; Cunha-Oliveira et al., 2008; Lim et al., 2018; Okon and George, 2008).

The potential mechanism by which psychostimulants and opioids exert their neurotoxic effects is suggested to be primarily linked to the direct or indirect action of the substance on neurotransmitter systems, particularly dopaminergic and glutamatergic (Cunha-Oliveira et al., 2008). Associated with the occurrence of addiction, the long-term intake of the drug can lead to several pathological processes, including alteration in neurotransmitter systems, mitochondrial dysfunction, increased oxidative stress and apoptosis, and glial/microglial cell overactivation (Cunha-Oliveira et al., 2008). It should be noted that although neuronal dysfunction induced by abuse of psychostimulants and opioids appears first in brain circuits and structures implicated in processing reward, prolonged use of the drug can affect other brain areas and lead to dysfunction in memory, learning, motivation/drive, executive control, and cognitive functions (Volkow et al., 2003).

Opioid abuse and overdoses, mostly attributed to the use of illicit fentanyl, are now considered one of the leading causes of death in the United States (Blanco and Volkow, 2019). However, the neurotoxicity of fentanyl compared to other opioid agonists, such as morphine and heroin, has been much less studied. In particular, the deleterious effect of chronic fentanyl use on the cerebral cortex is still not well investigated. Therefore, in the present study, we examined the effects of chronic administration of fentanyl on the cerebral cortex in Balb/c mice. We assessed various neurotoxic parameters, including apoptosis, oxidative stress, and neuroinflammation. The harmful effects of fentanyl were further demonstrated by corresponding histopathological changes in the cerebral cortex.

2. Materials and methods

2.1. Animals

Thirty adult males of Balb/c mice (25–35 g) were individually housed in a controlled room with 21–25 °C temperature and a 12:12 h light/dark cycle with ad libitum access to food and water. Mice were divided randomly into three groups. The first experimental group (n = 10) received a dialy intraperitoneal injections of 0.05 mg/kg of fentanyl (Cayman Chemical, Michigan, USA) dissolved in a vehicle (3 % dimethyl sulfoxide in PBS) for 5 consecutive weeks (35 days). The second experimental group (n = 10) received a daily injection of 0.1 mg/kg of fentanyl for the same period. Low and high doses of analgesic fentanyl were selected based on the literature (Miao et al., 2015; Raleigh et al., 2019). The control group (n =10) received a daily intraperitoneal injection of vehicle (3 % dimethyl sulfoxide in PBS) for 5 consecutive weeks. One day after the last injection, animals were sacrificed by decapitation, their brains were isolated, and the cortex was dissected.

2.2. Tissue processing and paraffin embedding

Whole or half fresh brains were fixed in 10 % paraformaldehyde (PFA, Sigma Aldrich, Poole, UK) for 24 hours at 4 oC and processed for paraffin embedding. Each fixed brain was first dehydrated in graded ethanol (70 %, 80 %, 90 %, and 100 %) for 90 minutes. Samples were then incubated in xylene for 2 h before embedding them in paraffin (Spin STP 120 tissue processor, Thermo Fisher Scientific, USA). Paraffin-embedded tissue was cut coronally into 8-um thick sections using a Leica RM 2135 microtome (Leica Biosystem, Germany) and mounted on coated slides using eosin and hematoxylin stain and TUNEL assay.

2.3. Hematoxylin and Eosin (H&E) Staining

Each Section was dewaxed in xylene for 4 min and rehydrated in graded ethanol (100 %, 90 %, and 70 %) for 1 min. Sections were then stained with Mayer's Hematoxylin (10 min), rinsed in tap water, and differentiated with 1 % acid alcohol solution (10 s). Sections were finally counter-stained with Eosin (1 min), dehydrated in graded ethanol, and coverslipped using the DPX Mountant (Techno Pharma Chem, India).

2.4. TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) assay was performed to detect DNA fragmentation generated during apoptotic cell death. The staining was performed using an in situ cell death detection kit (DeadEnd™ Colorimetric TUNEL System kit, Promega, Madison, USA) according to the manufacturer's instructions. Quantitative assessment of the apoptosis index was performed by manually counting the number of TUNEL-positive cells from randomly selected 5 microscopic fields (at X 400 magnification) taken from three brains for each group. The number of total cells and the number of TUNEL-positive cells within each section were counted using the Adobe Photoshop CS6 software, and the percentage of TUNEL-positive cells in the cortex of the three groups was calculated.

2.5. Total RNA isolation and cDNA synthesis

Using a total RNA isolation kit (JenaBioscience, Germany), total RNA was extracted from cortical samples following the manufacturer's instructions. The QuantiFluor RNA System and Quantus Fluorometer from Promega, Madison, USA, were used to determine the quantity and purity of the RNA (Promega, Madison, USA).

Additionally, first-strand cDNA was produced from RNA using a RevertAid First-strand cDNA synthesis kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions using 1 ug of total RNA. Before real-time PCR was employed to evaluate gene expression, these samples were frozen at −80°C.

2.6. Quantitative Real-Time PCR (qPCR)

Using the quantitative real-time PCR (qPCR) method, the expression levels of the mRNAs were determined. qPCR was performed using a Line-Gene 9600 Real-Time PCR equipment (Bioer Technology, Bingjiang, China). The primer sets (shown in Table 1) utilized for various genes were created using the Primer 3 software (Whitehead Institute for Biomedical Research). Following the instructions provided by the manufacturer, the qPCR reaction was carried out using the SYBR-PCR

Table 1
Primers' sequences used for qPCR.

Gene	Forward primer	Reverse primer
Bax	5′- CTGAGCTGACCTTGGAGC-3′	5′- GACTCCAGCCACAAAGATG-3
Bcl-2	5′- GTGGATGACTGAGTACCT −3′	5′- CCAGGAGAAATCAAACAGAG −3′
NOX4	5′-TCATTTGGCTGTCCCTAAACG-3′	5′-AAGGATGAGGCTGCAGTTGAG-3′
NOX2	5′- CTGGTGTGGTTGGGGCTGAATGTC-3′	5′- CAGAGCCAGTGCTGACCCAAGGAGT-3′
iNOS	5′-ATGGACCAGTATAAGGCAAGC-3′	5′-GCTCTGGATGAGCCTATATTG-3′
NCF1	5′-TCCCTGCATCCTATCTGGAG-3′	5′-TCCAGGAGCTTATGAATGACC-3′
TNF-α	5′-AAGCCTGTAGCCCACGTCGTA-3′	5′-AGGTACAACCCATCGGCTGG-3’
IL-1β	5’-AACCTGCTGGTGTGTGACGTTC-3	5’-CAGCACGAGGCTTTTTTGTTGT-3′
IL-6	5’-ACAACCACGGCCTTCCCTACTT-3′	5’-CACGATTTCCCAGAGAACATGTG-3′
EGF	5′-CTGCCAAGGCACAAGTAACA-3′	5′-ATTGGGACAGCTTGGATCAC-3′
Grin1	5’-GCGACACGGCTCTTGGAAG-3’	5’-GTGGTACGGTGCGAAGGAAG-3’
Grin2a	5’-GGGAGGGATGAAGGCTGTAAAC-3’	5’-GATGAAGGTGATGAGGCTGAGG-3’
Grin2b	5’-TCCGCAGCACTATTGAGAACAG-3’	5’-GAAGGCACCGTGTCCGTATC-3’
D1	5′-AACTGTATGGTGCCCTTCTGTGG-3′	5′-CATTCGTAGTTGTTGTTGCCCCG-3′
D2	5′-CACTCCGCCACTTCTTGACATACA-3′	5′-TCTCCTCCGACACCTACCCCGA-3′

master mix (FirePol qPCR Master Mix). To normalize the expression for the mRNA levels, the mean of housekeeping gene GAPDH was used as an internal reference. Each sample was examined in triplicate. The fold expression was calculated according to the 2-ΔΔCt method.

2.7. Malondialdehyde assay

The level of malondialdehyde (MDA) in the cortex was measured as marker of lipid peroxidation using commercially available TBARS assay kit (R&D systems, KGE013) and normalized to protein concentration. The assay procedure was performed following the manufacturer's instructions. The colored product of the reaction of MDA with thiobarbituric acid was measured spectrophotometrically at 532 nm. The MDA content was expressed as nmol/mg protein.

2.8. Western Blot analysis

The protein levels for Iba-1 and EGF were measured by western blotting. Briefly, total protein was extracted using RIPA lysis buffer, and its concentration was measured by the Bradford method. A 35 μg of extracted protein was loaded onto SDS-PAGE. Following electrophoresis, proteins were transferred to the PVDF membrane and incubated with appropriate primary and secondary antibodies. The membranes were developed using Evolution Capt Edge of FUSION SOLO X and bands were quantified using ImageJ software. The bands of the primary antibodies were normalized to the corresponding b- actin band, and the expression percentages were plotted.

2.9. Statistical analysis

All statistical analyses were conducted using GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, CA, USA).

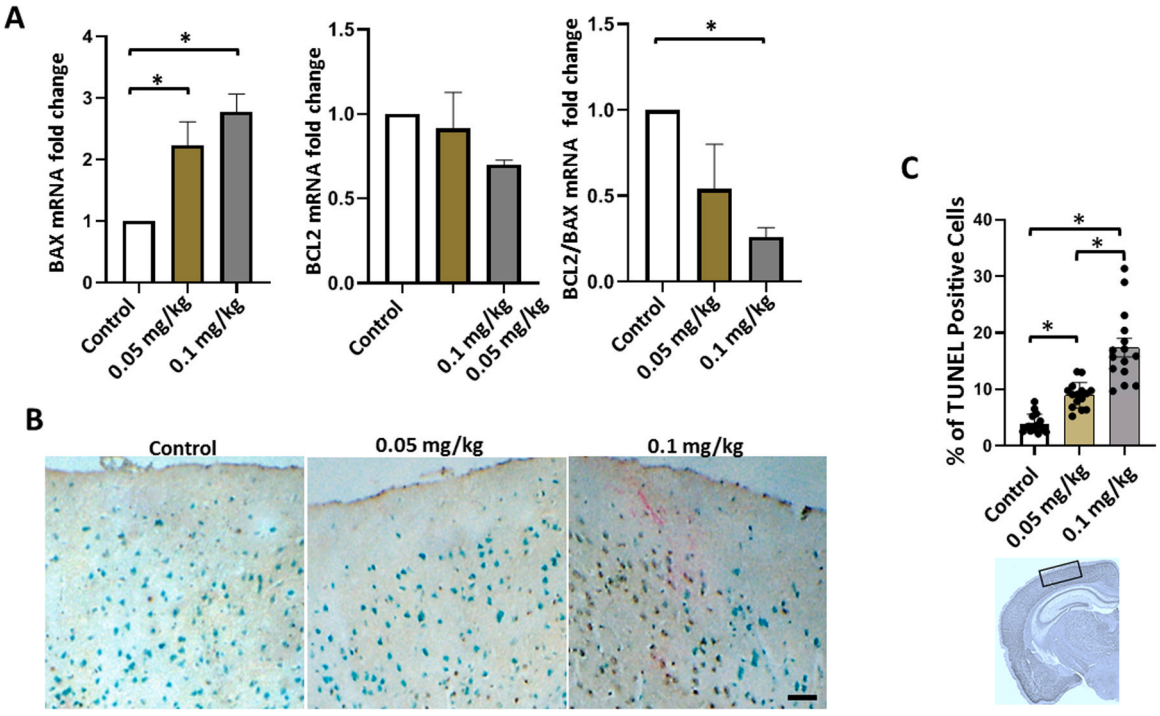

Fig. 1. (A) The relative expression levels of BAX and BCL2 mRNA, and BCL2/BAX ratio in the cerebral cortex of low-dose (0.05 mg/kg) and high-dose (0.1 mg/kg) fentanyl-treated mice compared with the control mice. (B) Representative images of TUNEL assay, TUNEL-positive nuclei appear dark brown. (C) Quantitative assessment of the percentage of TUNEL positive cells in the cerebral cortex (n=3) for each group. The frame in the coronal brain section indicates the area of interest for the cells counting in TUNEL assay analysis. Data presented as mean ± SEM. * p-value< 0.05; scale bar (in B): 50 μm.

The results were presented as mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. $p < 0.05$ considered a significant difference. For more detailed statistical results, please refer to the Supplementary Material file.

3. Results

3.1. Analysis of apoptosis

To investigate the effect of chronic fentanyl treatment on apoptosis in the cerebral cortex, we investigated the mRNA expression of pro-apoptotic marker Bax, anti-apoptotic marker Bcl-2. As shown in Fig. 1A, the expression of Bax significantly increased in both low and high-dose groups compared to the control group. However, significant decrease in the Bcl2/Bax was only observed in high-dose group. These results indicate potential susceptibility of apoptosis induction in the cerebral cortex.

To further prove increased apoptosis in the cerebral cortex, apoptosis-related DNA fragmentation was also evaluated using the TUNEL assay. As shown in Fig. 1B, a higher number of apoptotic nuclei were detected in the cortex of the mice group treated with high-dose fentanyl compared to the low-dose treated group, and a few positively stained nuclei in the cortex of vehicle control mice. Similarly, quantitative assessment of TUNEL-positive cells showed a significant increase in apoptosis index of low and high-dose groups compared to the control group; furthermore, a significant difference was found between the low-dose group and high-dose group (low-dose group vs high-dose group (Fig. 1C).

3.2. Measurement of oxidative stress

The mRNA level of oxidative stress-related genes NOX2, NOX4, iNOS, and NCF1 was evaluated in this study. The expression of NOX2 and iNOS showed a consistent significant elevation in the cortex of high-dose group compared to the low dose and control groups. On the contrary, no significant increase was observed in the expression of NOX4 and NCF1 in low and high dose groups compared to the control group (Fig. 2A).

The level of lipid peroxidation, as a marker of oxidative stress, was also evaluated by measuring malondialdehyde (MDA) levels. As shown in Fig. 2B, our results showed that although fentanyl treatments tended to increase MDA levels in the cortex, a substantial up-surging was only seen in the high-dose group compared to the vehicle control group.

3.3. Measurement of neuroinflammatory response

The potential impact of fentanyl treatment on immune response in the cerebral cortex was also assessed in this study. The mRNA levels of proinflammatory cytokines interleukin IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) were evaluated. Our results showed that the expression of these three markers was significantly elevated in the cortex of high-dose group compared to the control group; however, only IL-1β

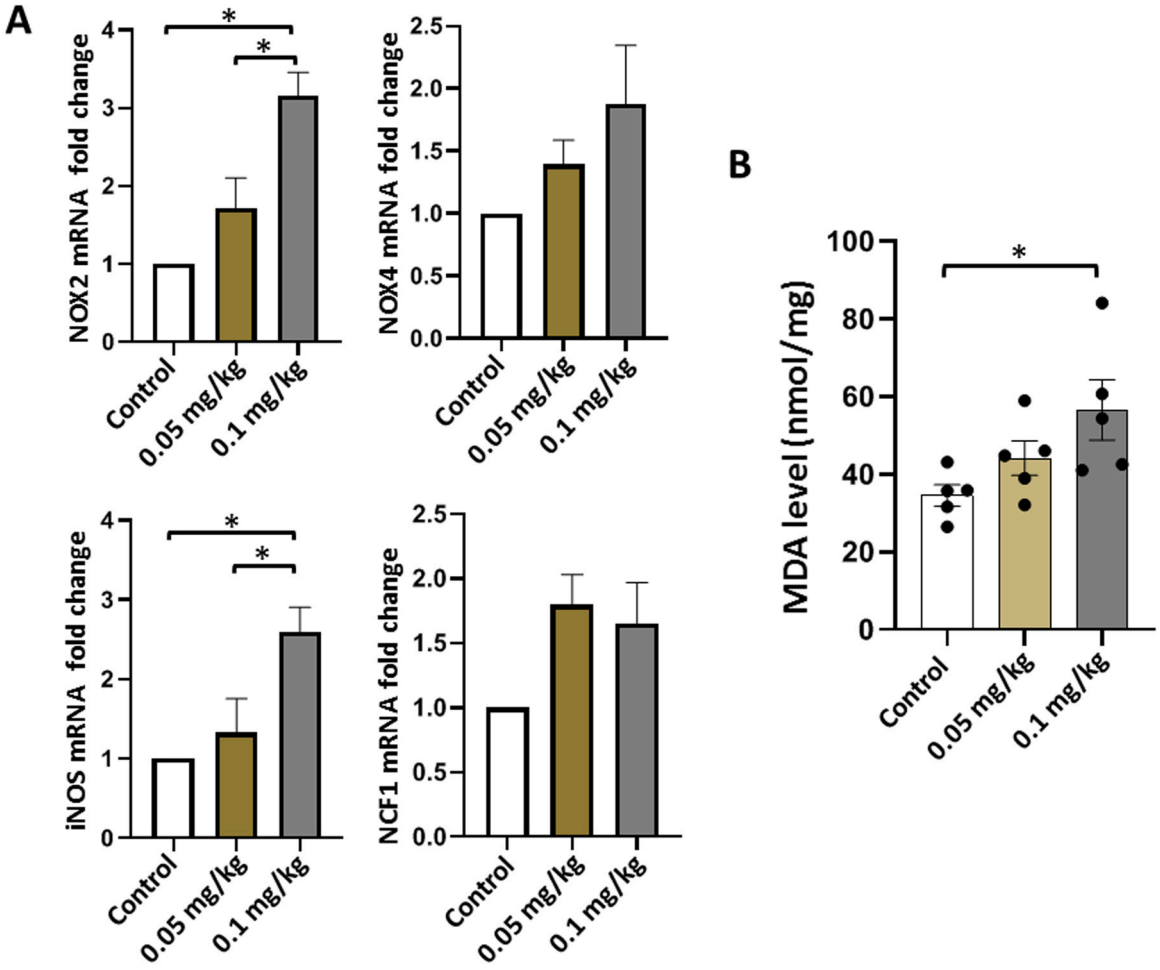

Fig. 2. (A) The relative expression levels of NOX2, NOX4, iNOS, and NCF1 mRNA in the cerebral cortex of low-dose (0.05 mg/kg) and high-dose (0.1 mg/kg) fentanyl-treated mice compared with the control mice. (B) The malondialdehyde (MDA) levels in the cerebral cortex (n=4) of the three groups. Data presented as mean ± SEM. * p-value< 0.05.

showed a significant increase in expression in the cortex of low-dose group compared to the control group. The significant difference in the expression of IL-1β, IL-6, and TNF-α between low-dose and high-dose groups indicates a dose-dependent activation of the immune response (Fig. 3A).

To confirm a correlation between fentanyl treatment and the neuroinflammatory response in the cerebral cortex, we performed a western blot analysis to investigate the expression of Iba-1, a potential sensitive marker for microglia activation. As shown in Fig. 3B, the protein expression of Iba-1 was significantly increased in the cortex of high-dose group compared to the control group. However, its expression wasn't significantly increased in the cortex of low-dose group compared to the control group.

3.4. Histopathology

To further confirm the corresponding deleterious effect of fentanyl on the brain, the treated mice with different doses were examined histopathologically and compared to the control group. As shown in Fig. 4 (A–D), the microscopic examination of the cortex of low-dose group revealed the presence of several histopathological changes such as apoptosis of neurocytes, increase in inflammatory cells mainly microglia and disorganized neurocytes admixed with microglia (epileptic focus). In addition, white matter fragmentation and aggregation of myelin surrounded by microglia and fasciculation were also demonstrated in low-dose group (Fig. 4 (E–G)). Severe degenerative changes were seen in the microscopic examination of the cortex of high-dose group, including wide areas of cortical necrosis and dense cortical inflammation; as well as extensive white matter necrosis (Fig. 5 (A–C)).

3.5. Expression of EGF in the cerebral cortex

As shown in Fig. 5A, the mRNA level of EGF exhibited a significant increase in expression in the cortex of both low and high-dose groups compared to the control group. The protein expression of EGF was also detected by western blot analysis. As shown in Fig. 5B, consistent with mRNA expression, the protein expression of EGF was revealed to be significantly elevated in the cortex of high-dose group compared to

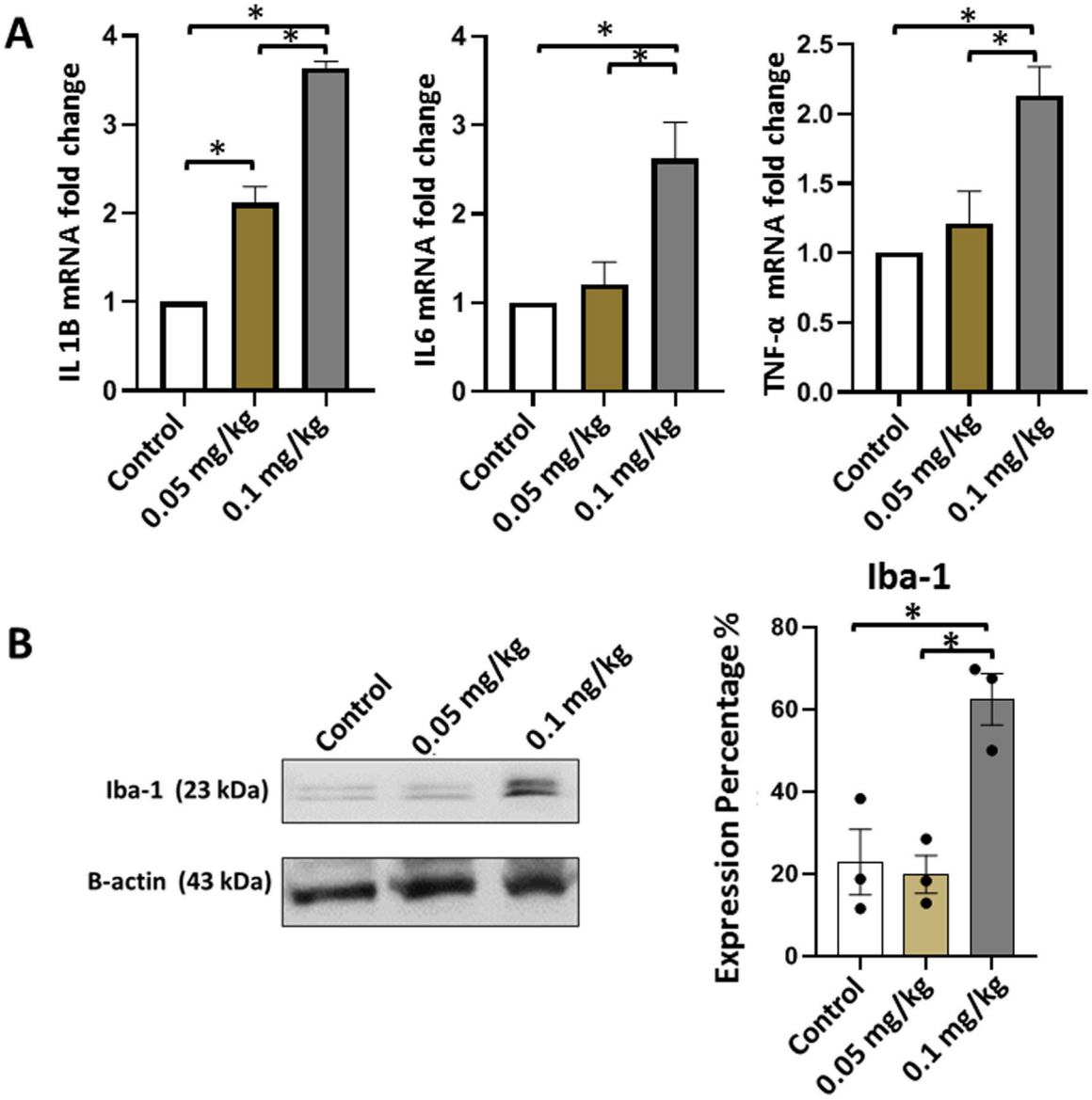

Fig. 3. (A) The relative expression levels of proinflammatory cytokines IL-1 Beta, IL-6, and TNF-α mRNA in the cerebral cortex of low-dose (0.05 mg/kg) and high-dose (0.1 mg/kg) fentanyl-treated mice compared with the control mice. (B) Western blot analysis for the expression of Iba-1 in the cerebral cortex of the three groups. Data presented as mean ± SEM. *, p-value< 0.05.

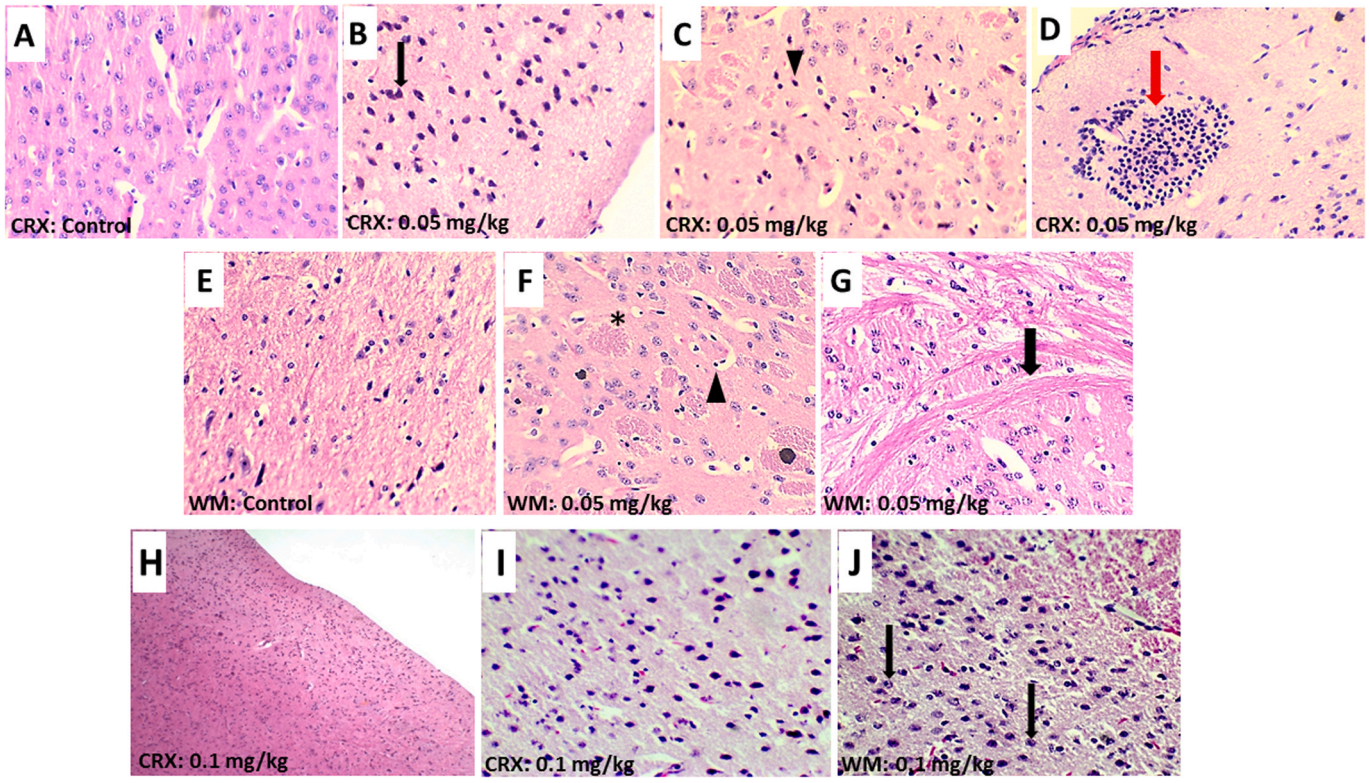

Fig. 4. Cortical (CRX) and white matter (WM) specimens stained with H&E. (A) Negative control showing normal cortex. (B) Cortex of mouse received 0.05 mg/kg fentanyl showing apoptosis of neurocytes (black arrow). (C) Cortex of mouse received 0.05 mg/kg fentanyl showing inflammation seen as increased microglia (arrow head). (D) Cortex of mouse received 0.05 mg/kg fentanyl showing epileptic focus formed of disorganized neurocytes admixed with microglia (red arrow). (E) Negative control showing normal white matter. (F) The white matter of mouse received 0.05 mg/kg fentanyl shows fragmentation and aggregation of myelin (asterisk) which surrounded by inflammatory cells formed of microglia (arrow head). (G) The white matter of mouse received 0.05 mg/kg fentanyl shows increased white matter with fasciculation (black arrow). (H and I) Cortex of mouse received 0.1 mg/kg fentanyl shows wide areas of necrosis consisting of granular structure with less background and more fragmented nuclei (karyorrhexis) infiltrated with dense inflammatory cells. (J) The white matter of mouse received 0.1 mg/kg fentanyl shows a less intense degree of necrosis and apoptosis with the presence of more viable oligodendrocytes (black arrows). A-G, I, and J: at X400 magnification; H: at X 100 magnification.

control group ($P = 0.0113$). However, the protein expression of EGF was significantly downregulated in the cortex of low-dose group compared to control group ($P = 0.034$).

3.6. *Expression of NMDA receptor subunits and dopamine receptors in the cerebral cortex*

The mRNA levels of N-Methyl-D-aspartate (NMDA) receptor subunits (Grin1, Grin2A, and Grin2B) and dopamine receptors (D1 and D2) were evaluated in this study. As shown in Fig. 6A, fentanyl treatment (low and high-dose groups) was associated with a remarkable decline in the expression of Grin1 and Grin2A compared to the control group, with no significant alterations observed in the mRNA level of Grin2B in the cortex.

The mRNA levels of dopamine receptors D1 and D2 were also evaluated, and the results showed that the expression of both D1 and D2 receptors was significantly downregulated in the cortex of low and high-dose groups compared to the control group (Fig. 6B).

4. Discussion

Fentanyl is a potent synthetic opioid that is commonly used for managing chronic pain in patients who develop tolerance to opiates such as morphine. Although it is a controlled medication and tightly regulated in clinical settings, this narcotic medication harbors a high risk of misuse and abuse which can be lethal. The number of fentanyl-related deaths due to extensive consumption and overdoses is increasing substantially worldwide. The cerebral cortex is responsible for processing higher and more sophisticated mental, behavioral, reasoning, memory, and decision-making functions. The endogenous opioid system in the cerebral cortex can be a potential target for opioids which can lead to structural and functional changes in the cerebral cortex, thus disturbing its normal functioning (Wang et al., 2016; van Steenbergen et al., 2019). Surprisingly, little is known about the deleterious effects of long-term exposure to fentanyl on the cerebral cortex. This study shows for the first time that chronic fentanyl usage produces many molecular and histological alterations in the cerebral cortex. Chronic fentanyl administration was found to be associated with histopathological findings of neural degeneration that are associated with elevated levels of apoptosis and increased oxidative stress and neuroinflammation. Furthermore, fentanyl administration was also demonstrated to be linked with altered expression of NMDA receptors and EGF levels in the cortex that may explain the psychotic symptoms related to long-term opioid use. The findings of this study are fundamental to anticipating the mechanisms of mental and behavioral changes that are associated with fentanyl addiction.

The present study found that chronic administration of fentanyl is associated with histopathological characteristics of neuronal degeneration and necrosis in the cerebral cortex. In line with these findings, fentanyl administration was also shown to be correlated with a decrease in Bcl-2/Bax mRNA ratio and a dose-dependent increase in the percentages of TUNEL-positive cells in the cerebral cortex, suggesting the activation of the apoptosis signaling pathway in response to fentanyl administration. These results are consistent with previous reports

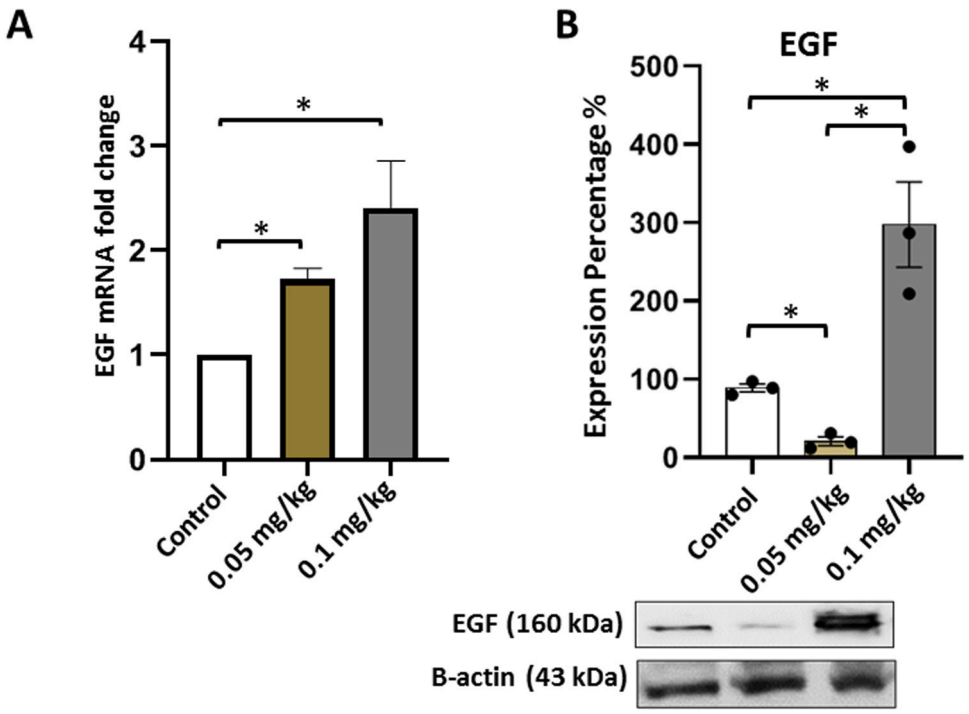

Fig. 5. (A) The relative expression levels of EGF mRNA in the cerebral cortex of low-dose (0.05 mg/kg) and high-dose (0.1 mg/kg) fentanyl-treated mice compared with the control mice. (B) Western blot analysis for the expression of EGF in the cerebral cortex of the three groups. Data presented as mean ± SEM. * p-value< 0.05.

demonstrating that chronic opioid exposure can induce apoptosis by several mechanisms, including caspase activation, decreased Bcl-2/Bax ratio, cytochrome c release, oxidative DNA damage, and increased expression of apoptosis-related proteins Fas, FasL, and Bad (Cunha-Oliveira et al., 2008, 2007; Hosseindoost et al., 2022; Mao et al., 2002; Tramullas et al., 2008). Along with apoptosis induction, our results showed that fentanyl was also associated with histological features of white matter injury and fasciculation, which is likely incongruent with recent reports showed that opioids can modulate the functions of oligodendrocytes, and leads to white matter injury and abnormal myelin structure (Merhar et al., 2019; Velasco et al., 2021). Therefore, the current study suggests that chronic fentanyl administration may be implicated in the structural integrity of the cerebral cortex, which provides a conceivable explanation of volume reduction in certain regions of the cortex upon long-term opioid use (Wang et al., 2016, 2012; Gardini and Venneri, 2012; Shi et al., 2020; Wollman et al., 2017).

To unravel the possible mechanistic explanation for the above-mentioned findings, we investigated whether chronic administration of fentanyl can induce oxidative stress as a potential mediator of opioids-induced neurotoxicity. The link between prolonged exposure to opioids and the induction of oxidative stress has been reported in several studies. Exposure to morphine and heroin was shown to be associated with impairment in the antioxidant defense system, including a reduction in the level of glutathione and various antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase (Guzman et al., 2006; Ozmen et al., 2007; Qiusheng et al., 2005; Xu et al., 2006). Recently, it was shown that opioids exposure increases the level of oxidative DNA damage level in prefrontal cortex that can be ameliorated by the treatment of reactive oxygen species (ROS) scavenger N-acetylcysteine (Wang et al., 2023). In line with these studies, our results revealed that fentanyl administration elevates the expression of major oxidative stress markers, and increases the cerebral cortex level of MDA, which is one of the end products of lipid peroxidation, and its overproduction is strongly correlated with intense oxidative stress in the brain. The neuronal membranes are known to have a rich profile of polyunsaturated fatty acids, which are particularly susceptible to free radical attack (Gadoth and Göbel, 2011). Although the mechanism by which opioid receptor agonists evoke toxicity and ROS in the brain is suggested to be primarily linked to an elevation in the synaptic concentrations of dopamine and increase in the extrasynaptic glutamate levels (Cunha-Oliveira et al., 2008; Jones et al., 2000; Rego and Oliveira, 2003), it is also possible that the respiratory depression associated with opioids use, particularly fentanyl, may increase the risk of brain hypoxia, which can trigger oxidative and nitrosative stress, and ultimately leads to cellular damage (Kiyatkin, 2019).

Besides the increase in oxidative stress level, data presented in this study revealed that fentanyl administration can incite neuroinflammatory response in the cerebral cortex, which is manifested by elevated mRNA expression of proinflammatory cytokines IL-1 Beta, IL-6, and TNF-α, increased levels of microglia activation-related protein Iba-1, as well as extensive microglia cells proliferation observed in the histological examination of the cortex in fentanyl treated mice. Herein, two potential mechanisms are suggested for the activation of neuroinflammatory response upon exposure to fentanyl, by direct activation of opioid receptors on the surface of microglia cells (Zhang et al., 2020) and as an indirect consequence of increased oxidative stress. In this regard, the accumulation of ROS in a chronic state of oxidative stress can potentiate the signaling that leads to the activation of the microglia cells and modulate the proinflammatory response (Solleiro-Villavicencio and Rivas-Arancibia, 2018).

Despite that opioids are not typically associated with psychosis, it has been described that opioids-dependent patients may present numerous psychotic symptoms (such as hallucinations, delusions, and irritability) while using opioids or after the sudden withdrawal (Fiorentini et al., 2021; Lozano-Lopez et al., 2021). However, little is known about the potential role of opioids, particularly fentanyl as a powerful synthetic opioid, in the pathogenesis of induced psychotic effects related to their chronic use. At this point, the results of this study suggested that fentanyl use may lead to numerous molecular changes in the cerebral cortex that could be interrelated with specific features of psychotic disorders. The downregulation of the NMDA receptor subunits may reflect potential hypofunction in NMDA receptor signaling, which is

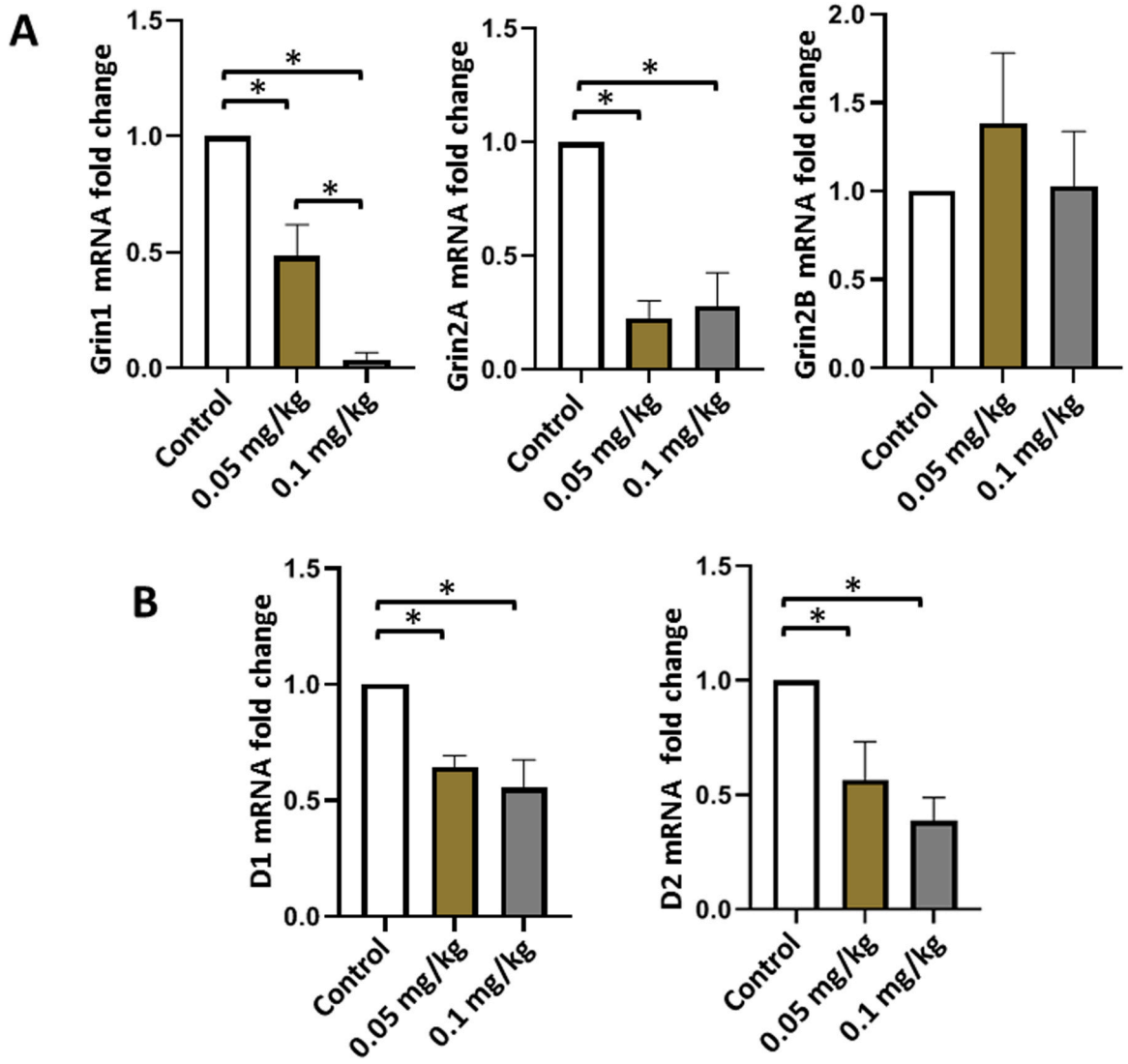

Fig. 6. (A) The relative expression levels of NMDA receptors subunit Grin1, Grin2a, and Grin2b mRNA in the cerebral cortex of low-dose (0.05 mg/kg) and high-dose (0.1 mg/kg) fentanyl-treated mice compared with the control mice. (B) The relative expression levels of dopamine receptors D1 and D2 mRNA in the cerebral cortex of low-dose (0.05 mg/kg) and high-dose (0.1 mg/kg) fentanyl-treated mice compared with the control mice. Data presented as mean ± SEM. * p-value< 0.05.

known as one of the main biological substrates of psychotic disorders (Balu, 2016; Espana et al., 2021). The expression of both dopamine receptors D1 and D2 was also demonstrated to be markedly down-regulated, indicating that fentanyl may be associated with increased dopamine activity, which is also described as an etiologic factor in triggering psychosis (Kupnicka et al., 2020; Claypool et al., 2022; Kesby et al., 2018). Furthermore, The EGF was shown to be conspicuously overexpressed in the cortex of fentanyl-treated mice. Existing piece of evidence shows that elevated levels of EGF are implicated in the emergence of psychotic symptoms primarily through the dysregulation of dopaminergic signaling (Koido et al., 2016; Sotoyama et al., 2013). Moreover, high levels of EGF were also found to alter the function of NMDA receptors (Chan et al., 2015; Tang et al., 2015). It is worth mentioning that the differential expression between EGF mRNA and protein when different doses of fentanyl were used can be explained by possible interference of fentanyl with the rate of EGF protein synthesis. Further, the lack of correlation between mRNA and its corresponding protein can be related to the type of stress existed, the coding region, the epigenetic differences, and the time lapse before starting protein synthesis (Liu, 2016). These findings need further investigation in future studies.

Our study holds some limitations, including the impact of gender on study findings as our study didn't include female mice. However, previous report revealed that females are usually more vulnerable to activating the reinforcement system by drugs of abuse and rapidly progressed to dependence than males (Lynch et al., 2002), thus using the same doses for males and females may be considered unsuitable and produced inconclusive results as females may require different doses to study the chronic effects of fentanyl administration. Future studies should investigate the effects of chronic fentanyl abuse on the cerebral cortex of female animals to provide better understanding and more depth analysis of the molecular changes associated with fentanyl abuse on the brain.

5. Conclusion

The rate of fentanyl abuse is increasing tremendously worldwide. Chronic fentanyl use is associated with many neurological symptoms that need further investigation, thus it is clinically important to understand the underlying mechanisms that lead to opioids-induced neurotoxicity. Chronic fentanyl ingestion produces many histopathological and molecular changes in the brain cortex that can explain the neurological-related symptoms reported in many studies. These pathological changes in the cortex are related to triggering intense oxidative

stress and neuroinflammation that can induce apoptosis. Psychosis risk in opioid chronic users can somehow be associated with decreased expression of NMDA receptors and elevated EGF levels. Targeting oxidative stress and neuroinflammation can repress the neurotoxic effects of fentanyl use and improve the treatment outcomes in addictive users.

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Ethical statement

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee for the use of animals in research at Yarmouk University (Approval No. IACUC/2022/6).

CRediT authorship contribution statement

Doaa S. Ghorab: Formal analysis, Data curation. **Waseem El-Huneidi:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Raed M. Al-Zoubi:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Ayman Alzu'bi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Worood Bani Baker:** Formal analysis, Data curation. **Bahaa Al-Trad:** Investigation, Formal analysis, Data curation. **Mazhar Salim Al Zoubi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Manal Isam AbuAlArjah:** Formal analysis, Data curation. **Ejlal Abu-El-Rub:** Formal analysis, Data curation. **Lena Tahat:** Formal analysis, Data curation. **Ahmed MNZ Helaly:** Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainresbull.2024.110917.

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