Toxicology Reports 12 (2024) 91–99

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Reciprocal crosslink among MeCP2/BDNF /CREB signaling pinpointed in autism spectrum disorder

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ARTICLE INFO

Handling Editor: Prof. L.H. Lash

Keywords:
Autism spectrum disorder
Neuquinon
Liposomal neuquinon
BDNF
CREB
MeCP2

ABSTRACT

Autism spectrum disorder, or individual disability (ID), is a condition characterized by complications in social interaction, restricted repetitive behavior, and difficulties in social communication. Neuquinon (NQ) possess a powerful therapeutic potential in various neurodegenerative disease. Nevertheless, contributing to NQ's low water solubility and bioavailability, its medicinal use has been constrained. Liposomes were supposed to be prospective drug-delivering agents for NQ, crossing the blood-brain barrier (BBB), and reaching the target organs. The current investigation aims to track the signaling pathways that govern NQ and liposomal neuquinon (LNQ) action in autistic models generated by ethyl formic acid. The neurotransmitters gamma amino-butyric acid (GABA), acetylcholine (ACh), and acetylcholinesterase (AChE) in addition to, the gene expressions of brain-derived neurotrophic factor (BDNF), cAMP response element-binding protein (CREB), and methyl-CpG-binding protein 2 (MeCP2) and the DNA damage COMET analysis at different time intervals of the study, were assessed. EFA in a dose of 500 mg/kg BW was used to induce autism in rats, and then NQ and LNQ were administered in 10 mg/kg and 2 mg/kg BW, respectively. The results revealed that NQ and LNQ significantly down-regulated BDNF, GABA, and AChE; on the other hand, they up-regulated MeCP2, CREB gene expressions, and ACh action. NQ and LNQ displayed improvement in DNA damage in almost all brain regions after EFA alterations; even better results were noticed post-LNQ therapy. Therefore, it may be concluded that neuquinon and liposomal-loaded neuquinon have a therapeutic index versus EFA-induced autism in a rat model.

1. Introduction

Autism spectrum disorder (ID) is a neurodevelopmental disorder resulting from prevalent abnormalities in social interaction, communication, repetitive behaviors, and restricted interests caused by improper neurodevelopmental conditions; it appears in children before three years old [13]. The etiology of autism stems from genetic, neurological, and environmental factors, with oxidative stress and neurotransmitters serving as a crucial pathway connecting these risk factors [53].

In the food industry, ethyl formic acid, a short-chain fatty acid produced metabolically by gut bacteria, is frequently employed as an anti-mold agent [42]. EFA can cause various central nervous system (CNS) disorders by penetrating physiological lipid barriers like intestinal-circulatory barriers, blood-brain barriers (BBB), cerebrospinal fluid, and neuronal cells. ID is accomplished with boosting neurotransmitter release and creating an intracellular acidosis known as ethyl-formic acidemia [4]. EFA causes reactive astrogliosis, oxidative stress, glutathione depletion, mitochondrial dysfunction, metabolic or immunological problems, and further changes in phospholipid and acylcarnitine levels [25]. In addition, EFA can also cause neurological impairment, affecting synaptic transmission and other types of neuronal activity [31] via altering brain fatty acid metabolism and disturbing antioxidant balance. Consequently, ethyl-formic acid was utilized as an autistic rat model [1].

Neurotransmitters and neuropeptides possess crucial role in normal brain development and contribute to memory, behavior, and motor activity regulation. Indeed, they influence neuronal cell migration, differentiation, synaptogenesis, apoptosis, and synaptic plasticity. Therefore, a neurotransmitters dysfunction can contribute to impairment in the process of brain development, leading to autism [7].

Acetylcholine is a neurotransmitter in the cholinergic system that is crucial for responding to environmental stimuli or threatening actions. ACh is active in the basal forebrain projections in the cortex and limbic structures. Therefore, cognitive functions of attention, memory,

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https://doi.org/10.1016/j.toxrep.2023.12.008
Received 2 September 2023; Received in revised form 1 December 2023; Accepted 22 December 2023
Available online 26 December 2023

learning, cognitive flexibility, and social interaction are all influenced by acetylcholine [39]. A low level of ACh is a potential contributor to ID symptomatology. ID patients' basal forebrain cholinergic nuclei revealed unusual size, number, and structure [20].

ID is caused by mutations in the methyl-CpG-binding protein encoded by MeCP2, which is essential for suppressing gene expression. Interestingly, other research studies have suggested the role of Methyl Cytosine Binding Protein (MeCP2) gene in the epigenetic regulation of genes expression in ID [51]. The BDNF gene is one of the targets of the MeCP2 gene. Increased expression of BDNF contributes to cell death and neurodegeneration in ID [60]. Based on its role in chromatin dynamics, MeCP2 could interfere with genes involved in antioxidant and radical scavengers' mechanisms in ID patients [23].

A cellular transcription factor called CREB (cAMP response element-binding protein) is involved in the generation of long-term memories and neuronal plasticity, proliferation, differentiation, survival, and long-term synaptic potentiation in the brain [40]. There is strong evidence that low-functioning CREB is linked to autism [10]. CREB is a crucial area of research since it is involved in signalling pathways that contribute to the etiology of some mental diseases, including schizophrenia [49].

Brain-derived neurotrophic factor (BDNF) is a small protein found in nearly all parts of the brain, CNS, and peripheral circulation. BDNF regulates several neurophysiological processes, including synaptogenesis, glycogenesis, and neurogenesis. It also plays a role in neuroprotection and the survival and differentiation of dopaminergic neurons during the development of the brain, which is directly related to the enhancement of learning and memory [21]. The gene plays a role in preventing apoptosis. Numerous experimental findings strongly imply BDNF's role in autism. ID patients reflected increased BDNF-immunoreactive protein levels [41].

Neuquinon (NQ) is a lipid-soluble benzoquinone an intracellular antioxidant that protects low-density lipoproteins, mitochondrial membrane proteins, and membrane phospholipids from free radical oxidative damage [35]. Neuquinon improves mitochondrial functions, such as electron transport, triggering ATP production [18]. NQ is used in psychiatric drugs to increase mitochondrial function and mental performance [12]. It possesses limited oral bioavailability due to its hydrophobicity and high molecular weight [11]. Due to its unsaturated double bond and weak chemical stability, it is also readily degraded by sunlight, ultraviolet light, and heat when exposed to the air. Liposomes have been extensively studied as drug carriers for several decades, because of their non-toxicity and non-immunogenicity. They can accommodate hydrophilic and lipophilic drugs and control the release of their encapsulated contents [16]. Thanks to their distinctive bilayer membrane structure, liposomes can cross the membrane and deliver NQ to the mitochondria [57]. In *vivo* and in *vitro*, liposomal encapsulation of NQ increased its water solubility and stability, which is a potential delivery method [16].

The goal of the current investigation is to determine whether the antioxidant power of NQ and LNQ can be linked to the neurotransmitters (GABA & ACh), brain Mecp2, BDNF, and CREB mRNA gene expression in the autistic rat model conducted via EFA.

2. Materials and methods

2.1. Chemicals

Ethyl-formic acid, NQ and LNQ were purchased from Alfa Aesar (Thermo Fisher (Kandel) GmbH. ErlenbAChweg 2.76870 Kandel, Germany), Amoun Pharmaceutical Company S.A.E (1st industrial zone-block 13015 EL Obour city-Cairo, Egypt) and Empirical Labs (1501 Academy, CT. Suite 5,Ft Collins, CO 80524), respectively. ELISA kits of GABA and ACh were provided from R&D Systems (MN, USA). RT-PCR kits and primers of BDNF, Mecp2 and CREB were purchased from the Qiagen Company (Germantown, USA). All other chemicals are of the highest analytical grade.

2.2. EFA preparation

Ethyl-formic acid sodium salt was dissolved in 0.1 M PBS and given subcutaneously (SC) in a dose of 500 mg/kg (0.26 M, pH 7.4) once a day, for five days [30].

2.3. Animals

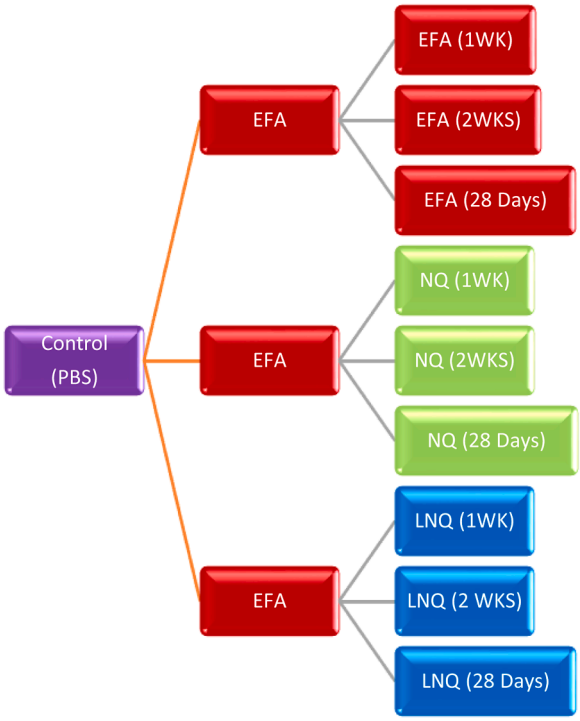
The National Research Centre Animal House provided us with 100 healthy male Wistar albino rats, each weighing between 80 and 100 gm. They dated back 21 days. We used every experimental technique available to judge ID development. Rats were divided into the EFA-intoxicated (ID) and control groups at random. Animals had unrestricted access to bottled water and regular rat food (Hyochang Science).

The National Research Centre's (19011) ethical principles and rules, as well as the US National Institutes of Health's Guide for Care and Use of Laboratory Animals, were rigorously followed in all procedures involving the care and treatment of animals.

2.4. Experimental design

After one week of acclimation, animals were divided into ten groups, each with ten animals: Phosphate buffered saline (PBS) was administered to the animals in Group 1 as a standard control. For 5 consecutive days, animal groups 2 to 10 got a daily dose of EFA (500 mg/kg) SC [24], after which the following protocol was used:

GII: EFA groups were sacrificed post 7 days.
GIII: EFA groups received NQ (10 mg/kg body weight) IP daily for 7 days [8].
GIV: EFA groups received liposomal loaded NQ (10 mg/kg body weight) IP daily for 7 days [16].
GV: EFA groups were sacrificed post 14 days.
GVI: EFA groups received NQ (10 mg/kg body weight) IP daily for 14 days.
GVII: EFA groups received liposomal loaded NQ (10 mg/kg body weight) IP daily for 14 days.
GVIII: EFA groups were sacrificed post 28 days.
GIX: EFA groups received NQ (10 mg/kg body weight) IP daily for 28 days.
GX: EFA groups received liposomal loaded NQ (10 mg/kg body weight) IP daily for 28 days.



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2.5. Brain tissue preparation

Rats were weighed, received carbon dioxide anesthetic to make them somewhat unconscious. Then, after cervical dislocation was performed, the brain tissues were carefully separated, dried, weighed, and homogenized in 5 volumes of pH 7.4 phosphate buffer using a Teflon homogenizer (Glass-Col homogenizer, Terre Haute, USA). This homogenate (20% w/v) was separated into different portions after being centrifuged at 4000 rpm for 15 min at 4 °C. GABA, ACh, and AChE analysis were done on the first supernatant part. The second brain part was used to evaluate BDNF, CREB, and Mecp2 mRNA gene expressions, while the third part was used to perform the COMET assay as a DNA damage marker.

3. Measured parameters

3.1. Determination of neurotransmitters

3.1.1. Determination of the brain gamma-aminobutyric acid (GABA) level

GABA content was assessed via ELISA kits (R &D systems MN, USA) regarding manufacturer's instructions using antigen-antibody sandwich technique. Then quantitative sandwich enzyme immunoassay was used to evaluate the assay. First, the microplate was pre-coated with specific antibodies. Then, the immobilized antibody that bound to GABA was added, and the wells were supplemented with the enzyme-linked secondary antibody specific for GABA. Afterward, the absorbance was determined at 450 nm.

3.1.2. Determination of the brain acetylcholine (ACh) level

The brain tissue was precisely weighted after it had thawed at room temperature. According to Deng et al. [19]] and Lin and Yu [34], the content of ACh in the rat brain tissue homogenate was identified using an ELISA reagent (MyBio-Source, Inc. San Diego, USA). First, the microplate was pre-coated with specific antibodies. Then, the immobilized antibody that bound to Ach was added, and the wells were supplemented with the enzyme-linked secondary antibody specific for Ach. The intensity of the color was measured (at 450 nm).

3.1.3. Determination of the brain acetylcholinesterase (AChE) activity

The technique of Khan et al. [32], which used acetylthiocholine iodide (ATCI) as a substrate, was used to measure the brain's AChE activity. Before adding the enzyme, the reaction's rate was subtracted to allow for any absorbance increase caused by the substrate's spontaneous hydrolysis. The absorbance was measured at 405 nm. Following the addition of 25 µl brain homogenates, the absorbance was once again measured after 5 min of incubation at room temperature. The AChE activity is represented as µmol/min/mg of tissue protein. All assessments were prepared twice and in triplicate.

3.1.4. Quantitative real time-polymerase chain reaction (qRT-PCR) for analysis of brain BDNF, CREB and Mecp2 mRNA gene expressions

The tissues of the brain were homogenized by using a Tissue Ruptor (Roche) with a QIAzol lysis reagent. Following the manufacturer's directions, Tripura Isolation Reagent (Roche) was used to isolate total RNA. According to the manufacturer's directions, complementary DNA (cDNA) was produced using Superscript Choice Systems (Life Technologies, Breda, Netherlands). Using SYBR green PCR Master Mix (Applied Biosystems, CA, USA) by the manufacturer's instructions, quantitative real-time PCR was carried out to evaluate the mRNA expression of GFAP, CACNA1C, BDNF, CREB, and Mecp2. In a 25- µl reaction volume, 5 µl of cDNA were added to 12.5 µl of 2 x SYBR Green Master Mix and 200 ng of each primer. Table 1 describes the primer sequences. The following describes the temperature profile: 94 °C for 3 min, 94°C for 20 s, 54°C for 20 s, and 72°C for 20 s for 35 cycles. The PCR cycle number (CT) at which the increased fluorescence curve crosses a threshold value was used to determine the expression level. The relative CT (ΔΔCT) technique was used to determine the relative expression of the target genes [2,36]. Table 1 lists the primers utilized in this investigation.

3.1.5. Investigation of DNA damage percentage by COMET examination in the brain tissues

Brain samples were subjected to the modified single-cell gel electrophoresis or comet assay. The comet assay is a straight forward method for quantifying strand breakage in deoxyribonucleic acid (DNA) in eukaryotic cells. Nucleoids are formed when cells placed in agarose on a microscope slide are lysed with detergent and high salt to generate supercoiled loops of DNA linked to the nuclear matrix. Fluorescence microscopy reveals comet-like structures formed by high-pH electrophoresis; the intensity of the comet tail relative to the head represents the amount of DNA breaks. The most likely explanation is that loops with a break lose supercoiling and are free to stretch toward the anode [9,6].

3.1.6. Statistical analysis of comet assay

The General Linear Models (GLM) procedure of the Statistical Analysis System (1982) was used to analyze all data, and the Scheffé -test was used to determine whether there were any notable group differences. Mean ± SEM is used to describe the values. Every assertion of significance was predicated on the likelihood of $P < 0.05$.

Table 1
Primer sequences.

Primer name	Primer sequence 5'….3'
BDNF	Forward primer, 5 '- GAGAAGAGTGATGACCATCCT –3' Reverse primer, 5 '- TCACGTGCTCAAAAGTGTCAG –3'
CREB	Forward primer, 5 '- AGACTCGAGATGACCATGGACTCTG - 3' Reverse primer, 5 '- ACGGAATTCTCCCAAATTAATCTGAC - 3'
Mecp2	Forward primer, 5'-ACA GCG CTC CATTAT C-3' Reverse Primer, 5'-CCC AGT TAC CGT GAA GTC AAA A-3'
β- actin	Forward primer, 5 '-CCCATCTATGAGGGTTACGC- 3' Reverse Primer, 5 '-TTTAATGTCACGCACGATTTC- 3'

3.2. Statistical analysis

Data were expressed as means ± S.E.M. Statistical analysis was performed using the Instat-3 computer program (Graph pad software Inc, San Diego, CA, USA). One way analysis of variance (ANOVA) by SPSS 12 program followed by Post HOC test was used to determine the differences between means of different groups. The level of significance was set at $P < 0.05$.

4. Results

4.1. Modulation of brain neurotransmitters

4.1.1. Brain GABA

Table (2) showed a significant increase in brain GABA levels after EFA intoxication compared to control values for all time intervals, with the most increase observed in the fourth week. On the other hand, NQ and LNQ supplementation reported a statistically significant reduction in brain GABA at all-time intervals. NQ treatment with a percentage of improvement of 18.5%, 31.61%, and 53.92% in the first, second, and fourth weeks, respectively. LNQ treatment revealed a percentage of improvement of 31.3%, 54.21%, and 73.31% in the first, the second and fourth week, respectively, with the most significant improvement in LNQ groups in the fourth week reflecting the superior effect of LNQ over NQ treatment.

4.1.2. Brain ACh

Data expressed in Table (2) revealed a significant reduction in brain ACh post-EFA intoxication as compared with the control value during all time intervals. ACh values recorded 156.5, 146.97, and 137.20 in the first, second, and fourth week, respectively, with the most significant reduction observed in the fourth week. Supplementation with NQ and LNQ resulted in a gradual but significant increase in brain ACh levels. CoQ10 led to a 2.5%, 11.63%, and 21.12% improvement in the first, second, and fourth weeks, respectively. Regarding LCoQ10 treatment, the percentage of improvement was 6.84%, 20.14%, and 31.56% during the first, second, and fourth week, respectively. With the most significant improvements in the fourth week for both treatments.

4.1.3. Brain AChE activity

According to Table 2, the results indicated a significant increase in brain AChE levels following EFA intoxication in all time intervals, with the most elevation observed in the fourth week compared to control values. Meanwhile, treatment with NQ and LNQ reported a statistically significant reduction in brain AChE along all-time intervals with the maximum improvements in the fourth week for both treatments, thus affecting the ACh level.

The percentage of improvement in NQ during the first, second, and fourth week was 66.87%, 105.75%, and 164.43%, respectively, and for LNQ treatment, were 91.54%, 129.90%, and 182.74%, respectively, showing the superior effect of LNQ over NQ in the fourth week of treatment.

4.2. Impact of EFA on brain BDNF, CREB and Mecp2 mRNA gene expressions

Brain CREB mRNA gene expression was significantly downregulated in EFA-intoxicated rats with the most significant reduction observed in the second week, showing a 4.5-fold reduction compared to the control value (Fig. 1c). However, NQ and LNQ-treated groups revealed statistically significant upregulation of brain CREB across all time points, with the group receiving liposomal-loaded NQ having the most significant impact by almost 4-fold when compared to the EFA-impaired group in the fourth week. Finally, brain Mecp2 gene expression showed a significant downregulation post-EFA intoxication, especially in the fourth week at almost 4.5-fold relative to the control value. Meanwhile, treatment with NQ and LNQ reported a significant upregulation in Mecp2 during all time intervals, with the liposomal NQ in the fourth week revealing the most significant effect (Fig. 1a).

Ethyl-formic acid-intoxicated rats showed a significant upregulation in brain BDNF mRNA gene expression throughout all time intervals, especially in the fourth week compared to the control value. Meanwhile, treated NQ and LNQ groups revealed significant amelioration in brain BDNF synchronization with EFA-intoxicated groups in the liposomal loaded NQ group at the fourth week demonstrated the most significant impact (Fig. 1b).

4.3. Impact of EFA on brain DNA damage (COMET assay)

Ethyl-formic acid-intoxicated rats exhibited a significant increase in brain DNA damage biomarkers throughout the experiment, with the highest levels in week four compared to controls. Meanwhile, treatment with NQ and LNQ revealed significant mitigation in brain DNA damage as compared with EFA intoxicated groups. The groups treated with LNQ showed a 140% improvement in the fourth week, while the NQ-treated groups showed a 120% improvement compared to the EFA-intoxicated groups (Figs. 2 and 3).

5. Discussion

Autism is a complicated condition with numerous etiologies, risk factors, and clinical manifestations. Mutations in genes linked to intellectual impairment influence transcription, expression, regulation, protein synthesis, translation, synapse formation/function, and cell migration. These mutations may influence neural development through various pathways. The current study examined how NQ and LNQ affected EFA-induced autism and the related signaling pathways. GABA levels increased significantly after EFA intoxication and decreased significantly following NQ and LNQ treatment, with the most pronounced effect after four weeks of treatment. This reduction may be due

Table 2
Influence of different time intervals of NQ and LNQ treatment on biochemical parameters in brain tissues of EFA intoxicated rats.

Groups										
Parameters	Control (zero time)	EFA (7 days)	NQ (7 days)	LNQ (7 days)	EFA (14 days)	NQ (14 days)	LNQ (14 days)	EFA (28 days)	NQ (28 days)	LNQ (28 days)
GABA (ng/ml protein)	31.1 ± 0.74^{a}	59.3 ± 0.45^{b}	53.57 ± 0.64^{c}	49.6 ± 0.84^{d}	60.5 ± 0.48^{b}	50.7 ± 0.46^{d}	43.67 ± 0.66^{e}	62.3 ± 1.09^{b}	45.53 ± 0.78^{d}	39.5 ± 0.78^{e}
ACh (μg/ g protein)	262.4 ± 3.2^{a}	156.5 ± 2.1^{b}	163.11 ± 3.0^{c}	174.47 ± 3.7^{d}	146.97 ± 3.1^{e}	177.5 ± 2.5^{d}	199.83 ± 2.5 ^{f}	137.20 ± 2.57 ^{g}	192.63 ± 3.36 ^{f}	220.03 ± 2.92 ^{g}
ACh E (μM/mint/mg protein)	11.4 ± 0.72^{a}	39.7 ± 0.38^{b}	31.4 ± 0.64^{c}	28.57 ± 0.66^{c}	40.6 ± 0.38^{b}	28.47 ± 0.35^{c}	25.7 ± 0.64^{c}	42.43 ± 0.71^{b}	23.57 ± 0.78^{c}	21.47 ± 0.49^{c}

Data are expressed as Mean± SEM at $P \leq 0.05$ by one way ANOVA test where ($n = 10$). A,b,c,d,e,f, and g refer to different groups. If both groups take the same letter, they aren't significantly different from each other but if they take different letters as a,b,c,., they are considered significantly different from each other at $P \leq 0.05$.

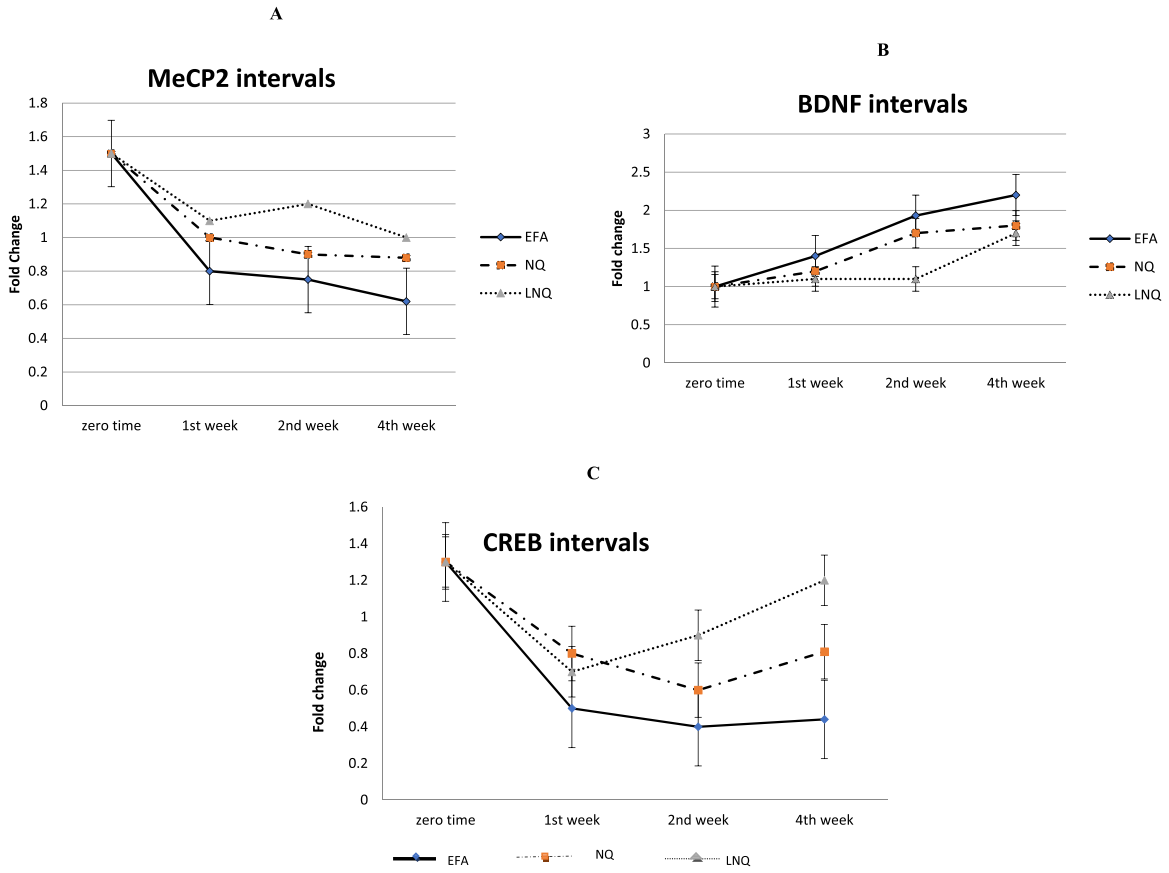

Fig. 1. : Figure A, B and C show the fold change in Mecp2, BDNF and CREB genes expression throughout the three time intervals after treatment with NQ and LNQ post EFA intoxication.

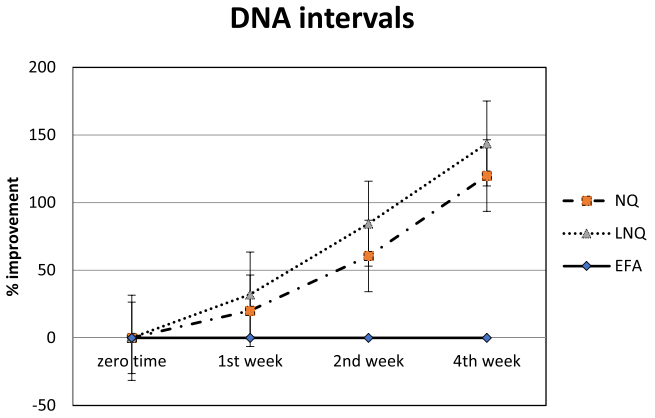

Fig. 2. : The improvement of DNA in brain tissues of male rat after different treatment.

to astrocyte deactivation. GABA is essential in glia-neuron interactions as it functions as a gliotransmitter and reactive astrocyte. High levels of GABA caused by Ca^{2+}-activated channels may be directly responsible for the memory impairment revealed in ID patients. An aberrant increase in GABA release from reactive astrocytes in the brain might be the source of this impairment. These interactions have an impact on neural networks in the diseased brain. The secretion of GABA activates GABAA and GABAB receptors in neurons that severely inhibit synaptic release. GABA is released from reactive astrocytes to prevent a spike, which affects synaptic plasticity and memory. According to Jo et al. [28], several neurological disorders such as Alzheimer's disease, Parkinson's disease, stroke, epilepsy, and traumatic brain injury (TBI) are associated with this phenomenon. In line with earlier findings, the EFA autistic rat model had considerably more GABA than the control group. According to Zieminska et al. [59], male rats have significantly higher levels of GABA in their hippocampus compared to control groups. Meanwhile, Montanari et al. [43] discovered that children with intellectual disabilities (ID) have elevated GABA levels in their bilateral visual cortex. This increase in GABA was associated with greater efficiency but also social impairments when compared to control groups.

On treating autistic models with NQ and LNQ, the level of the neurotransmitter GABA was reduced. It could be due to decreased oxidative stress, which improves astrocyte health, reduces reactivity, and lowers GABA production. Additionally, it may increase the GABA catabolic enzyme (GABA decarboxylase) and inhibit the receptors involved in the GABAergic system; this is a key factor in the imbalance in the E/I ratio that led to the emergence of ID. The finding explanation agrees with the study suggested by [58].

In the current study, EFA significantly reduced brain ACh levels and elevated AChE activity. On the other hand, treatment with NQ and LNQ significantly modulated these deviated parameters, with the superiority of LNQ in the fourth week Table 3.

AChE is an enzyme that breaks down acetylcholine, a specific neurotransmitter in the cholinergic system. This system is often associated with neurodegenerative diseases. AChE inhibitors (AChEI) are commonly used to treat these diseases. During neurodegenerative disorders, inflammation, increased oxidative stress, and apoptosis occur in cells that express AChE, leading to the loss of neurons. Acetylcholine is active in the basal forebrain projections into the cortex and limbic structures and therefore has a role in cognitive function [55].

Ethyl-formic acid, a strong brain cholinotoxin, causes the brain's

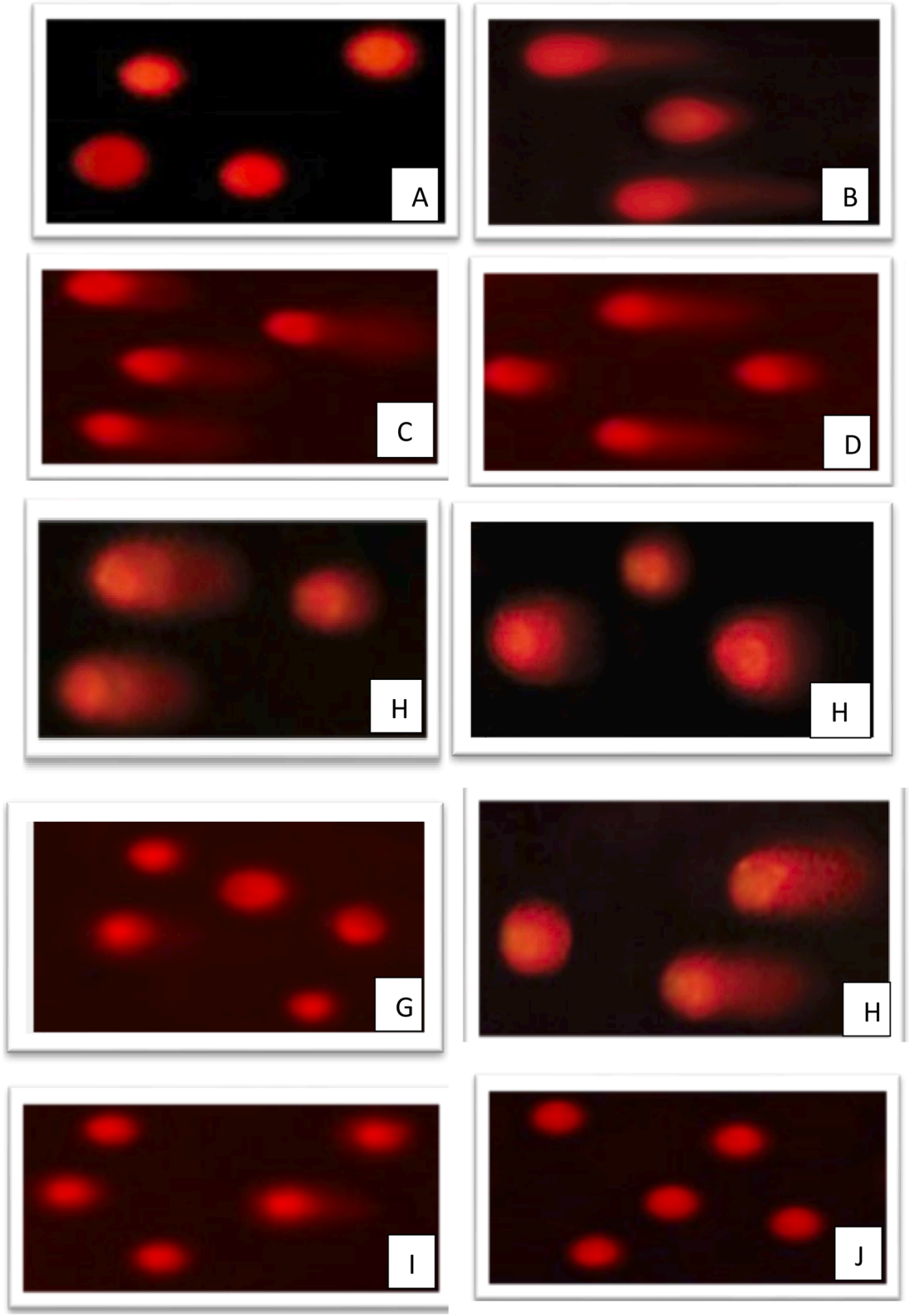

Fig. 3. : COMET Assay expressing DNA damage for different time intervals of NQ and LNQ treatment in EFA intoxicated rats. **(A)** normal DNA (class 0). **(B)** EFA - (7 days) representing classes 1 and 3 of DNA damage. **(C)** NQ (7 days) representing classes 2 and 3 of DNA damage. **(D)** LNQ (7 days) representing classes 1, 2 and 3 of DNA damage. **(E)** EFA- (14 days) representing classes 1 and 3 of DNA damage. **(F)** NQ (14 days) representing classes1 and 2 of DNA damage. **(G)** LNQ (14 days) representing classes 1 and 2 of DNA damage. **(H)** EFA- (28 days) representing classes 1 and 3 of DNA damage. **(I)** NQ (28 days) representing classes 0, 1 and 2 of DNA damage. **(J)** LNQ (28 days) representing class 1 of DNA damage. Class 0 = no tail; 1 = tail length < diameter of nucleus; 2 = tail length between 1X and 2X the diameter of nucleus; and 3 = tail length > 2X the diameter of nucleus. (*): No of cells analyzed were 100 per an animal (photo with 300 pixels).

Table 3
Rate of DNA damage in brain tissues of male mice exposed to different treatment using comet assay.

Treatment	No. of cells		Class[a] of comet				DNA damaged cells (mean ± SEM)
	Analyzed	Total comets	0	1	2	3	
Negative control	300	25	276	21	4	0	8.31 ± 0.33^{d}
EFA (1 week)	300	69	232	24	29	16	22.92 ± 1.22^{a}
NQ (1 week)	300	64	237	28	23	13	21.26 ± 0.89^{ab}
LNQ (1 week)	300	61	240	30	22	9	20.27 ± 0.67^{ab}
EFA (2 week)	300	72	229	23	31	18	23.94 ± 1.15^{a}
NQ (2 week)	300	57	244	27	24	6	18.91 ± 0.58^{b}
LNQ (2 week)	300	51	250	19	22	10	16.93 ± 1.16^{bc}
EFA (4 week)	300	77	224	25	28	24	25.58 ± 0.87^{a}
NQ (4 week)	300	47	254	20	19	8	15.62 ± 1.20^{c}
LNQ (4 week)	300	41	260	22	15	4	13.64 ± 0.89^{c}

[a] : Class 0 = no tail; 1 = tail length < diameter of nucleus; 2 = tail length between 1X and 2X the diameter of nucleus; and 3 = tail length > 2X the diameter of nucleus (*): No of cells analyzed were 100 per an animal.

cholinergic neurons to undergo apoptosis. Cognitive function is impaired by ACh hydrolysis brought on by increased AChE activity. The brain's fatty acid metabolism is altered by EFA levels exceeding a particular threshold. This lowers choline and ACh level, and linked to the severity of autism. Systemic AChEI therapy preserved the autistic-like phenotype. Apoptosis is reduced by enhancing cholinergic transmission, that is achieved either by raising ACh synthesis/release or by blocking AChE activity and mitigating these effects. The pro-cholinergic and anti-cholinesterase potentials were indicated by NQ and LNQ therapy. In the long run, this stops neurocognitive decline. The neuroprotective impact of LNQ is directly associated with its ability to cross the blood-brain barrier. This opens the door for the treatment of ID by elucidating the function of LNQ as a therapeutic method in EFA-induced neurotoxicity [5]. The results are matched with [27]. ID has also been linked to malfunctioning of the brain's cholinergic system [22] which resulted in low levels of ACh as a potential contributor to ID symptomatology. Inflammation, cell apoptosis, and increased oxidative stress are observed during neurodegenerative diseases and depressive disorders [50]. According to earlier studies, injections of 3-nitroethyl-formic acid (3-NEFA) were associated with increased oxidative stress, astrocyte activation, and AChE activity in the hippocampus, cerebral cortex, and striatum [55]. Numerous studies have supported the neuroprotective abilities of NQ in degenerative disorders like Alzheimer's, Huntington's, and Parkinson's disease. Previous research has indicated a direct connection between the development of hippocampus neuronal death and an excess production of ROS/RONS.

After the investigation, EFA reduced MeCP2 gene expression while NQ and LNQ therapies increased it, with LNQ showing the greatest impact in the fourth week of treatment.

MeCP2 is an important regulator of synaptic and neuronal plasticity in the brain [44]. It can also be a transcriptional activator by recruiting CREB. Further, it is essential for neural development by regulating BDNF gene expression. The phosphorylated CREB can bind to the BDNF promoter, which triggers its transcription. In ID, the BDNF-ERK-CREB signaling pathway is crucial for cell survival, synaptic organization, and plasticity [37].

Defects in excitatory neurotransmission and long-term potentiation as a result of the abnormally deficient MeCP2 protein expression in the autistic rat model resulted in some cognitive and behavioral abnormalities. This study result is in parallel with a study previously reported in ID frontal cortex [15]. NQ altered this effect and increased MeCP2 expression. Moreover, LNQ's ability to penetrate the BBB and target brain tissue makes it more efficient [57]. As previously discussed, traditional NQ formulations are not satisfactory due to their poor water solubility, photosensitivity, and thermolability [46].

In the current study, the level of BDNF gene expression in rats challenged with EFA resulted in a marked upregulation as compared with the control value. However, the administration of NQ and LNQ showed a significant downregulation in its gene expression compared with the EFA-intoxicated rats, indicating the superiority of LNQ in the fourth week.

The most prevalent neurotrophin in the brain, BDNF is crucial for peripheral and central neurons' survival, differentiation, synaptic plasticity, and axonal extension throughout adulthood [26]. The expression of BDNF in the hippocampus was dramatically increased following EFA-intoxication. Increased BDNF protein and mRNA levels in prenatal brains have been observed in studies using EFA animal models of autism. Additionally, several researchers claimed that NF-B, a specific protein in the neuroinflammatory cascade, up-regulates BDNF. Numerous research on autism have noted a characteristic called neuroinflammation and increased NF-B levels. Increased BDNF transcript levels may function as a balancing mechanism downstream of the neuroinflammation linked to the EFA model of autism. One study found that the pro-BDNF/BDNF ratio in autistic people's brains was out of balance. Additionally, BDNF overexpression improved survival and locomotor utility, which reduced MeCP2. A recognized BDNF transcriptional repressor is the protein MeCP2 [3].

According to Zhang et al. [56], patients with ID have anomalies in the structure and function of the brain. Depending on some researchers, the neurotrophic effects of BDNF may interfere with the 5-hydroxytryptamine nerve system, influence patients with IDs' cerebral cortex and cortex-thalamus, and result in dysontogenesis, which may lead to the development of autism. This impact was confirmed in a male Mecp2-deficient by [21]. Autism is a neurodevelopmental condition characterized by an altered MeCP2 gene, which transcriptionally inhibits BDNF via the MeCP2 protein [17].

CREB is a constitutively expressed nuclear transcription factor playing a vital role in cognition and neuronal survival. Its regulation is primarily through phosphorylation via several signaling kinases and thus responds to numerous extracellular signals. The current investigation showed that EFA dramatically decreased the expression of the CREB gene. NQ and LNQ treatment significantly increased its expression, with LNQ reflecting the most impact in the fourth week. This result is consistent with a previous research reflecting that oxidative stress reduces CREB expression in cultured rat hippocampal neurons. Patients with ID may experience exacerbated neurodegeneration due to reduced CREB gene expression [48].

Numerous genes that have been linked to ID and associated in neurotransmitter systems, neuronal cell adhesion molecules, oxidative stress, inflammation, lipid metabolism, and mitochondrial function were affected by ethanolic acid. EFA's histone deacetylase inhibitor can affect the expression of the CREB gene. Tyrosine hydroxylase (TH) gene transcriptional activity required CREB transcription factor(s). EFA caused accumulation of TH mRNA and protein, at lower CREB concentrations, indicative of increased cell capacity to produce catecholamines. Adults with Asperger's produce and store more dopamine in the striatum and frontal cortex, and autistic children have increased striatal dopamine D2 receptor binding, suggesting an overactive dopaminergic system. The orbitofrontal cortex plays a vital role in regulating emotions through a network of interconnected brain regions [45]. Catecholamines (CAs) are neurotransmitters and neuromodulators essential for learning, motor control, regulating emotions, reward processing, and biorhythms [33]. Mutations in the TH gene can cause Congenital TH deficiency

(THD, OMIM #605407). The symptoms of this condition can range from L-Dopa-responsive dystonia (DRD) and infantile parkinsonism to severe and complicated encephalopathy with neonatal onset. Furthermore, Parkinson's disease is characterized by a deficit in striatal TH [14]. Phosphorylated CREB interacts with a critical Ca2 + response element (CRE) in the BDNF gene to trigger transcription of BDNF, which is initiated by Ca2 + influx in cortical neurons. Mutations in either BDNF or the surrounding new regulatory region prevented CREB activity, in addition to causing a significant decrease in BDNF transcription and an increase in autism spectrum disorders. Belonging to the CREB family, the BDNF gene controls adaptive neuronal responses at synapses through its protein product. According to Martínez de Paz et al. [38], MeCP2 functions as a transcriptional regulator by interacting with methylation DNA surrounding the BDNF promoter.

NQ plays a significant role as an antioxidant in lipid membranes and mitochondria. Since deficiencies in energy metabolism and oxidative damage contribute to the etiology of neurodegenerative disorders, NQ therapy may have a variety of therapeutic advantages. LNQ was chosen because nanoparticles may easily interact with biomolecules both outside and inside of cells, increasing the native compound's bioavailability. A prior study found that the amelioration of the altered expression of P-CREB and BDNF proteins, the reduction of lipid peroxidation and brain NO, and the decrease in serum IL-1 and TNF levels suggested a possible role for carnitine and LNQ combination therapy as neuroprotective medications against EFA toxicity in rats.

According to the current study, EFA considerably increases DNA abnormalities. Treatment with NQ and LNQ significantly decreased DNA damage (Fig. 2) caused by EFA oxidative stress, which chemically alters proteins, lipids, DNA, and RNA. Additionally, the genome's integrity and stability are impaired (genotoxic effect) through the induction of single- and double-strand DNA breaks. Numerous genomic modifications, DNA-DNA and DNA-protein crosslinks, as well as base-specific modifications, may result from oxidative stress. It was suggested that ROS attacks double bonds and DNA, producing free radicals and adducts. This increases lysosomal enzymes, causing DNA and membrane damage, with lipid peroxidation leading to loss of membrane integrity and DNA damage. The results suggest that genomic instability and DNA repair capacity may have a significant impact on ID. Previous studies have demonstrated that the effectiveness of NQ and LNQ in preventing EFA neurotoxicity is attributed to their antioxidant properties. NQ is an efficient antioxidant method for neuroprotective diseases by reducing lipid peroxidation, DNA oxidative damage, and mitochondrial dysfunction [47].In cell culture and rodent animals, NQ treatment prevents DNA fragmentation [54].

Liposomes are the most widely studied and commonly used carrier for targeted medication delivery. They have been extensively researched and are prevalent in the field. They have targeted therapies for a range of biomedical applications by stabilizing therapeutic molecules, eliminating obstacles to cellular and tissue absorption, and increasing the biodistribution of compounds to specific locations in *vivo* [29,52].

6. Conclusion

Liposomal loaded NQ could be a promising candidate in the strategy of ID treatment via regulating Mecp2, BDNF and CREB signaling pathways and via its neuromodulator impact.

Ethics approval and consent to participate

Ethics number is (19011) in NRC institute.

Funding

This work has no financial support.

CRediT authorship contribution statement

Kadry Mai O: Conceptualization, Data curation, Investigation, Supervision, Writing – review & editing. **Elhefnawei Doaa M.:** Formal analysis, Methodology, Writing – original draft. **EL-Desouky Mohamed A.:** Visualization. **Mahmoud Ahlam H.:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft.

Consent for publication

Ethics number is (19011).

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the corresponding author is the sole contact for the editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions and final approval.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

Acknowledgement

Is directed to the National Research Center Egypt for the great support.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2023.12.008.

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