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Inhibition of Hippocampal Microstructural Alterations in Nickel Chloride-Exposed Rats Pretreated with Rutin: Role of NRF-2, Caspase-3, AChE and BDNF

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ABSTRACT

Background: Nickel chloride ($NiCl_2$) induces neurotoxicity by triggering harmful effects in the nervous system, thereby promoting oxidative stress, apoptosis, and ultimately neuronal death. Rutin, a dietary flavonoid, acts as a potent antioxidant, protecting cells from oxidative damage. Accordingly, this study investigated the activity of rutin in the hippocampus of $NiCl_2$-exposed rats.
Materials and Methods: Forty-eight Wistar rats, randomly distributed into six groups (n=8), were treated for twenty-eight days as follows: Group A – Control; Group B - 5 mg/kg body weight (bw) $NiCl_2$; Group C - 50 mg/kg bw rutin and 5 mg/kg bw $NiCl_2$; Group D - 100 mg/kg bw rutin and 5 mg/kg bw $NiCl_2$; Group E - 50 mg/kg bw rutin; Group F – 100 mg/kg bw rutin. Thereafter, neurobehavioural, antioxidant, lipid peroxidation, histological, gene expression, and *in-silico* assessments were done.
Results: The findings showed that the $NiCl_2$ caused a significant decrease ($P<0.05$) in spontaneous alternation and discrimination index, as well as antioxidant enzymes, following comparison to control. A significant increase ($P<0.05$) was noticed in lipid peroxidation and microstructural alterations in the hippocampus of $NiCl_2$-treated rats. Furthermore, a significant downregulation ($P<0.05$) in NRF-2 expression and a significant upregulation ($P<0.05$) in Caspase-3 expression were observed following $NiCl_2$ exposure. However, these effects were inhibited in the $NiCl_2$-exposed rats pretreated with rutin. Also, *in-silico* docking results revealed that rutin had a strong binding affinity with AChE and BDNF, thus demonstrating its therapeutic potential against cognitive disorders.
Conclusion: Taken together, these findings demonstrate that rutin attenuated $NiCl_2$ toxicity in the hippocampus of rats, possibly via its ability to modulate NRF-2, AChE and BDNF activity.

Keywords: Nickel chloride, Rutin, Hippocampus, NRF-2, Caspase-3, AChE, BDNF.

INTRODUCTION

Metals play vital functions within the human body, such as upholding cell structure, regulating gene expression, facilitating antioxidant response, and enabling neurotransmission (1). Elevated metal absorption in the nervous system poses risks as it can induce oxidative stress, interfere with mitochondrial function, and hinder the operation of diverse enzymes (2). Nickel, a recognized heavy metal, exists in the environment at very low concentrations. It is present in all types of soil, meteorites, and is emitted from volcanic activities. In the environment, nickel primarily binds with oxygen or sulfur, forming oxides or sulfides in the earth's crust (3). The extensive industrial utilization of nickel in its production, recycling, and disposal has resulted in widespread environmental contamination.

Nickel is released into the atmosphere through nickel mining and various industrial processes, including power plants, incinerators, rubber and plastic industries, nickel-cadmium battery industries, and electroplating industries (3). The extensive use of nickel in various industries, as well as occupational exposure, poses significant risks to human health. Heavy metals like nickel have the potential to generate free radicals from diatomic molecules through a double-step process, leading to the production of superoxide anions (4). Free radicals are molecular structures characterized by one or more unpaired electrons in atomic or molecular orbitals. These harmful free radicals instigate a chain reaction and initiate lipid peroxidation in membrane-rich structures containing phospholipids, such as mitochondria and endoplasmic reticulum, leading to oxidative, mitochondrial, and endoplasmic reticulum stress. In addition, free radicals instigate various biological processes, including apoptosis, necrosis, ferroptosis, and autophagy (5).

Antioxidants inhibit the oxidation of species, thereby controlling the generation of free radicals (6, 7). Cellular antioxidant enzyme systems, such as superoxide dismutase,

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catalase, glutathione peroxidases/reductase, as well as non-enzymatic antioxidants like tocopherols, vitamin C, and glutathione, among others, along with various dietary components, play crucial roles in safeguarding cells and organisms against the harmful effects of free radicals (8). Polyphenolic compounds, including flavonoids, phenolic acids, and anthocyanins, are well-known for their ability to scavenge free radicals, act as antioxidants, and chelate iron (9). Rutin is a distinctive antioxidant flavonoid predominantly present in fruits, vegetables, cereals, and various other plant-based components of human diets (10). Rutin is known for its strong antioxidant and anti-inflammatory properties (10, 11). Pharmacological studies have documented the beneficial effects of rutin in numerous disease conditions, showcasing its therapeutic potential in various models of neurodegenerative diseases (12-14). These effects include the reduction of proinflammatory cytokines, enhancement of antioxidant enzyme activities, activation of the mitogen-activated protein kinase cascade, downregulation of proapoptotic genes, upregulation of antiapoptotic genes, and restoration of the activities of mitochondrial complex enzymes (10). Rutin and/or its metabolites possess the capability to traverse the blood-brain barrier, and can alter cognitive and behavioral symptoms associated with neurodegenerative disorders (15). The current study addresses a notable gap in scientific literature regarding the role of Rutin in mitigating nickel-induced toxicity in Wistar rats, and findings from this study will provide significant insights into the neuroprotective effects of Rutin. Such insights could be instrumental in the development of novel neuroprotective drugs aimed at effectively managing nickel neurotoxicity and its associated neurological disorders.

MATERIALS AND METHODS

Chemicals and reagents

Rutin ($C_{27}H_{30}O_{16}$; purity ≥94% HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and Nickel Chloride ($NiCl_2.6H_2O$; purity ≥98%) was manufactured by Molychem, Mumbai, India. Other reagents were all of the analytical grade.

Care and Management of Experimental Animals

A total of Forty-eight Wistar rats were bought and kept in the Department of Anatomy animal holdings. After acclimatization for two weeks, the experimental procedures followed the guidelines of the Research Ethics Committee of the College of Medical Sciences, University of Benin, Nigeria, with approval number CMS/REC/2024/576.

Experimental design

The rats were distributed to six (6) different groups (n=8). The experimental design was as follows:

- Group A (control) - 1 ml of distilled water
- Group B ($NiCl_2$) - 5 mg/kg body weight (BW) of Nickel Chloride ($NiCl_2$) only.
- Group C (RU1 + $NiCl_2$) - 50 mg/kg BW/day of rutin (RU) and 5 mg/kg BW of $NiCl_2$.
- Group D (RU2 + $NiCl_2$) - 100 mg/kg BW/day of rutin and 5 mg/kg BW of $NiCl_2$.
- Group E (RU1) - 50 mg/kg BW/day of rutin.
- Group F (RU2) - 100 mg/kg BW/day of rutin.

Rutin and $NiCl_2$ were administered orally and intraperitoneally, respectively, for 28 days.

Neurobehavioural Evaluation

Novel Object Recognition Test: This test was carried out in a wooden open box device (80 × 60 × 40 cm), as previously described (16, 17). The discrimination was determined by equating the time spent exploring the familiar and Novel Object (16, 17). For quality control, a discrimination index (DI) was calculated as follows:

Discrimination Index = Novel Object – Familiar Object 1 / Novel Object + Familiar Object 1.

Y-Maze test: This test was carried out in a wooden apparatus consisting of three identical arms (33×11×12cm each) which are symmetrically separated at 120° with an equilateral triangular central area, as previously described (18, 19). An arm entry was recorded when the hind paws of the rat were completely within the arm, and spontaneous alternation behaviour was defined as three consecutive entries in three different arms (18, 19). The percentage of alternation was calculated as the total of alternations / (total arm entries – 2×100).

Evaluation of biochemical parameters

The hippocampus was homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.4), and the homogenate was then centrifuged at 10,000 g for 10 min at 4°C (20, 21). The supernatant was collected and evaluated for Catalase – CAT (22), Superoxide dismutase – SOD (23), and Malondialdehyde – MDA (24).

Histological evaluation

After suitable fixation of the hippocampus in 10% buffered formal saline for 72 h, processing through the paraffin wax embedding and the Hematoxylin and Eosin staining method was done as previously described (25).

Gene Expression Assessment

Using real-time quantitative reverse transcription PCR, an assessment of Caspase-3 and NRF-2 gene expression was done as previously described (26). Briefly, using freshly excised hippocampi, total RNA was extracted and DNA was purified following DNAse I treatment (NEB, Cat: M0303S) according to the manufacturer's instructions. Purified DNA-free RNA was converted to cDNA immediately using the M-MuLV

Reverse Transcriptase Kit (NEB, Cat: M0253S) (26). PCR amplification was done using OneTaq® 2X Master Mix (NEB) with the primer set shown below (Table 1).

Molecular docking study

In-silico molecular docking of rutin was performed on Acetylcholinesterase (AChE) and Brain-Derived Neurotrophic Factor (BDNF). The structures (PDB ID: 4pqe, 1b8m, respectively) were obtained from the Protein Data Bank. Using Auto Dock Vina Software, a docking investigation was carried out as previously described (28), and the binding affinities/energies were reported in Kcal/mol. The renderings for the 2D diagrams and 3D (surface) view of the interactions were computed using the BIOVIA Discovery Studio 2019 and the PyMOL Molecular Graphics Software, respectively, as previously reported (29).

Table 1: Experimental Genes and Primers

Primer Name	Primer Sequence (5′-3′)	Gene Accession Number
NRF-2	Forward: GTCAGCTACTCCCAGGTTGC Reverse: ATATCCAGGGCAAGCGACTG	NM_001399173.1
Caspase-3	Forward: GAGCTTGGAACGCGAAGAAA Reverse: CCATTTTGTAACTGCTGTCCAGA	NM_012922.2

Gel density quantification was carried out using Image-J software, and data were reported relative to the β-actin gene (27).

Statistical analysis

Analysis of data was carried out using the GraphPad Prism Software V9. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post hoc test was utilized to determine statistical significance ($p < 0.05$). Values are presented as Mean ± Standard Error of Mean (SEM).

RESULTS

Effect of treatment on Neurobehaviour

A significant decrease (P<0.05) was observed in the discrimination index of the $NiCl_2$-treated group B rats when compared to control. However, a significant increase (P<0.05) was observed in the rutin pretreated rats (RUT1 + $NiCl_2$ and RUT2 + $NiCl_2$) when compared to the $NiCl_2$-treated group B rats (Figure 1).

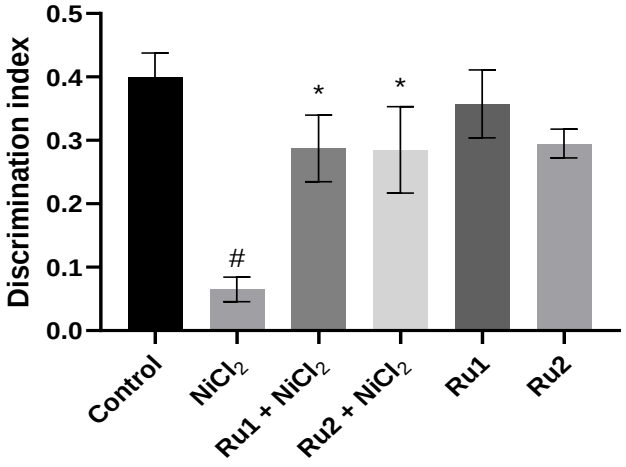

Figure 1: Discrimination index of control and treatment groups after 28 days. # $p< 0.05$ compared with the control group; * $p<0.05$ compared with the $NiCl_2$-alone group.

In addition, there was a significant decrease (P<0.05) in the number of total alternations and spontaneous alternations in the $NiCl_2$-treated group B rats when compared to control, however, a significant increase (P<0.05) was observed in the rutin pretreated rats (RU1 + $NiCl_2$ and RU2 + $NiCl_2$) when compared to the $NiCl_2$-treated group B rats (Figure 2).

Effect of Treatment on Oxidative Stress

Here, a significant decrease (P<0.05) in SOD and CAT was observed in the $NiCl_2$-treated group B rats when compared to control. However, a significant increase (P<0.05) was observed in the rutin pretreated rats (RU1 + $NiCl_2$ and RU2 + $NiCl_2$) for CAT, and in the rutin pretreated rats group D (RU2 + $NiCl_2$) for SOD, following comparisons to the $NiCl_2$-treated group B rats (Figure 3). For MDA, a significant increase (P<0.05) was observed in the $NiCl_2$-treated group B rats when compared to control. However, a significant decrease (P<0.05) was observed in the rutin pretreated rats (RU1 + $NiCl_2$ and RU2 + $NiCl_2$) when compared to the $NiCl_2$-treated group B rats (Figure 3).

Effect of Treatment on Hippocampal Histology

Plate 1A-F shows the representative histology of the hippocampus CA1 in control and treatment rats. Plate 1A (Control group) revealed the normal structure of pyramidal cells and astrocytes. Plate 1B ($NiCl_2$ treated) showed atrophy and vacuolated pyramidal cells and astrocytes. Plates 1C & 1D (RU1+ $NiCl_2$ and RU2 + $NiCl_2$) showed fewer vacuolations and relatively normal pyramidal cells. Plates 1E & 1F (RU1 and RU2) showed relatively normal pyramidal cells and astrocytes.

Effect of Treatment on Gene Expression

There was a significant decrease (P<0.05) in NRF2 expression in the hippocampus of $NiCl_2$-treated group B rats when compared to control. However, a significant increase (P<0.05) was observed in the hippocampus of rutin pretreated rats (RU1

+ $NiCl_2$ and RU2 + $NiCl_2$) when compared to the $NiCl_2$-treated group B rats. Also, there was a significant increase ($P<0.05$) in Caspase-3 expression in the hippocampus of $NiCl_2$-treated group B rats when compared to control. However, a significant decrease ($P<0.05$) was observed in the hippocampus of rutin pretreated rats (RU1 + $NiCl_2$ and RU2 + $NiCl_2$) when compared to the $NiCl_2$-treated group B rats.

In-silico Findings

Table 2 represents the binding energy and interaction of rutin against NF-kB. Also, Figures 5 and 6 show the 2D and 3D (active site) views of rutin against NF-kB.

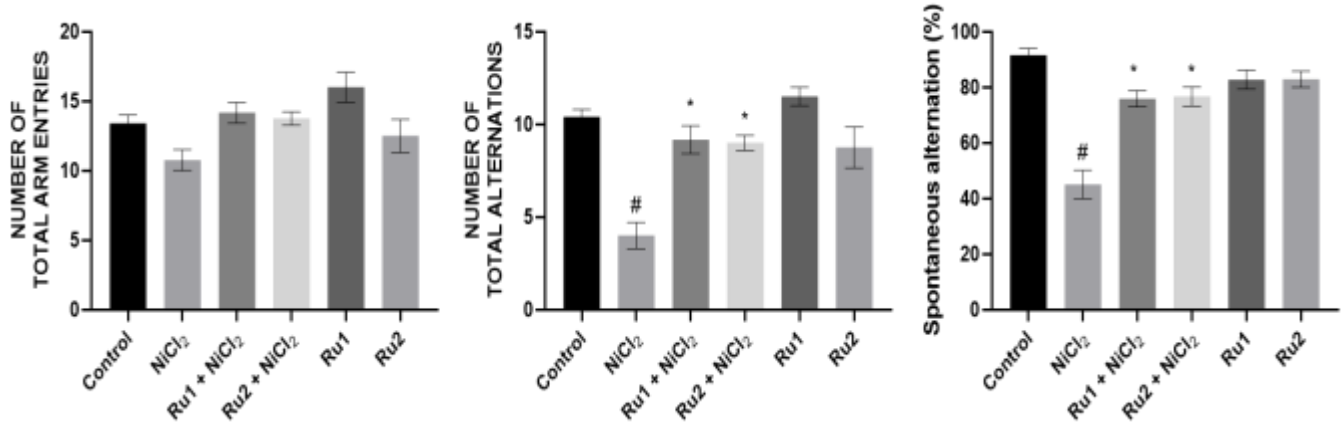

Figure 2: Y-maze parameters of control and treatment groups after 28 days. # $p< 0.05$ compared with the control group; * $p<0.05$ compared with the $NiCl_2$-alone group.

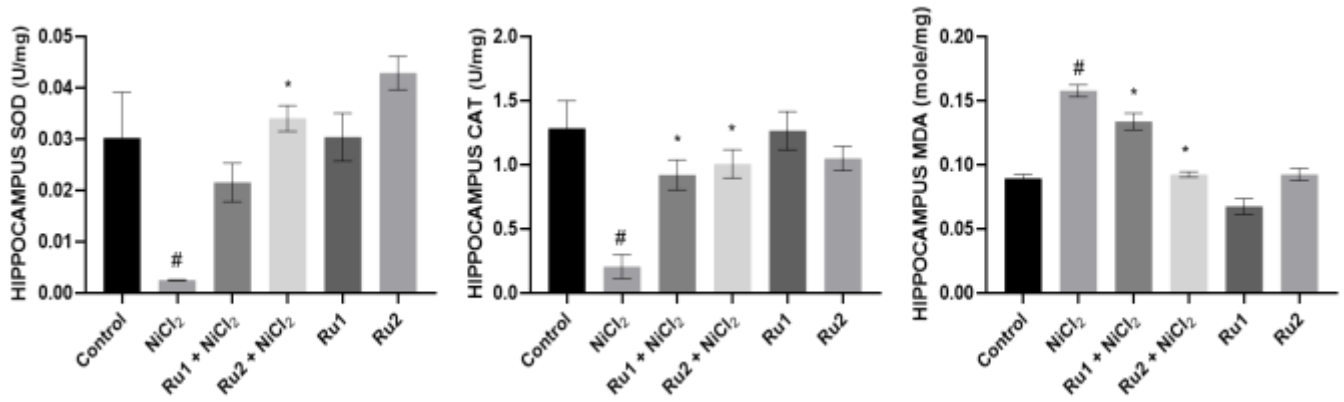

Figure 3: Oxidative stress assessment in the hippocampus of control and treatment groups after 28 days. # $p< 0.05$ compared with the control group; * $p<0.05$ compared with the $NiCl_2$-alone group

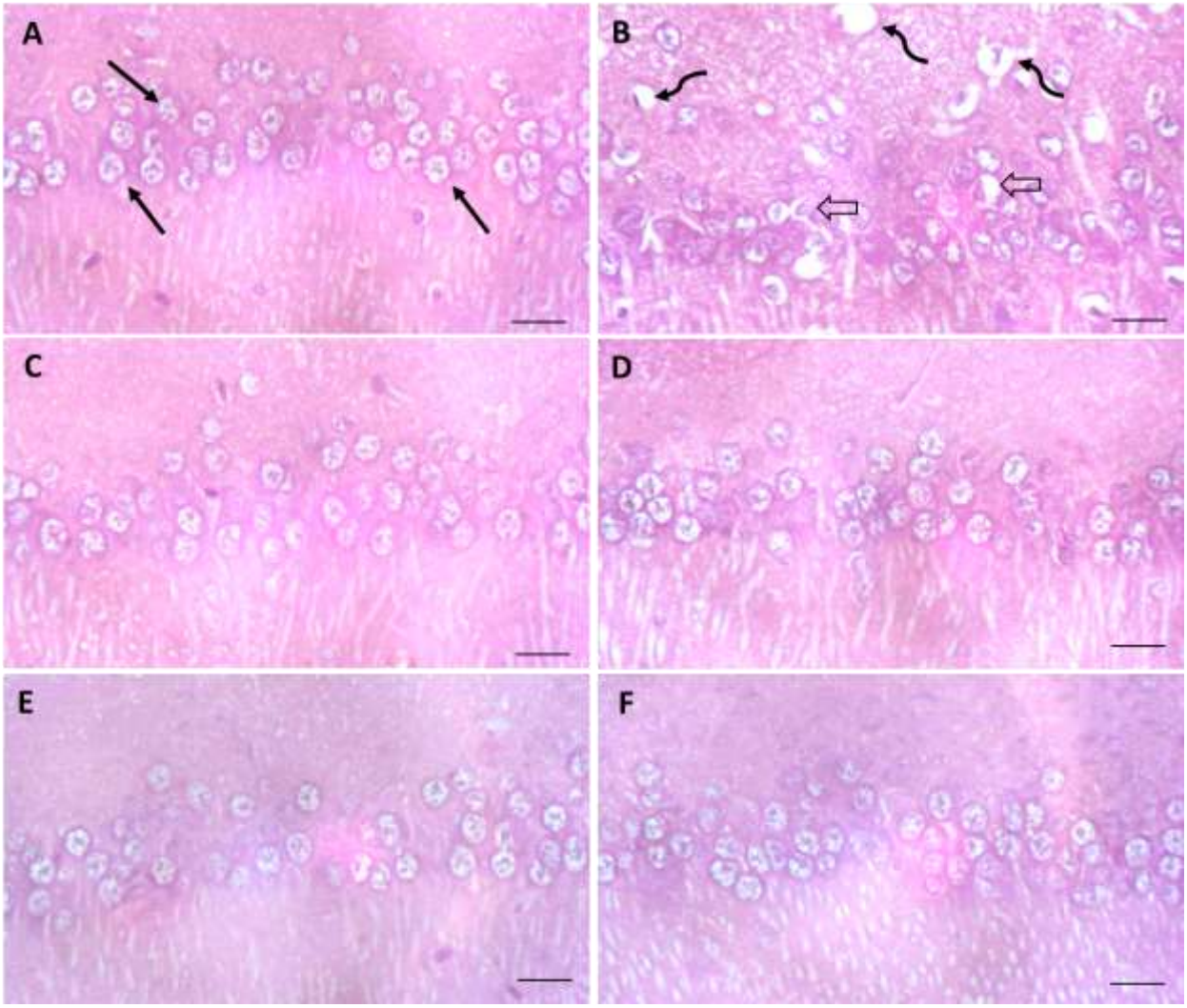

Plate 1: Representative Histology of the Hippocampus across experimental groups. (A) Control - normal Pyramidal cells [arrows]. (B) $NiCl_2$ group - atrophy and vacuolated pyramidal cells [double arrows] and astrocytes [curved arrows] (C) RU1 + $NiCl_2$ - normal Pyramidal cells (V) (D) RU2 + $NiCl_2$ - normal Pyramidal cells (E) RU1 - normal Pyramidal cells (F) RU2 - normal Pyramidal cells (H&E; 400x; Scale bar: 25µm

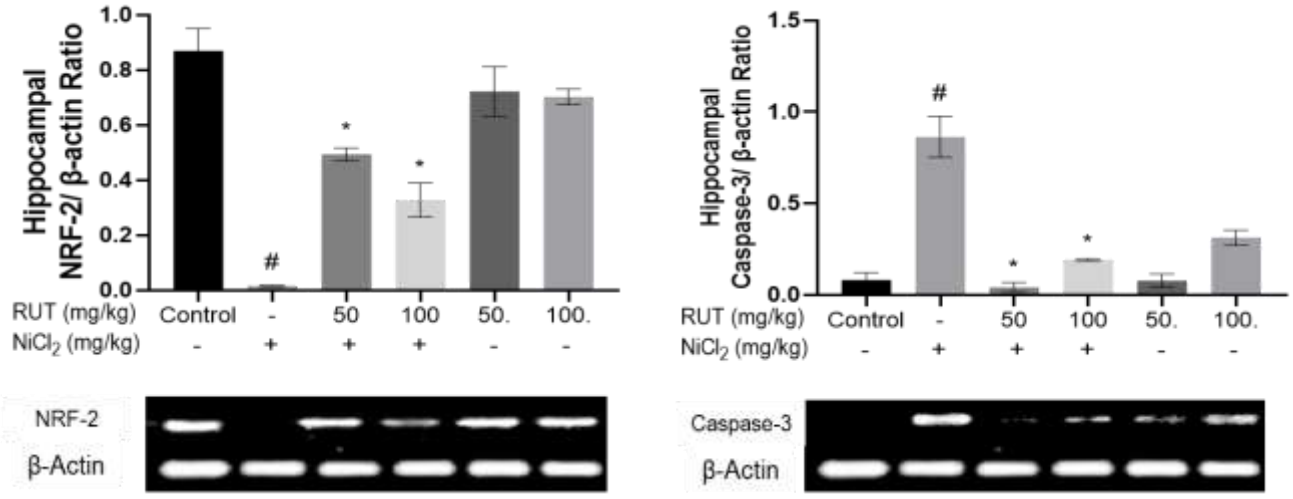

Figure 4: Expression of NRF-2 and Caspase-3 in the Hippocampus of rats across experimental groups. $^{\#}$ $p<0.05$ compared with the control group; * $p<0.05$ compared with the $NiCl_2$ group.

Table 2: Binding energy against IL-6

Compound	AChE (Kcal/mol)	BDNF (Kcal/mol)
Rutin	- 8.6	- 7.4

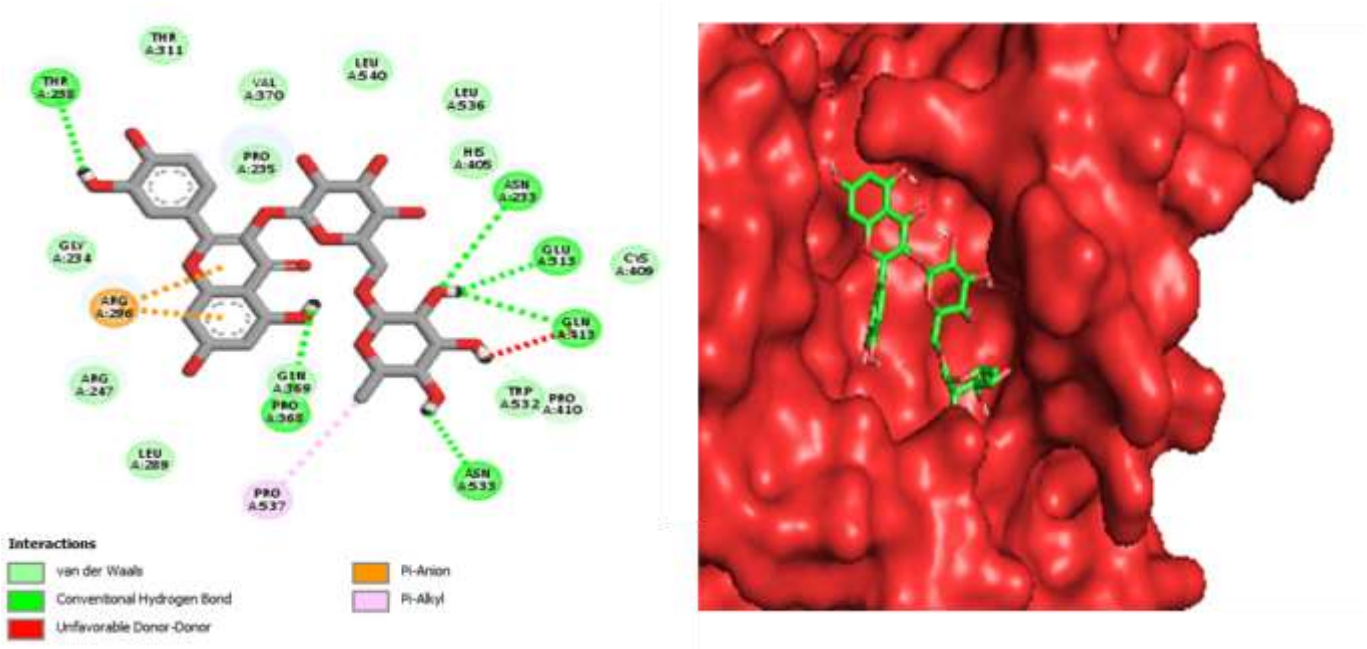

Figure 5: 2D and 3D surface view of rutin at the active site of AChE.

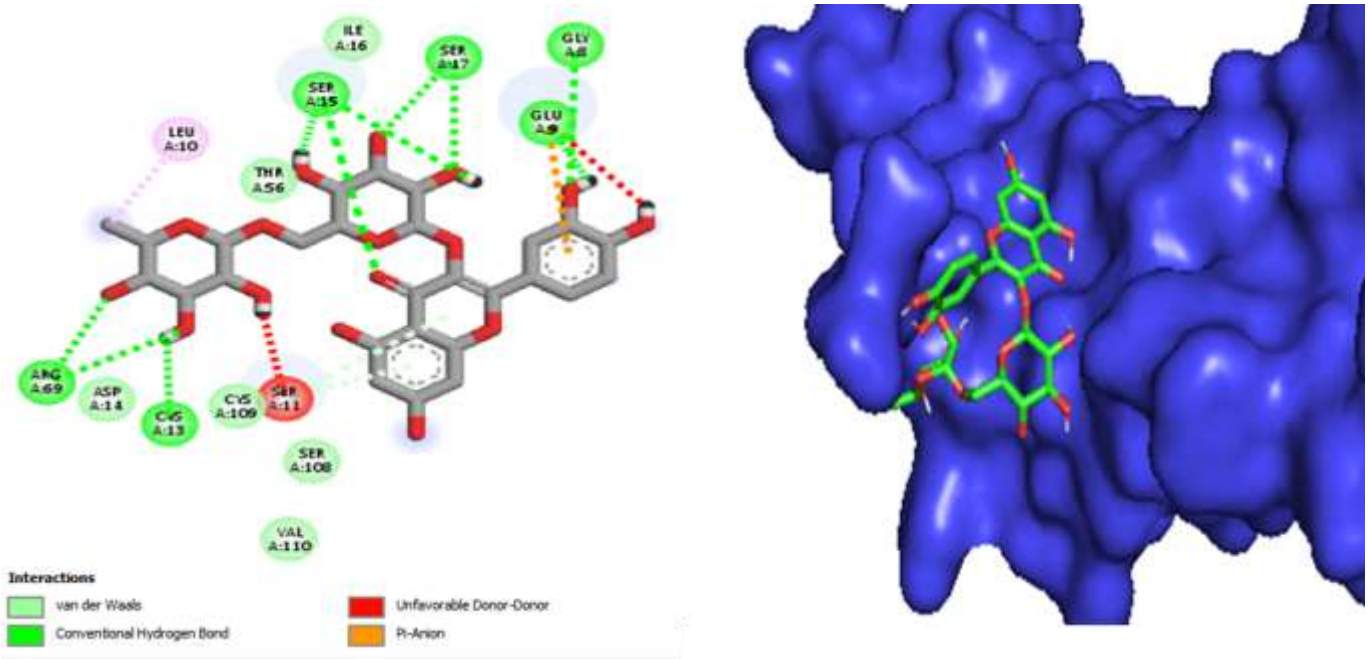

Figure 6: 2D and 3D surface view of rutin at the active site of BDNF.

DISCUSSION

Exposure to nickel in both environmental and occupational settings can lead to toxicity in multiple organs such as the liver, kidneys, lungs, skin, gonads, and brain. Notably, exposure to nickel specifically targets the nervous system, leading to a range of neurological symptoms (30). This study examined the impact of $NiCl_2$ on neurobehavior, oxidative stress, hippocampal histomorphology, and gene expression levels of NRF-2 and Caspase-3, as well as the protective role of rutin pretreatment in Wistar rats.

The hippocampus constitutes a vital component of the limbic lobe, playing a crucial role in memory processing, learning, spatial navigation, and emotions. It is instrumental in spatial navigation and the consolidation of information from short-term memory into long-term memory (31). Hippocampal neurons encode various physical variables, such as space or auditory frequency, within cognitive maps. The presence of integrated cognitive maps implies that the hippocampus engages in a fundamental computation, generating task-specific low-dimensional structures that encapsulate a geometric representation of acquired knowledge (32). The Y-maze test is utilized as an assessment of spatial working memory, employed to measure cognitive impairment, particularly in learning and memory domains (18, 19, 33). This assessment relies on observing animals' spontaneous alternation behavior, which indicates their cognitive abilities (34). In this study, a significant decrease in total alternation and spontaneous alternation behavior was noted in the rats exposed to $NiCl_2$ when compared to the control group, indicating cognitive impairment. This finding aligns with previous studies demonstrating that toxicity from $NiCl_2$ results in cognitive impairment (35, 36). The novel object recognition test (NOR) is a two-trial cognitive paradigm used to assess recognition memory (37, 38). In this study, the rats exposed solely to $NiCl_2$ exhibited significantly diminished discrimination index when compared to the control rats. The $NiCl_2$-exposed rats exhibited a very low discrimination index, suggesting an inability to distinguish between familiar and novel objects. These findings correlate with previous studies reporting that $NiCl_2$ toxicity causes a decline in hippocampal function and cognitive ability (39, 40). However, pretreatment of $NiCl_2$-exposed rats with rutin, a significantly higher spontaneous alternation and discrimination index was observed in the rats, demonstrating its capacity to enhance cognition and memory.

Reactive oxygen species are closely associated with declining health and neurological disorders such as Alzheimer's and Parkinson's diseases. They are known by-products of cellular processes, particularly mitochondrial respiration, and their increased reactivity is linked to the damage of macromolecules such as proteins, lipids, and DNA (41). Prolonged exposure to both endogenous and exogenous reactive oxygen species leads to structural and oxidative changes in crucial biomolecules. Chronic oxidative stress is correlated with modifications occurring in key biomolecules, including lipid peroxidation, protein carbonylation, and DNA damage, such as strand breaks or nucleobase oxidation. This oxidative stress is intimately associated with neurodegenerative processes (10, 42). Antioxidant enzymes stabilize or deactivate free radicals before they harm cellular components by reducing their energy or donating electrons to render them stable. They also intervene in the oxidizing chain reaction to mitigate damage caused by free radicals (43). This study showed a significant decrease in the antioxidant enzymes activity of SOD and CAT in the hippocampus of $NiCl_2$-exposed rats. This indicates that $NiCl_2$ exposure can alter antioxidant functions by inhibiting antioxidant enzymes activity and promoting the generation of reactive oxygen species, in agreement with previous studies (40, 44). However, pre-treatment with rutin was able to mitigate the dysregulation of antioxidant enzymes in nickel-exposed rats, thus demonstrating the potent antioxidant activity of rutin. The extent of lipid peroxidation in biological samples can be assessed by measuring the concentration of malondialdehyde (MDA). MDA is an end product of lipid peroxidation and is widely recognized as one of the most prominent and dependable biomarkers for lipid peroxidation and oxidative stress (45). The study demonstrated a notable increase in MDA activity in the hippocampus of rats treated solely with $NiCl_2$, and aligns with findings from other studies (35, 40). However, rutin was effective in mitigating this effect, thus suggesting its ability to protect against $NiCl_2$-induced oxidative stress.

Memory formation relies significantly on the hippocampus (46). The hippocampal CA1 field integrates a wide array of subcortical and cortical inputs. It exhibits excitatory properties, targeting dendritic spines, and displays a characteristic macular shape across different layers examined (47). CA1 is instrumental in the retention of social memory, particularly in behavioral neuroscience and neurophysiology, elucidating its functional role in encoding and storing social experiences (48). In this study, the CA1 region of the hippocampus in control rats revealed normal architecture with intact pyramidal cells and astrocytes. In contrast, $NiCl_2$-treated rats exhibited altered morphology, characterized by vacuolation of pyramidal cells and astrocytes, as well as pyknotic nuclei within the pyramidal cells. These morphological changes align with previous neuropathological findings on the toxic effects of $NiCl_2$ on brain structure (40, 44, 49). Specifically, these alterations are associated with impairments in both short-term and long-term memory functions. In rats pretreated with rutin, the hippocampus displayed little or no vacuolations and normal pyramidal cells when compared to those treated with $NiCl_2$ alone, thus indicating its protective activity. Additionally, rats treated with rutin alone exhibited hippocampal histology similar to the control group, suggesting that rutin was not toxic to the rats.

The Nuclear factor erythroid 2-related factor 2 (NRF-2), acting as a master regulator of redox homeostasis, serves as a key transcription factor overseeing the expression of numerous genes encoding antioxidant and detoxification enzymes (50). NRF-2 governs the physiological balance of cellular redox status and manages responses to stress and inflammation (51). It responds to oxidative stress by promoting the expression of numerous cytoprotective genes, including those governing mitochondrial and non-mitochondrial antioxidant proteins (52). In this study, a significant downregulation in the expression of NRF-2 was observed in the hippocampus of rats exposed to $NiCl_2$. This is consistent with previous studies demonstrating reduced expression of NRF-2 with nickel

exposure in the liver and kidney (53, 54). Nevertheless, pre-treatment with rutin was able to upregulate the expression of NRF-2 in the hippocampus of rats, thus affirming its protective effects against oxidative stress. Caspase-3, a commonly found member of a conserved protein family, is widely acknowledged for its activated proteolytic functions in driving apoptosis (55). This process occurs in cells responding to various extrinsic or intrinsic triggers of programmed cell death. Neuronal apoptosis occurs through excessive production of free radicals, calcium overload, and excitotoxicity, leading to the opening of mitochondrial permeability transition pores and facilitating the translocation of apoptogenic proteins such as cytochrome c into the cytosol (56). Cytochrome c interacts with apoptotic protease-activating factor-1, activating pro-caspase-9, which subsequently activates caspase-3, essential for apoptosis execution. In this study, the expression of caspase-3 was significantly upregulated in the hippocampus of $NiCl_2$-treated rats; this is consistent with previous studies demonstrating elevated caspase-3 expression upon exposure to nickel (44, 57). However, rutin significantly inhibited the expression of caspase-3, thus demonstrating its anti-apoptotic effects against $NiCl_2$ toxicity.

Findings from the *in-silico* docking analysis revealed that rutin interacted with AChE, the enzyme responsible for breaking down the neurotransmitter acetylcholine, which plays a role in the development and progression of neurodegenerative diseases like Alzheimer's. Reports indicate that overactivity of AChE causes a decrease in acetylcholine, contributing to the degeneration of the cholinergic system, which is crucial for cognitive function (37, 58). AChE also interacts with amyloid-beta (Aβ) peptides, which are key components of the plaques found in Alzheimer's, potentially accelerating their aggregation and increasing neurotoxicity (59). Understanding the role of AChE in neurodegenerative diseases highlights the potential of targeting this enzyme for therapeutic interventions. This strong interaction with AChE aligns with previous studies indicating that rutin inhibits the excessive activity of AChE (60, 61). Inhibition of Brain-Derived Neurotrophic Factor (BDNF) signaling is implicated in several neurodegenerative diseases, such as Alzheimer's and Parkinson's (62). Reduced BDNF levels are associated with neuronal loss, altered synaptic function, increased neuroinflammation, tau protein phosphorylation, amyloid (Aβ) accumulation, and neuronal apoptosis (63). BDNF levels are often decreased in Parkinson's, and lower levels are correlated with cognitive impairment and depression. The strong and stable interaction with BDNF aligns with previous studies indicating that rutin upregulates the expression of BDNF (64, 65), thus demonstrating its cognition-enhancing effects and its role as a possible therapeutic agent against cognitive disorders.

Conclusion: The results of this study suggest that rutin possesses strong neuroprotective properties and may have potential applications in the treatment and management of neurological disorders associated with nickel exposure. Additional investigations in other experimental models are encouraged to corroborate these findings. Furthermore, there is a need for studies into the potential synergistic effects of rutin in combination with other neuroprotective compounds.

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