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Original Article

Beneficial role of rosemary extract on oxidative stress-mediated neuronal apoptosis in rotenone-induced attention deficit hyperactivity disease in juvenile rat model

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Abstract. *Background and aim***:** Attention deficit hyperactivity disorder (ADHD) is heterogeneous neurobehavioral disorders that co-exist with cognitive and learning deficits affecting 3-7% of children. We study the role of rosemary in the protection of the prefrontal cortical neurons against rotenone-induced ADHD in juvenile rats. *Methods:* Twenty-four juvenile rats were divided into four groups (n=6): control group, received olive oil 0.5 ml/kg/day/ I.P. for 4 weeks, rosemary group received rosemary 75 mg/kg/day/ I.P. for 4 weeks, rotenone group received rotenone 1 mg/kg/day/ I.P. dissolved in olive oil for 4 days and combined group received rotenone 1 mg/kg/day/ I.P. for 4 days and rosemary 75 mg/kg/day/ I.P. for 4 weeks. *Results:* Rotenone group showed higher impulsivity with reduction in the recognition index and total locomotor activity. However, combined group showed significant improvement in the recognition index and the total locomotor activity. Neurochemical analysis disclosed that rotenone decreased levels of GSH and significantly increased lipid peroxidation and oxidative stress. The administration of rosemary amended these neurochemical changes. Rotenone caused a significant increase in serum amyloid protein A and C-reactive protein levels indicating a marked state of inflammation. Rosemary ameliorated these biochemical changes. The immunohistochemical expression of tyrosine hydroxylase was decreased in the rotenone group. On the other hand, caspase-3 was increased in the rotenone group. PCR confirmed immunohistochemical results for gene expression. *Conclusions:* The findings of the behavioral, neurochemical, biochemical, immunohistochemical and molecular outcomes suggested that rosemary could fight oxidative stress, inflammation and apoptosis in the prefrontal cortex of rotenone-induced ADHD in juvenile rats. (www.actabiomedica.it)

Key words: ADHD, Rotenone, Rosemary, Tyrosine Hydroxylase, Caspase-3

Introduction

Attention deficit hyperactivity disorder (ADHD) is a largely predominant childhood-onset neurodevelopmental disorder characterized by developmental extremes and impaired symptoms, including intellectual and behavioral symptoms such as hyperkinesia, inattention, confusion, and impulsivity, occurring before age 12 and often continuing into adulthood (1-4). ADHD occurs when children have many troubles in focusing, behaving appropriately, and doing their usual tasks. This can lead to problems in their schoolwork

and day-to-day lives (5). Though ADHD was previously thought to be a childhood disorder, longitudinal studies have shown that most patients' ADHD symptoms persist into adulthood (6). ADHD animal models were produced using rotenone, 6-hydroxydopamine and bisphenol-A to display similar behaviors and genetic abnormalities to those found in human cases of ADHD, furthermore, those models can be used to identify the causes and pathology of ADHD (7, 8).

Rotenone, a plant-derived pesticide, is the most potent neurotoxin that belongs to the rotenoid family. Due to its lipophilic nature, rotenone can easily cross the blood-brain resulting in suppression of mitochondrial Complex I of the electron transport chain (9, 10). Previous studies suggested that rotenone may affect the central nervous system of both children and adults by oxidative stress and thus triggering neuronal cell death (11). High doses (16 mg/kg) or repeated low doses of rotenone (1 mg/kg/day for 4 days) cause general restlessness and excessive movement in juvenile rats (8). In neonatal rat models, rotenone causes a functional disability involving the dopaminergic system, thus proposing the likelihood of inflammation-induced neurological dysfunction (12). Due to changes in the brain's micro- and macrostructure, a hypothesis was made stating that an adult ADHD diagnosis is correlated with structural abnormalities in the frontal, temporal, basal ganglia, and parietal regions (13).

Due to their few side effects, natural medications with strong anti-inflammatory and antioxidant qualities are utilized as the favored treatment for ADHD since the disorder exerts oxidative stress-mediated dopaminergic neurotoxicity (14). Rosmarinus officinalis L. is an aromatic plant with an amiable smell, belonging to the Lamiaceae family. The plant has derivatives like ursolic acid, rosmarinic acid, micromeric acid, and oleanolic acid making it a possible natural antioxidants source (15). Rosmarinic acid acts as an immune regulator in various ways, such as an antioxidant, antiangiogenic, anti-apoptotic, anti-fibrotic, chemo-preventive, and Alzheimer's disease preventive (16). Although these natural substances possess antioxidant and anti-inflammatory characteristics, their neuroprotective effects are deficient. Hence, in the current study, we analyze the properties of rosemary leaf extract in a model of ADHD induced by rotenone in juvenile rats. Because rosemary extract contains several biologically active compounds, we believe it has neuroprotective mechanisms through its antioxidant and anti-inflammatory properties in a rotenone-induced ADHD rat model.

Materials and methods

Animals

Twenty-four juvenile albino rats (5-days old) were bought from the Medical Experiment Research Centre (MERC) of Mansoura University. The rats were weaned at postnatal day 21 (P21) so they would grow and behave normally in the experiment. After weaning, every two to three rats were kept in the same cage and given drinking water and standard rodent chow. All rats were kept at constant temperature and humidity (22°C and 55%, respectively) and on a diurnal rhythm of 12-hour light/dark cycle. Every effort was made to reduce animal suffering.

Chemicals used

Rotenone and rosemary extract were obtained in powder forms from Sigma -Aldrich™ (Saint Louis, MO, USA). Olive oil was secured from Al Nasr Company (Egypt) for chemical industries. Antibodies against TH and caspase-3 were bought from Abcam (Egypt).

Study design

The rats were divided randomly into 4 groups (n = 6/group):

- Group I (Vehicle group): control group received olive oil at a dose of 0.5 ml/kg/day through intraperitoneal (i.p.) injections for 4 weeks.
- Group II (Rosemary group): received rosemary extract at s dose of 75 mg/kg/day i.p. for 4 weeks (16).
- Group III (Rotenone group): received rotenone at a dose of 1 mg/kg/d for 4 days i.p. dissolved in olive oil (8).
- Group IV (Rotenone + Rosemary group): received

both rotenone at a dose of 1 mg/kg/day i.p for 4 days and rosemary extract at 75 mg/kg/day i.p for 4 weeks.

Behavioral assessment

The assessment began on day 29 after the last intraperitoneal injection. The rats were accustomed to the test room 30 minutes before the start of the analyses. The handling, adaptation, and behavioral testing ensued during the lights-off period, under low light. The rats were subjected to three behavioral tests:

1. Object-based attention test (OBA) (17)

The Object-Based Attention test (OBA) is used to test attention. On the first day, the habituation phase, the rats were placed for 5 minutes in each of the exploration and testing chambers. Then the second day, the acquisition phase, the rats were also kept for 5 minutes in each chamber: in the exploration chamber with 5 novel wooden objects and in the testing chamber with 2 wooden objects, one novel and one familiar. Finally, on the third day, the rats were familiarized with each chamber again for 3 minutes. The preference for the novel object was measured by recording the time spent engaged with each object (novel vs. familiar). The enclosed area and objects were cleaned after each animal to prevent odor cues from interfering.

2. Cliff avoidance reflex test (CAR) (18)

The Cliff Avoidance Reflex test (CAR) is used to report impulsive behavior. CAR was evaluated using a round wooden podium (diameter, 20 cm; thickness, 2 cm), held up by an iron rod (height, 50 cm). The floor below the platform was covered with carpet to avoid injury to the animals in case they fell. The test started by gently putting a rat on the wooden podium so that its forelimbs were close to the verge. If the rat fell from the podium, it was considered to have a compromised CAR, the test was repeated over a course of 60 minutes for each rat. Rats that did not fall from podiums were also tested for the same period.

3. Open-field test (19)

The open-field test is used to measure hyperactivity. An open field apparatus measuring 72 x 72 cm^2 was constructed of white plywood with walls measuring 36 cm high. One of the walls was clear, allowing the rats to be seen inside the apparatus. Blue lines were drawn dividing the floor into sixteen squares, each measuring18 x 18 cm. In the center of the open field, an 18 x 18 cm^2 central square was created. For 5 minutes, the rats were allowed to explore the apparatus then they were sent back to their home cages after the exploration period. The open field was cleansed with 70% ethyl alcohol and left to dry between tests. The number of lines crossed, the frequency of rearing, and the number of central square entries was the factor chosen for the measurement of the rats' hyperactivity. Each rat was assigned a score based on the sum of those factors.

The sacrifice of rats and specimens' collection

The rats were sacrificed by cervical dislocation at the appointed time. The blood samples were obtained from the tail vein. The prefrontal cortex (PFC) was dissected; the PFC of one hemisphere was fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.05% phosphate buffer saline (PBS: pH 7.4) for histological and immunohistochemical studies. The PFC of the other hemisphere was divided equally into two halves. One half was maintained in a flask with RNA later and kept at -80°C to be used for gene expression. The other half was kept at -20°C for homogenate preparation.

Preparation of PFC homogenates

Prefrontal cortex homogenates were weighed in an analytical balance after being gently marked between the folds of filter paper to prepare 10% homogenate in 0.05 M phosphate buffer (pH 7) a polytron homogenizer was used at 4°C. For 20 minutes, the homogenates were centrifuged at 10,000 rpm to remove any cell debris, unbroken cells, erythrocytes, nuclei, and mitochondria. For further analysis, the supernatant was aliquoted and stored at -80 °C.

Assessments of oxidative stress and lipid peroxidation markers

Following the manufacturer's instructions, levels of nitric oxide (NO), malondialdehyde (MDA), and

reduced glutathione (GSH) were detected in PFC homogenates using commercial colorimetric kits from the Biodiagnostic Company (Cairo, Egypt).

Assessments of inflammatory markers in the serum

Measurement of serum C-reactive protein (CRP) was performed using the CRP ELISA kit (ab99995; Abcam, Cambridge, MA) based on the method described by Li et al. (20). Moreover, serum levels of amyloid protein A (SAA) were measured using Human SAA ELISA Kit (SAA1) (ab100635; Abcam, Cambridge, MA) concurring with the modified method described by Chang et al. (21).

Processing the specimens for pathological examination

PFC specimens were dehydrated in increasing concentrations of alcohol (70%, 95%, and 100% alcohol), cleaned in xylene, and permeated with two paraffin changes (70 °C, 2 hours each). Then the tissues were fixed in paraffin wax and cut at 5 μm intervals. Finally, staining of the sections was done with Hematoxylin (H3136, Sigma-Aldrich) and Eosin (230,251, Sigma- Aldrich) (H & E) (22) for histopathological assessment.

Immunohistochemical determination of caspase- 3 and tyrosine hydroxylase

To eliminate endogenous peroxidase activity prior to immunohistochemical staining, prefrontal cortical (PFC) tissue sections were treated with 3% hydrogen peroxide. For antigen retrieval, the slides were rinsed three times in PBS (pH 7.4) and then inoculated in sodium citrate buffer (0.01 M, pH 6.0) for 30 minutes in a water bath (95 °C). The slides were inoculated with bovine serum albumin (BSA) (1%, 1 hour) after reaching room temperature, and subsequently with the principal antibodies (4 C, overnight): anti-caspase-3 (ab2302, Abcam- Cambridge, UK, 1:100) (ab2302, Abcam- Cambridge, UK, 1:100) (23) or recombinant anti-tyrosine hydroxylase antibody (ab137869, Abcam, Cambridge-UK, 1:200) (24). Secondary antibodies conjugated with horseradish peroxidase were incubated on the sections for 0.5 hours at 37 °C, followed by labeled streptavidin-biotin (0.5 h) (DETHP1000, Sigma-Aldrich). Finally, we used hematoxylin as a counterstain and diaminobenzidine (DAB: 3 min) to envision the reaction.

Quantitative measurement of area % of positive TH and caspase-3 immune reaction

Four nonconsecutive scattered sections (5μm thick) were examined for each rat in all the groups. Images were captured with a light microscope (Olympus model BX53, Tokyo, Japan) connected to a digital camera (Toup Cam model BX53, Japan) linked to a computer. The area fraction of immune expression of TH and caspase-3 was analyzed using a 40× objective (area: 0.071mm2). Examination of the immune-positive reaction was done using the computerized image analysis system Image-j (version1.48). Immune expression was visible in nearly all layers of every section as a distinct brownish coloration. Color deconvolution plugin was used to separate each image's color content by measuring the brown color in immune-stained sections and the threshold was accustomed to be more accurate (25).

mRNA quantification by quantitative real-time PCR (qRT-PCR)

Tissue sections were collected from the PFC of every rat in the four groups studied and homogenized with four strokes of liquid nitrogen. Total cellular RNA was isolated according to the manufacturer's instructions using the QIAzol reagent (Qiagen, Germany). Thermo Scientific Nano Drop 2000 (USA) was used to test the RNA yield for concentration using 260 nm absorbance and purity using 260/280 and 260/230 ratios. Reverse transcription of 1μg of RNA was done using Sensi FAST™ cDNA Synthesis Kit (Bioline, UK) by means of the following program: 10 minutes at 25°C for primer annealing, 15 minutes at 42°C for reverse transcription, and 5 minutes at 85°C for inactivation on Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA).

A real-time PCR instrument (Applied Biosystems 7500) was used to amplify cDNA templates. The amplification reaction was carried out in a 20 l total volume mixture [10 l of HERA SYBR green PCR

Master Mix (Willowfort, UK), 2 l of cDNA template, 2 l (10 pmol) gene primer, and 6 l of nuclease-free water] using the following program: 95°C for 2 minutes, then 40 cycles of 10 seconds at 95°C and 30 seconds at 60°C. The used primer pairs' sequences are supplied in (Table 1); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The Primer 3 software (v.4.1.0) [http://primer3.ut.ee] was used to identify primer sets, and the Primer-BLAST program [https://www.ncbi.nlm.nih.gov/tools/primer-blast/] was used to determine primer specificity. Melting curve analysis was used to examine the specificity of the PCR products, and Vivantis synthesised primer sets (Vivantis Technologies, Malaysia). Ct = Ct target gene– Ct housekeeping gene was employed to represent relative gene expression levels fold change of gene expression was calculated using the 2^{-CT} method (26).

Statistical analysis

Data analysis was done by IBM SPSS Corp. Released in 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. Numbers and percentages were used to describe qualitative data. After testing normality with the Shapiro-Wilk test, quantitative data were described using both mean and standard deviation for parametric, or medians and interquartile ranges for nonparametric data. Kruskal Wallis test was used to compare more than 2 independent groups with Mann Whitney U test to detect pair-wise comparison. The significance of the obtained results was judged at the (P: 0.05) level.

Ethical approval

This study was approved by the Institutional Research Board (IRB) (Code number: R.21.03.1241. R1.R2 - 2021/03/22), Faculty of Medicine, Mansoura University. All experiments were managed in accordance with the National Institutes of Health (NIH) guide for the use and care of laboratory animals. (NIH Publications No. 8023, revised 1978).

Results

The findings of this study on rotenone-induced ADHD in rat models suggest that rosemary extract may have protective properties.

Histopathological assessment results

Hematoxylin and eosin-stained sections from the control group showed the normal appearance of the prefrontal cortex (PFC); the granule cells had rounded vesicular nuclei with prominent nucleoli and the pyramidal cells had vesicular nuclei and long peripheral processes (Figure 1). Moreover, the rosemary-treated group showed normal appearing granule cells and pyramidal cells (Figure 2A). Unsurprisingly, rotenone-treated rat brain sections revealed shrunken neurons with dark basophilic cytoplasm and pyknotic nuclei. There were also many deeply stained neurons with pyknotic nuclei surrounded by halos (Figure 2B). Sections obtained from rats receiving a co-administration of rotenone and rosemary extract restored the normal

Table 1. The sequence of rat primers used in qRT-PCR analysis.

Gene	Sequence	Product length	Reference sequence
Caspase 3	**Forward primer:** GTGGAACTGACGATGATATGGC **Reverse primer:** CGCAAAGTGACTGGATGAACC	135 bp	NM_012922.2
Tyrosine hydroxylase (TH)	**Forward primer:** GTCCGCCCGTGATTTTCTG **Reverse primer:** AGATGCAAGTCCAATGTCCTG	184 bp	NM_012740.4
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	**Forward primer:** TGCCACTCAGAAGACTGTG **Reverse primer:** GGATGCAGGGATGATGTTC	85 bp	NM_017008.4

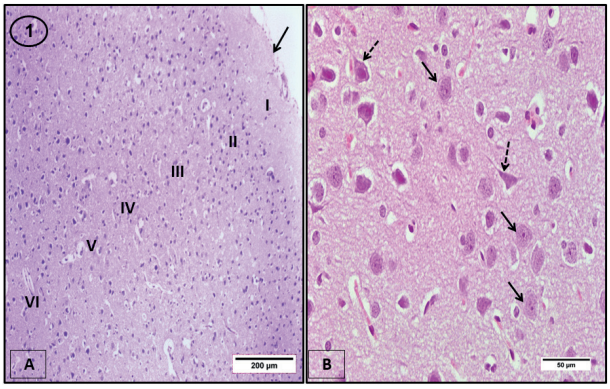

Figure 1. Photomicrographs of prefrontal cortex (PFC) sections of control rats: (A) The PFC showed its six-layer arrangement. Layers are numbered from I to VI. (B) The granule cells showed rounded vesicular nuclei with prominent nucleoli (arrows). The pyramidal cells had vesicular nuclei and long peripheral processes (dotted arrows) (H & E, A x 100, B x 400).

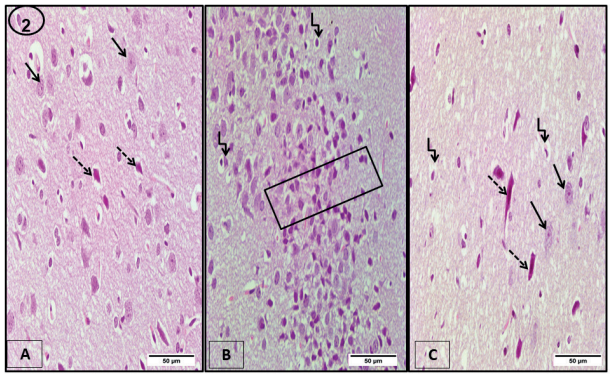

Figure 2. Photomicrograph of PFC sections of rats: (A): Rosemary-treated rats with normal appearing granule cells (arrows) and pyramidal cells (dotted arrows). (B) Rotenone-treated rats showed condensation of the cortical neurons (rectangle). There were many deeply stained neurons with pyknotic nuclei surrounded by halos (curved arrows). (C): Combined rotenone and rosemary-treated rats showed restoration of PFC. Granule cells (arrows) and pyramidal cells (dotted arrows) appeared normal. However, some deeply stained neurons with pyknotic nuclei surrounded by halos were still present (curved arrows) (H & E x 400).

appearance of neuronal cells, except for some neurons appearing deeply stained with pyknotic nuclei surrounded by halos (Figure 2C).

Immunohistochemistry assessment results

The effect of rosemary extract on Caspase-3 (apoptotic marker) immunohistochemistry: PFC Slides stained with caspase-3 antibody from both control and rosemary-treated groups showed weak positive caspase-3 immunostaining in some cortical neurons (Figure 3A, 3B). On the other hand, PFC sections from rotenone–treated rats showed strong positive caspase-3 immunostaining in most of the cortical neurons (Figure 3C). The effect of rotenone decreased with the administration of rosemary extract exhibiting weak positive caspase-3 immunostaining in some cortical neurons (Figure 3D).

Results of quantification of area % of positive caspase-3 immune reaction in the PFC: There was a significant rise in the area % of caspase-3 positive reaction in the PFC of the rotenone-treated group in comparison with both the control and the rosemary-treated groups. The reaction showed a significant decline in area % of caspase-3 during the administration of rosemary to the rotenone-treated group (P: 0.001) (Figure 3E).

The effect of rosemary extract on tyrosine hydroxylase (dopaminergic marker) immunohistochemistry: PFC sections stained with TH from the control and rosemary-treated groups showed many strong positive fibers (Figure 4A, 4B). However, sections from the rotenone-treated group displayed weak positive TH immunostaining (Figure 4C). For the group receiving both rotenone and rosemary, there was an increase in TH-positive fibers in comparison to those in the rotenone-treated group (Figure 4D).

Results of quantification of area % of positive TH immune reaction in the PFC: In addition, the area % of TH-positive reaction in PFC was significantly decreased in the rotenone-treated group in contrast to the rest of the groups, control, rosemary, rotenone + rosemary (P: 0.001) (Figure 4E).

Behavioral assessment results

1. Object recognition test: In comparison to the control group, time spent exploring both novel and familiar objects were significantly reduced in the rotenone group, with a significantly impaired recognition index. When comparing the rotenone-treated group to the group receiving both rotenone and rosemary; time exploring novel objects was statistically reduced with a significantly lower recognition index in the former group. There was no statistical difference between the

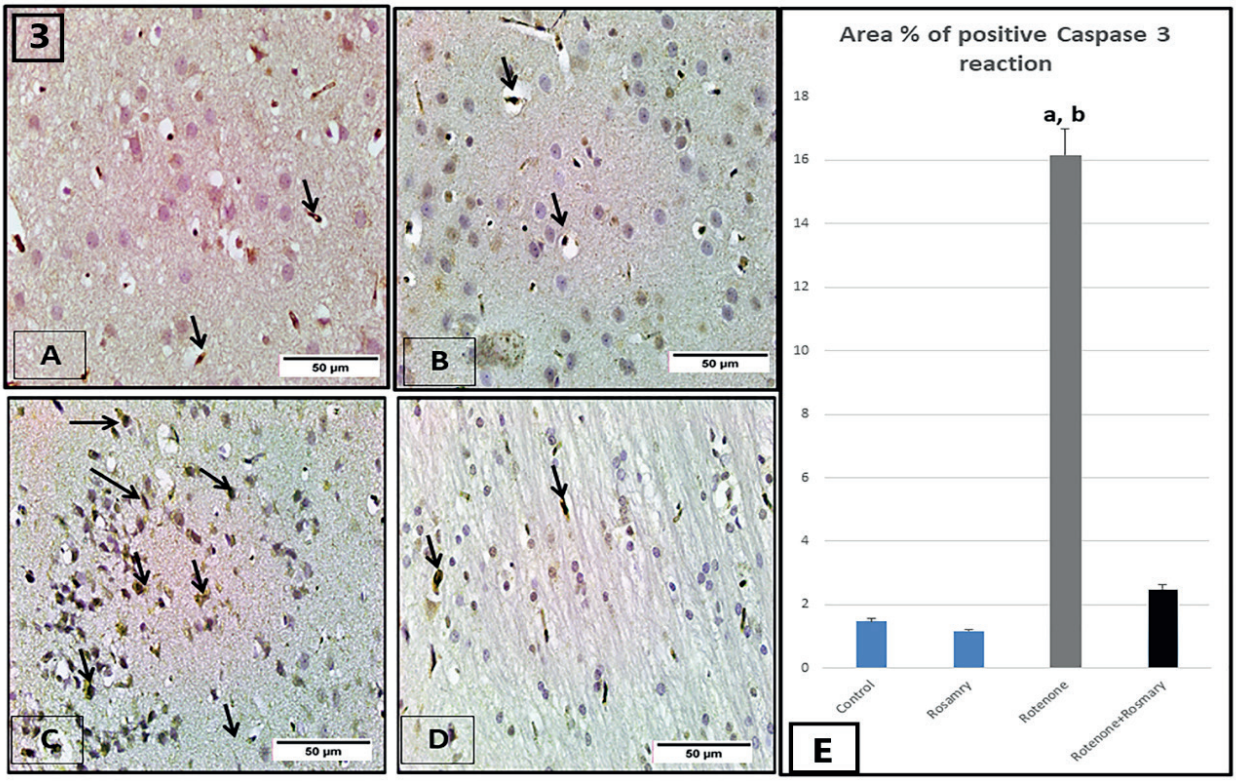

Figure 3. Photomicrographs of PFC sections of rats: (A), (B): Control and rosemary-treated rats respectively showed weak positive caspase-3 immunostaining in some cortical neurons (arrows). (C): Rotenone-treated rats showed strong positive caspase-3 immunostaining in most of the cortical neurons (arrows). (D): Combined rotenone and rosemary-treated rats showed weak positive caspase-3 immunostaining in some cortical neurons (arrows). (E): a: significant difference between rotenone-group and control or rosemary group (P value= 0.001). b: significant difference between rotenone-group and combined group (P: 0.001). There was no significant difference between the control and rosemary groups (Caspase-3 x 400).

controls and the groups that received both rotenone and rosemary.

2. Open field test: The rotenone + rosemary treated group showed significantly more line crossing in comparison to the control group (P: 0.05). On the contrary, the group receiving rotenone presented significantly reduced line crossing in comparison to the rest of the groups: the control group, rosemary group, and rotenone + rosemary treated group (P: 0.05, 0.003, 0.004, respectively). Moreover, the group receiving rotenone showed significantly reduced central zone entries in comparison to the rosemary group and rotenone + rosemary treated group (P: 0.01, 0.004, respectively). The score for total locomotor activity was significantly lower in the rotenone-treated group as opposed to the rest of the groups: control group, rosemary-treated group, and rotenone + rosemary-treated group (P: 0.05, 0.003, 0.004, respectively) (Table 2).

3. Cliff avoidance test: Rats in the rotenone group had a significantly higher tendency to fall from the cliff in comparison to the controls (P: 0.003) and the group receiving both rotenone and rosemary (P: 0.004). Though those receiving rosemary had a significantly lower tendency to fall compared to the control (P: 0.019) and rosemary with rotenone groups (P: 0.006).

Neurochemical assessment results

1. Rosemary's effect on oxidative stress markers: To better understand the role of oxidative stress in rotenone-induced neurodegeneration and the ameliorative effect of rosemary, we measured tissue levels of MDA,

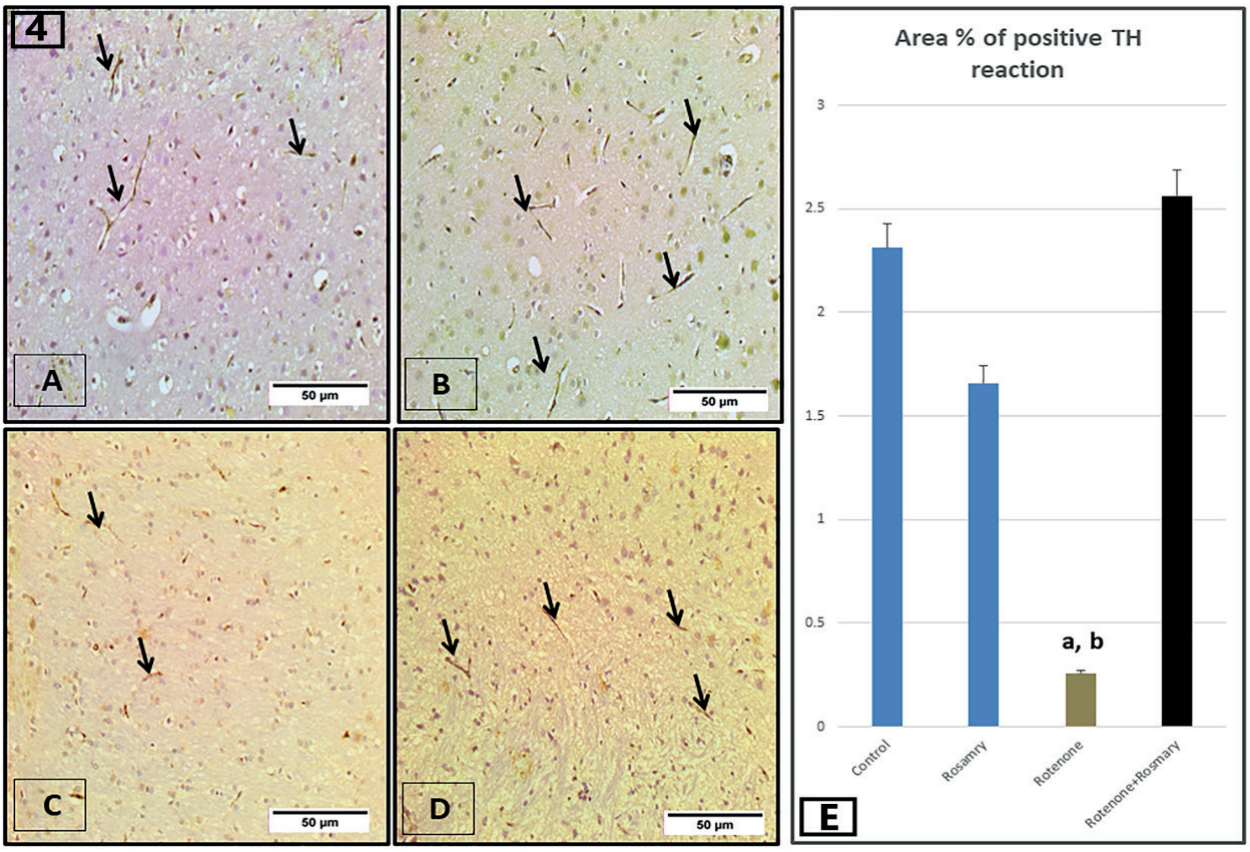

Figure 4. Photomicrographs of PFC sections of rats: (A), (B): Control and rosemary-treated rats respectively showed many strong positive TH-positive fibers (arrows). (C): Rotenone-treated rats showed a reduction in TH-positive fibers (arrows). (D): Combined rotenone and rosemary-treated rats exhibited an increase in TH-positive fibers (arrows) more than those of the rotenone-treated group. (E): a: significant difference between rotenone-group and control or rosemary group (P: 0.001). b: significant difference between rotenone-group and combined group (P: 0.001). There was no significant difference between the control group and rosemary group (TH x 400).

NO, and GSH. In contrast to the other groups studied, rotenone treatment showed a significant increase in tissue MDA and NO and a significant drop in GSH concentrations (P: 0.001). Nonetheless, when compared to the rotenone group, the rotenone + rosemary group demonstrated a significant decrease in tissue MDA and NO concentrations as well as a significant increase in GSH concentrations (P:0.001).

2. The effect of rosemary on inflammatory markers: Rotenone-only treated rats exhibited a significant rise in serum amyloid protein A and CRP levels to their corresponding vehicle control (P: 0.003 and P: 0.003, respectively). Rosemary treatment significantly decreased serum amyloid protein A and CRP levels (P: 0.003 and P: 0.006, respectively).

Gene expression assessment results

To further illuminate the rotenone toxicity's molecular mechanism and rosemary's protective effects, we measured the cortical caspase- 3 and tyrosine hydroxylase expression.

1. The effect of rosemary on cortical caspase- 3 gene expression: Rotenone treatment induced a significant increase in expression for caspase- 3 in contrast to the control group (P: 0.002). When compared to the rotenone group, the rotenone + rosemary group had a significant decrease in caspase-3 expression (P: 0.003).
2. The effect of rosemary on cortical tyrosine hydroxylase gene expression: In addition, there is a significant decrease in the expression of tyrosine hydroxylase in the

Table 2. Comparison of open field test between studied groups:

Open field test	Control	Rosmary	Rotenone	Rotenone + Rosemary	KW	Within group significance	
Line Crossing	**61 (45-76)**	**76 (58-94)**	**32 (27-53)**	**80 (68-89)**	**P=0.004***	P1=0.138 P2=0.05* P3=0.05*	P4=0.003* P5=1.000 P6=0.004*
Rearing	**16 (12-20)**	**20 (14-25)**	**7 (2-16)**	**19 (14-21)**	**P=0.061**	P1=0.138 P2=0.05* P3=0.623	P4=0.05 P5=1.00 P6=0.023*
Center Zone	**1 (0-2)**	**3 (1-5)**	**0 (0-1)**	**2 (1.0-3)**	**P=0.018***	P1=0.138 P2=0.279 P3=0.105	P4=0.01* P5=0.802 P6=0.004*
SUM	**78 (57-98)**	**99 (73-124)**	**39 (29-70)**	**101 (84-115)**	**P=0.004***	P1=0.138 P2=0.05* P3=0.05*	P4=0.003* P5=1.00 P6=0.004*

Legend: KW: Kruskal Wallis test. * Statistically significant if p≤ 0.05. Parameters are described as median (interquartile range). P1: the difference between Control & Rosemary, P2: the difference between control & Rotenone, P3: the difference between control & Rotenone +rosemary, P4: the difference between Rosemary & Rotenone, P5: the difference between Rosemary & Rotenone +rosemary and P6: Rotenone & Rotenone +rosemary.

rotenone-treated group compared with the control group (P: 0.002). The rotenone and rosemary group showed a significant increase in the expression of tyrosine hydroxylase as opposed to the rotenone group (P: 0.003). A graphical abstract was designed to summarize all results of the current study (Figure 5).

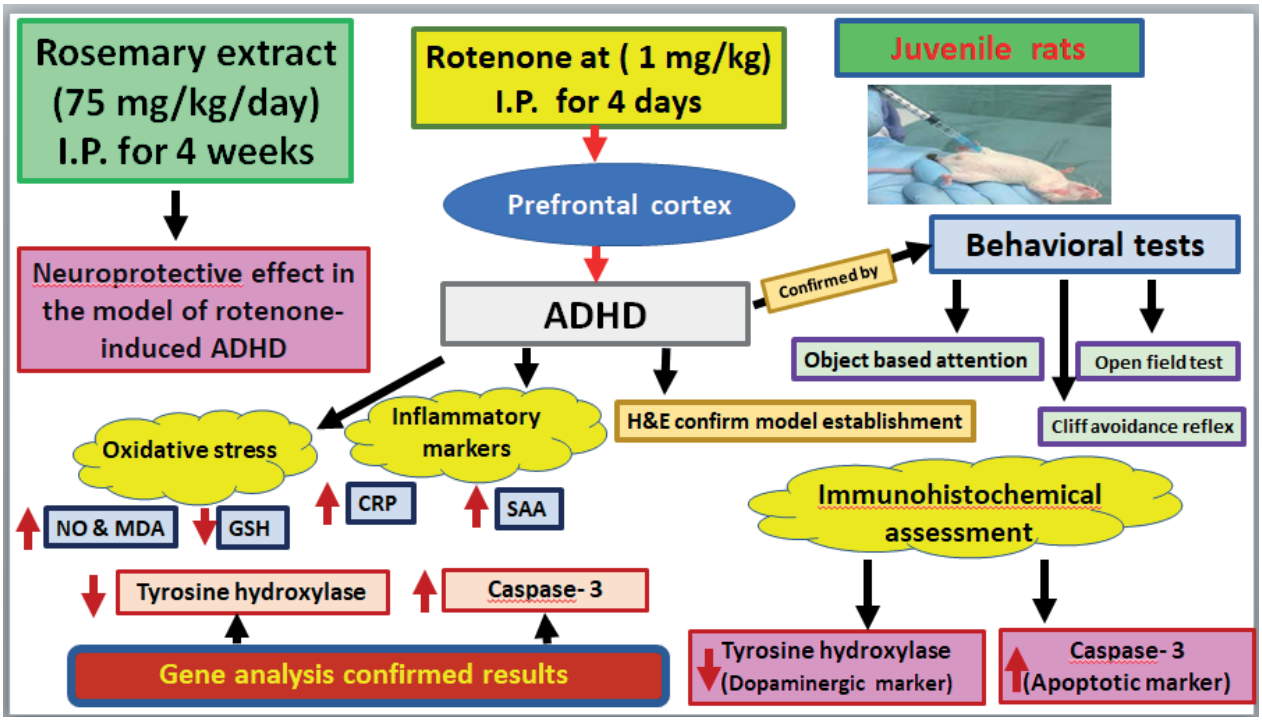

Figure 5. Graphical abstract. Rosemary extract alleviated the cortical neurotoxicity induced by rotenone administration assessed by the behavioral tests, histochemical changes, oxidative stress marker (NO, MDA, GSH), inflammatory marker (CRP, SAA), dopaminergic marker (tyrosine hydroxylase) and the apoptotic marker (caspase -3). (ADHD) Attention deficit hyperactivity disorder, (I.P.) intraperitoneal, (CRP) C-reactive protein, (SAA) serum amyloid protein A, (GSH) Reduced glutathione, (NO) Nitric oxide and (MDA) Malondialdehyde.

Discussion

This study was designed to establish an experimental model of ADHD in juvenile rats using rotenone and assess rosemary leaf extract's potential neuroprotective effects. The results obtained from the present study were of particular interest as they provided clear evidence of the protective and preventative potentials of rosemary leaf extract against neurotoxic effects associated with rotenone exposure in juvenile rats.

ADHD is a childhood-onset neurodevelopmental disorder that is associated with a broad range of mental disorders, including affective disorders, antisocial personality, and self-harm (27,28). Unfortunately, current ADHD treatments cannot alter the pathological features and fix the underlying cause of the disorder, but are just used for symptomatic relief and to alleviate the symptoms associated with the disorder. Since current treatments are noticeably inadequate, an alternative approach focusing on the use of phytochemicals that can offer neuroprotection through their anti-oxidative and anti-apoptotic activities is being investigated as a potential new strategy for the treatment of ADHD. Rosemary leaf extract is a natural neuroprotective substance, capable of crossing the blood-brain barrier, that acts by decreasing lipid peroxidation and hydroxyl anion radical and hydrogen peroxide activities in rat brain tissues (29,30).

Rosemary as a traditional herb has neuropharmacological properties in addition to significant antimicrobial, antioxidant, anti-inflammatory, and anti-apoptotic properties. Furthermore, it shows essential clinical effects on mood, memory, learning pain, sleep, and anxiety (31, 32). Moreover, rosemary and its phenolic derivatives increase antioxidant enzyme activity, thus reducing free radical formation. This has been a point of interest to researche rs as a possible protective measure in diseases involving oxidative stress (33-35). Besides rosemary essential oil has been shown to improve mood and cognition in healthy adults (36).

Rotenone, a common insecticide, is a mitochondrial complex I inhibitor generated from plants. It is lipophilic in nature and thus crosses biological membranes easily. Rotenone is a dopaminergic toxin and is capable of causing Parkinson's disease in adult rats (37,38). Rotenone exposure during the developmental period of rats causes hyperactivity, peaking at around the juvenile or adulthood period, due to dopaminergic lesions; the pivotal point of both behavioral phenotypes was found to be between three weeks and four weeks of age (8). Previous studies have shown that rotenone causes dopaminergic neurodegeneration by generating free radicals, leading to oxidative stress-mediated neuroinflammation, -synuclein aggregation, impaired autophagy, and nigrostriatal neuron loss (39-41).

The pathophysiology of ADHD is thought to be associated with oxidative stress, neuroinflammation, mitochondrial dysfunction, and enhanced dopamine metabolism, as a result, excessive hydrogen oxide and other reactive oxygen species (ROS) are produced. In ADHD, oxidative stress is alleged to have an important role in the degeneration of neurons and the death of substantia nigra cells (42-44).

Complementary results from our biochemical analyses showed that lipid peroxidation markers MDA and NO were increasing and GSH was decreasing in the rotenone-treated group, which is in agreement with other studies (45-48). In light of our observations, high levels of MDA have been reported in children with ADHD (49,50). As well as high levels of NO were observed in ADHD patients (49, 51, 52). There were statistically lower levels of GSH in patients with ADHD in comparison to the normal population (50,53). On the contrary, Varol et al. (54) found that patients with ADHD had lower levels of blood nitric oxide; in addition to other studies that revealed statistically lower levels of MDA in children with ADHD compared to the normal population (55, 56).

Rotenone decreases the activity of mitochondrial complex I, resulting in an increase in ROS and peroxynitrite. ROS and peroxynitrite production causes damage to all cell components, including mitochondrial membrane structure, proteins, lipids, and, DNA resulting in neuronal death (57-59). Rosemary has been found to help protect cells from the harmful effects of ROS by decreasing oxidative stress and neuroinflammatory response and acting as an anti-apoptotic agent. We reported that rosemary has antioxidant action on prefrontal cortical tissues by lowering MDA and NO levels as well as limiting the decrease in GSH in the rotenone model of ADHD. This study was in

accordance with other studies that revealed that rosemary leaf extract possesses antioxidant effects (60-62).

Additionally, our study showed that CRP levels were higher in the rats exposed to rotenone, whereas they were lower in the rosemary-treated rats. In concordance, one study has reported an increase in CRP in children with ADHD (63). While another study found no association between ADHD and non-ADHD CRP samples (64). Chudal et al. (65) showed that maternal CRP during early pregnancy had no significant association with offspring diagnosed with ADHD.

Both prefrontal association cortices, of the two hemispheres, play an important role in regulating behavior, attention, and emotion; moreover, the cortex of the right hemisphere is specialized in behavioral inhibition. The PFC is more susceptible to rotenone toxicity because some studies have found that there is reduced blood flow and metabolic activity in the prefrontal cortex of ADHD patients. Moreover, impaired function and structure of the prefrontal cortex circuits are associated with poor cognitive function (66,67). The histological findings in the present study showed that rosemary leaf extract protected PFC as well as cortical neurons against rotenone-induced neuronal degeneration and apoptosis. These findings support the results of previous studies that have shown that administering rotenone can produce neurotoxicity in the striatum and prefrontal cortex (68,69). Lee et al. (70) reported that rosemary extract has an antioxidant effect on dopaminergic neurons and believes that rosemarinic acid, a component of rosemary leaf extract, plays a neuroprotective role on neuronal tissues.

Caspase-3, the active form of procaspase-3, is the enzyme most commonly implicated in neuronal apoptosis. Caspases can regulate apoptotic neuronal death as well as inflammation in the central nervous system. Also, It can mediate synaptic depression in other brain regions and/or regulate attentional processes in response to different demands (71-73). Based on the immunohistochemical staining Caspase 3 activity rose in the PFC of rats treated with rotenone, and apoptotic cells expanded and spread throughout the cortical layers. Those findings are in concordance with other studies (74-76). It is generally believed that rotenone intoxication results in the production and release of mitochondrial cytochrome c to the cytosol, activating the caspase-3 cascade, and thus resulting in apoptotic cell death (77-79). The rosemary-treated group had very little caspase-3 positive cell expression, indicating that the extract inhibits rotenone-induced neurodegeneration, possibly by interfering with caspase-3 activation.

Neurochemical alterations involving dopamine have been considered to play a critical role in the pathophysiology of ADHD as well as tyrosine hydroxylase (TH) which is regarded as the rate-limiting enzyme in the biosynthesis of dopamine (80). Several studies have found that patients with ADHD have lower levels of dopamine activity (81,82). The results of this experiment revealed many strong positive TH-positive fibers in the control group through cortical layers by immunohistochemical staining, and there was a decrease in TH-positive nerve fibers in the rotenone groups. This result was confirmed by earlier studies that reported a decrease in TH expression in the substantia nigra, striatum, and hippocampus in ADHD model rats treated with rotenone (83-85). These findings are due to dopaminergic neuro- degeneration caused by mitochondrial electron transport chain inhibition and nigrostriatal degeneration, as well as a decrease in GSH generation, which acts as a protective agent for dopaminergic cells (86). Low levels of dopamine storage in the mPFC of ADHD patients compared with healthy subjects suggest that their dopamine system is not working properly. Immunostaining for tyrosine hydroxylase in PFC indicated that treatment with rosemary prevented the loss of TH-immunoreactivity caused by rotenone. In accordance, other studies reported that carnosol, the major oxidation product of carnosic acid, significantly increased the amount of tyrosine hydroxylase due to its antioxidative and anti-apoptotic properties (87,88). Many compounds that possess neuroprotective effects have been shown to prevent the loss of dopaminergic neurons and TH-positive cells caused by dopaminergic neurotoxins (89-91).

In this model, we demonstrated the typical presentation of ADHD in the form of impulsivity and inattention in the juvenile rats (4-week-old rats; approximately equivalent to 6–8-year-old children) (92) , induced by repeated exposure to small doses of rotenone during the neonatal period. In contrast to the expected, we found that this exposure produced a marked

decrease in rats' activity which is not consistent with ADHD and is different from the obtained results in previous research. This verdict might be related to the difference in the timing at which behavioral tests were performed and the used method for assessment of the rats' activity. In the current study, We used the open field test to assess activity at 29 days of age, whereas Ishido et al. (38) used the Supermex system to investigate the behavioral effects of rotenone and found hyperactivity to be evident with the same repeated dose of rotenone at the age of 5 weeks during the nocturnal phase rather than the diurnal phase.

Because gene expression profiling is a fundamental method for demonstrating molecular changes in behavior, we investigated the cellular effects of rotenone on tyrosine hydroxylase and caspase 3 gene expression. Rotenone-treated rats revealed a significant reduction in gene expression of tyrosine hydroxylase in comparison to controls, while the group treated with rotenone and rosemary demonstrated improved expression of the same gene denoting the protective effect of rosemary. On the contrary, expression of caspase 3 was significantly enhanced in the rotenone-treated rats and reduced in the groups receiving rosemary or both rotenone and rosemary. Lo et al. (93) reported that deletion of the caspase-3 gene, encoding apoptosis exhibited behavior traits bears a striking resemblance to the triad of core symptoms of ADHD.

Our results support that rotenone exerts a toxic effect on dopamine synthesis as evidenced by reduced expression of tyrosine hydroxylase since tyrosine hydroxylase activity modulation is the primary mechanism for controlling the rate of dopamine synthesis in dopamine neurons (94). Enhanced expression of caspase 3 indicates that apoptotic mechanisms are also involved in the neurotoxic effect of rotenone; as Caspase-3 is a cysteine-aspartate protease that is best known for its destructive role in apoptotic cell death (95).

Conclusion

Taken as a whole, the current study's findings confirmed not only the protective but also the therapeutic impact of rosemary extract in rotenone-treated juvinail rats. These assertions were supported by significant improvements in attention and impulsivity, as well as a reduction in oxidative stress and preservation against dopaminergic neuron death, demonstrating that rosemary has a neuroprotective impact in the model of rotenone-induced ADHD.

Limitations of the study

To fully understand how rosemary extract affects Attention Deficit Hyperactivity Disease in various experimental circumstances, more researches are necessary.

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Conflict of Interest: Each author declares that she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Authors Contribution: Eman Abdelrazik and Eman A. Abdulhai designed the study and established the model of the study. Eman Hamza and Marwa H. Elnagdy performed and interpreted the biochemical and molecular results. Hend M. Hassan examined the cortical tissue specimens, interpreted the histological and immunohistochemical and morphometric results. Data collection and analysis were performed by Eman Abdelrazik and Eman A. Abdulhai. All authors wrote, revised the manuscript, and approved the final manuscript.

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