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Acetamiprid induced oxidative stress and genotoxicity in male albino rats: Attenuation by ambroxol

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Abstract

The present study was undertaken to evaluate the oxidative stress and genotoxicity induced by 43.40 mg/kg/day of acetamiprid (ACE) in male rats and the protective role of 30 mg/kg/day of ambroxol (AMB) in male rats for consecutive 28 days. The results showed that the administration of ACE caused significant increments in the levels of catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), lipid peroxidation (LPO), the frequencies of chromosomal aberrations (CAs) and the total micronucleated polychromatic erythrocytes (MNPCEs), while GSH content and mitotic index (MI) were reduced. However, the co-administration of AMB to rats intoxicated with ACE attenuated the tested oxidative stress and genotoxic parameters. In conclusion, ambroxol can be used to ameliorate the toxicity of certain neonicotinoid insecticides such as acetamiprid.

Introduction

Neonicotinoids (NEOs), nicotine-derived insecticides, are a new class of insecticides used as an alternative to organophosphate in agriculture and domestic purposes [1,2], where they constitute nearly 30% of the pesticide market due to their selectivity on insects [3] acting as agonists on nicotinic acetylcholine receptor (nAChR) [4]. The toxicity of NEOs in mammals is suspected to be centrally mediated because their symptoms of poisoning are similar to that produced by nicotine [5] and do not easily pass the blood-brain barrier [6]. Although their toxicity to mammals and other vertebrates is low because of the distinct features of the target receptor sites in insects [7], they are considered potential inductors of adverse effects in mammals [8] and can disturb the neuronal distribution leading to neurotoxicity [2].

Acetamiprid (ACE) is one of the most widely used NEOs, commonly used as a systemic and a contact compound to control of Hemiptera, Thysanoptera, and Lepidoptera by soil and foliar application on a wide range of crops, especially vegetables, fruit, and tea [9-11] as well as against animal parasites [12]. ACE has been reported to accumulate in plants and contaminates water causing a potential risk for organisms [13-15]. It has been found to b potential toxic to the male reproductive system [16] and produces pathological hematological and immunological changes [17-19], hepatotoxicity [3], alteration in the structural and biochemical profiles of the liver due to the loss of integrity of cell membranes [20], and oxidative stress [21].

Oxidative stress is one of the underlying mechanisms for many diseases [22], and it plays a role in liver and kidney injury [23] resulting from either the diminishing antioxidants and/

or increasing the production of reactive oxygen species (ROS). These ROS are derived from either the normal metabolic processes or/and external sources such as exposure to cigarettes, ozone, X-rays, air pollutants, and industrial chemicals such as pesticides [24]. The excessive ROS reduce the antioxidant content and increase the lipid peroxidation, DNA damage, and genotoxicity [25-27].

Ambroxol, AMB [trans-4-[(2,4-dibromanilin-6-yl)-methyamino]-cyclohexanol], an aromatic amine, is derived from vasicine, naturally found in *Adhatoda vasica* [28], of which extracts were used for centuries in India to treat bronchitis, asthma, and rheumatism [29]. Therefore, it is considered a novel anti-allergic drug used as a secretolytic agent in the treatment of chronic bronchitis [30], and suitable for clinical studies to prevent corona virus disease 2019 with almost no side effects [31]. AMB easily penetrates into the brain and reached a maximum concentration in the striatum at approximately 60 min after low- and high-dose treatment [32]; therefore, clinical neurologists have prescribed AMB as an adjunctive treatment for Parkinson's disease [32] because it delays or even halts the evolution of neurological manifestations [33]. Beyond the mucokinetic and secretagogue effects, AMB shows great antioxidant and anti-inflammatory activities [34], improves mitochondrial dysfunction [35], protects lung tissue from oxidant-induced injury [36], and effective as pharmacological chaperone of the lysosomal enzyme glucocerebrosidase [37]. However, the mode of action of AMB and related analogues is also unknown in terms of explaining the blockade of both degranulatory processes (e.g. histamine release) and *de novo* mediator generation (e.g. IL-4 synthesis) [30]. Over the last years, the extensive use of NEOs has been criticized due to the risks associated with their toxicity to mammals, therefore, the present study aimed to evaluate the toxic effects of ACE on some oxidative stress and genetic parameters of male Albino rats as well as to study the possible role of AMB to attenuate these effects.

Methods

Chemicals

Acetamiprid ((E)-N-[(6-chloro-3-pyridinyl) methyl]-N'-cyano-N-methylethanimidamide) was obtained from Starchem Company, Egypt, with purity of 97%, while ambroxol (2-amino-3,5-dibromo-N-[trans-4- hydroxycyclohexyl] benzylamine) with purity of 100% was obtained from Glaxo Smith Kline Company, Egypt. Cyclophosphamide (CYC), with purity of 99% was purchased from Sigma-Aldrich Company. All other chemicals used were of the highest purity grade available from Sigma and Merck Chemical Companies.

Animals

Albino male rats weighing 180–200 g were obtained from the Institute of Graduate Studies and Research, Alexandria University, Egypt, kept in plastic cages at a temperature of 22 °C with light/dark cycle 12/12 h, provided with standard rat pellet and tap water *ad libitum*, and left to acclimatize under standard conditions for 14 days before the commencement of experiments. The local ethics committee approved the design of the experiments, and the protocol conforms to the guidelines of the National Institute of Health (NIH) and Alexandria University, Egypt (Approval number AU08181231306).

Experimental design

Thirty rats were randomly divided into 5 groups (6 animals for each) and orally treated for consecutive 28 days as follows: Group I received corn oil (1 ml/kg/day) and kept as a negative control, group II received 25 mg/kg/day of CYC prepared in distilled water and kept as a positive control for genotoxic studies, group III received ACE at a dose level of 43.40 mg/kg/day in corn oil (equivalent to 1/5 the LD_{50} value) [9], group IV received AMB at a dose of 30 mg/kg/day in distilled water, and group V received 30 mg/kg/day of AMB and then after one hour treated with 43.40 mg/kg/day of ACE. The selection of AMB dose was based on its ability to reverse the effect of the noxious stimulus [38], where relatively high doses of AMB should be used in rodent studies to observe the beneficial effects of it [38,39].

Sample preparation

Twenty-four hours after the last treatment, blood samples were collected from retro-orbital venous and centrifuged at 3000 ×g for 15 min at 4°C using a Janetzki type K23 refrigerated centrifuge, (Germany) to separate the serum. The clear sera were collected using a Pasteur pipette and then stored at −20°C for determination of the biochemical parameters. After withdrawing blood samples, rats were intravenously injected with 4 mg/kg of colchicine 3 hrs before sacrificing, dissected, then femorals of each rat were separated, flushed with 0.075M KCl and kept frozen for genotoxic studies.

Determination of oxidative stress parameters

Superoxide dismutase (SOD) activity was determined based on the ability of the enzyme to inhibit the autoxidation of pyrogallol and the activity was expressed as unit/mg protein [40], while glutathione s-transferase (GST) was determined using 1-chloro-2,4- dinitrobenzene (CDNB) as a substrate, and the activity was expressed as µmole of conjugated CDNB/min/mg protein [41]. Catalase (CAT) activity was assayed according to the method of Beers and Sizer and expressed as U/mg protein [42]. The level of reduced glutathione (GSH) as a non-enzymatic antioxidant was measured at 412 nm using 5,5-dithio-bis-2-nitrobenzoic acid as a colour reagent and expressed as µg GSH/g tissue [43]. Lipid peroxidation levels (LPO) were determined based on the reaction with thiobarbituric acid (TBA) and trichloroacetic acid (TCA) and the level of malondialdehyde (MDA) as a marker of LPO was expressed as nmole of MDA/ ml. [44] Protein contents in serum were determined by the method of Lowry et al. using bovine serum albumin as the standard [45].

Genotoxic studies

Chromosomal aberrations

The bone marrow of rat was prepared for the determination of chromosomal aberrations and then stained with 5% Giemsa stain [46]. A total of 100 cells spread metaphases were counted to record the chromosomal aberrations. The mitotic index was determined by counting the number of dividing cells in 6000 bone marrow cells from each rat.

Micronucleus assay

The bone marrow was flushed into a tube containing tri-sodium citrate (3 ml), centrifuged at 3,000 rpm for 10 min, and

then smears were spread on the slides, air-dried, and stained by Giemsa [47]. A total of 12,000 polychromatic erythrocytes (PCE) from each rat were analyzed to estimate the frequencies of micronucleated polychromatic erythrocytes (MNPCE) and the ratio of PCE /normochromatic erythrocytes (NCE).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. The least significant difference (LSD) multiple comparison tests were used to compare between treatments at a level of 0.05. Statistical analyses were done using Statistical Package for Social Sciences (SPSS, version 25). Also, statistical correlations between the tested variables were calculated using the Pearson correlation analysis method.

Results and discussion

ACE induced oxidative stress

Effect on SOD: The effect of 43.40 mg/kg/day of ACE on the activity of SOD in serum of male rats treated for 28 days is shown in Table 1. It was found that the activity of SOD significantly increased by 28% of control when rats were treated only with ACE compared to untreated control. However, when AMB was administrated to ACE-treated rats, the activity of SOD was non-significantly increased compared with rats treated only with ACE.

Effect on GST: As shown in Table 1 significant increases by 49 and 24% in the activity of GST were observed after ACE and AMB treatment compared to the control group, respectively. However, the co-administration of AMB with ACE modulated the activity of GST to be non-significantly differed from control value.

Effect on CAT: As presented in Table 1, a significant ($p<0.05$) elevation in serum CAT activity in ACE-treated rats for by 17% of control group. On the contrary, when AMB was administered to ACE-intoxicated rats, the enzyme activity was restored to the normal value.

Effect on LPO: Treatment of rats with ACE at a dose of 43.40 mg/kg/day for 28 days resulted in a significant increase in serum LPO level by 63% compared with control rats, while the administration of AMB only to rats reduced the activity by 10%. However, the co-administered AMB with ACE significantly modulated the MDA levels to be 66% of the levels in rats treated only with ACE (Table 1).

Effect on GSH: Data in Table 1 illustrate a significant ($p<0.05$) decrease in GSH level of ACE-treated rats by 33% compared with untreated rats, whereas the GSH level was restored to the normal value of control when AMB was co-administered to rats treated with ACE to be 95% of control value.

ACE is a selective agonist of type-2 nicotinic acetylcholine receptors in insects [3] with low agonist effects on mammalian neuronal nAChRs indicating its relative safety for mammals [2]. However, some researchers reported that ACE causes neurodegenerative diseases by disturbing neuronal distribution and/or inducing oxidative stress, leading to neurotoxicity [2,48,49]. Also, ACE was found to significantly alter the structural and biochemical profiles of the liver due to the loss of integrity of cell membranes [20]. However, tissues and cells of organisms contain systems for detoxification of the biological reactive intermediates to prevent the damage to cells [50]. CAT is one of the cellular defense mechanisms which acts as an antioxidant defense enzyme that catalyzes the breakdown of H_2O_2 to H_2O and molecular oxygen [51], while SOD metabolizes the superoxide anion to hydrogen [52]. Also, GST is involved in xenobiotic metabolism and catalyzes the nucleophilic attack of GSH on electrophilic toxic compounds [53,54]. In the present, the increase in the activities of CAT, SOD, and GST as antioxidant enzymes in rats treated with 43.40 mg/kg/day for 28 days showed effective scavengers of reactive species such as hydrogen peroxide and superoxide anion [55]. The recorded SOD/CAT ratios were 8.7, 9.52, 12.02, and 9.98 for control, ACE, AMB, and ACE+ AMB, respectively. The increase in the ratio SOD/CAT may reflect the more availability of H_2O_2 which can form the most potent prooxidant molecule, hydroxyl radical, by Fenton reaction [55,56].

In addition, the present results revealed that LPO level was significantly increased following ACE treatment as reported by many researchers, where ACE increased the MDA level, a marker of membrane lipid peroxidation in different tissues [20,57,58] due to the overproduction of ROS by ACE. The damage to the membrane alters the properties of the membrane leading to a loss of cellular homeostasis [59], and subsequently induces various types of cell death [60].

Regarding the GSH content, a significant reduction was recorded following the ACE treatment. GSH is a tripeptide and one of the major components of overall antioxidant defenses [61] which reacts enzymatically or non-enzymatically with the toxic compounds to form GSH conjugates [62]. The conjugation with electrophiles consumes a substantial portion of cellular GSH [62]. Our results confirm the findings of many investigators who reported that exposure of rats to ACE caused a reduction in levels of GSH in liver, kidney, spleen, brain, and testes of a rat [16,23]. Some studies revealed that the reduction in GSH and the increment in activities of GST and CAT are concomitant with raised LPO levels after exposure of rats to ACP suggesting hepatic oxidative stress and brain damage [20,58,64,68]. However, when rats received only 30 mg/kg/day of AMB or co-administered with ACE for 28 days, the activities of antioxidants enzymes (SOD, CAT, and GST), and GSH content non-significantly differed from the negative control indicating recoveries.

Genotoxicity of ACE and the protective role of AMB

The present investigation aimed to evaluate the genotoxic effects of ACE and the role of AMB to attenuate its effects on male rats. Battery tests for genotoxicity were studied to detect the potential effects of ACE.

Chromosomal aberrations (CAs)

Data in Table 2 show that both CYC (positive control) and ACE significantly ($p < 0.05$) induced CAs (breaks, gaps, deletions, ring, stickiness, and multiple aberrations) in rat bone marrow, when rats were treated either with 25 or 43.40 mg/kg/day for 28 days, respectively, compared to the negative control group. The recorded frequencies of CAs induced by CYC and ACE were 1.90 and 1.38 fold the untreated rats, respectively. However, the administration of 30 mg/kg AMB alone or combined with ACE resulted in non-significant alterations in the frequencies of CAs, where the recoded frequencies equaled 0.92 and 1.13 fold the control value, respectively. This means that the frequency of different kinds of CAs was reduced by 60% of rats given ACE.

Mitotic index

The mitotic index (MI) is used to quantify the degree of cytotoxicity in the cellular system and reflects the fraction of divid-

ing cells and cell proliferation [66]. The role of AMB to mitigate the effect of 43.40 mg/kg/day of ACE on male rats treated for 28 consecutive days was studied (Table 1). The percentages of mitotic index (MI) were reduced either in CYC- or ACE-treated groups to be 83 and 92, respectively, indicating the cytotoxic potential of ACE as CYC. However, when rats were given only 30 mg/kg/day of AMB or co-administered with ACE for 28 days, the percentages of MI were 102 and 98 and non-significantly differed from the untreated control indicating partial recoveries in the number of dividing cells.

Micronuclei in bone marrow of rat

The micronucleus test (MN), is frequently used to quantitatively determine the *in vivo* or *in vitro* harmful effects of chemical mutagens on the cellular system, where it gives true results to detect the clastogenic and genotoxic effects of xenobiotics [67,68] including structural mutations in chromosomes known as chromosomal aberration, abnormal nuclei in erythrocyte known as micronucleus and the changes in genetic materials [69] to explore the mechanism of action of known or suspected carcinogens [70,71]. Therefore, the MN test was used to investigate the effects of ACE genotoxic agent in rats and the role of AMB to ameliorate these effects (Table 1). Results showed that CYC was the more potent to increase the MNPCE followed by ACE ≈ ACE+AMB, and then AMB. ACE-induced clastogenic effect in the bone marrow of intoxicated rats as evidenced by the significant increase in the total number of bone marrow MNPCE by 0.22 fold compared with the negative control. Unfortunately, the co-administration of AMB with ACE did not show significant differences in MNPCEs as compared to ACE-treated rats. Regarding the PCE/NCE ratio, all tested treatments showed ratios more than the unit except rats treated with CYC, where the obtained ratio was 0.53. It is documented that genotoxic effects are the most serious of the possible side effects of pesticides [72] and the prolonged exposure to such pesticides may lead to heritable genetic diseases, carcinogenesis, and birth defects [73]. Our results exhibit that, ACE initiated different types of CAs and increased the MNPCE in rat bone marrow as well as decreased MI indicating a cytotoxic potential of ACE as CYC (The positive control). The present findings are in coincide with the studies of many investigators who reported that NEOs have been linked to genotoxic damage [74-77]. In addition, ACE has been found to cause chromatid changes in human peripheral lymphocyte cultures, micronucleus formation in blood lymphocytes, and chromosomal anomalies [78]. The ROS generation causes DNA damage [79] indicating the genotoxic potential of the pesticide [26]. It can be concluded that, because ACE is a cyanoamide derivative, therefore lower doses of it may cause more genotoxic effects [80].

Correlation analysis for biochemical and genetic markers

In a simple correlation analysis (Table 3), SOD showed a significant positive correlation with LPO (r= 0.856), GST (r = 0.827), CAT (r =0.679), CA (r = 0.907), and MNPCE (r = 0.828), while LPO showed a significant positive correlation with GST (r = 0.823), CAT (r = 0.836), CA (r= 0.971), and MNPCE (r = 0.715). Also, GST showed a significant positive correlation with CAT (r = 0.391), CA (r = 0.735), and MNPCE (r = 0.398). CAT showed a significant positive correlation with CA (r = 0.905) and MNPCE (r = 0.872). GSH showed a significant positive correlation only with MI (r = 0.559), while CA showed a significant positive correlation with MNPCE (r = 0.862). These findings are consistent with many investigators who reported that biomarkers of oxidative stress positively correlated with the increasing of pesticide toxicity [20,27,77,81-84] and genotoxicity which is associated with the apoptosis in different tissues [85,86].

AMB, a metabolite of bromhexine, has been widely prescribed to treat respiratory diseases associated with increased mucus production because of its anti-inflammatory and antioxidant properties [35]. In the present study, the role of AMB against the sublethal doses of ACE at a dose of 43.40 mg/kg/day for 28 days was documented, and supplementation of AMB to rats treated with ACE attenuated the toxic effect of ACE with reduction in the chromosomal abnormalities and partial recovery in the number of dividing cells compared to rats treated only with ACP. AMB acts as a precursor for the synthesis of GSH and stimulates the activity GST which enhancing the rate of GSH regeneration. AMB was found to increase the levels of GSH and SOD [87] and prevents the elevation of MDA and tumor necrosis factor-alpha (TNF-α) induced by CYC [88]. The good and direct oxidant-reducing capabilities of AMB may be directly related to the aromatic moiety of the molecule which acts as a scavenger for reactive ROS such as O_2^- [89] and decreasing the lipopolysaccharide-induced synthesis of cytokines, superoxide anion, and hydrogen peroxide production in rat alveolar macrophages [90]. Moreover, certain studies have suggested that AMB diminishes pain-like behaviors in chronic, inflammatory, and neuropathic pain models in rats due to its neuronal Na^+ and Ca^{2+} channels blocker activity [39,91-93] and CI^-/HCO_3^- exchangers in human epithelial cell lines [94,95] and blocks glutamate receptors [91] due to its antioxidant and free radical scavenging properties [89,96].

Table 1: *In vivo* effect of ACE and AMB and/or their combination on the oxidative markers of male rats after 28 daily doses.

Treatments	SOD (U /mg protein)	CAT (U /mg protein)	GST (µmol CDNB/min/mg protein)	LPO (nmol MDA /ml)	GSH (µg GSH/ml)
Control	9.36 ± 0.45c	1.08 ± 0.19ab	2.80 ± 0.58c	2.08 ± 0.25b	9.38 ± 0.45a
ACE	12.00 ± 0.34a	1.26 ± 0.40a	4.18 ± 0.41a	3.40 ± 0.34a	6.40 ± 1.08c
AMB	9.98 ± 0.65bc	0.83 ± 0.16b	3.46 ± 0.39b	1.90 ± 0.23b	8.00 ± 1.38b
ACE+AMB	11.08 ± 1.55ab	1.11 ± 0.19ab	3.18 ± 0.43b	2.25 ± 0.76b	8.90 ± 1.50a

Data are represented as mean ±S.D. (n=6)

Means in the same column followed by the same letters are not significantly different at $p \leq 0.05$.

Table 2: *In vivo* effect of ACE and AMB and/or their combination on the genotoxic markers of male rats after 28 daily doses.

	Chromosomal aberrations[2]		Mitotic index[3]		Micronucleated polychromatic erythrocytes[4]	
Treatment	Total chromosomal aberrations	% CAs	Number of dividing cells	%MI	% of total MNPCE	PCE/NCE
Control	31.50	5.25 ± 1.05cd	459	7.60 ± 2.34bc	1.80 ± 0.14bc	1.33 ± 0.03b
Positive control[1]	59.83	9.97 ± 1.32a	380	6.29 ± 2.10e	4.00 ± 0.16a	0.70 ± 0.02c
ACE	43.50	7.25 ± 0.72b	422	7.02 ± 1.20d	2.20 ± 0.13b	1.54 ± 0.02a
AMB	28.83	4.81 ± 1.10d	470	7.78 ± 2.23ab	1.60 ± 0.15c	1.35 ± 0.02b
ACE+AMB	35.66	5.94 ± 1.22c	450	7.46 ± 1.60c	2.20 ± 0.12b	1.50 ± 0.02a

Data are represented as mean ±S.D. (n=6)

Means in the same column followed by the same letters are not significantly different at $p \leq 0.05$.

[1.] Rats treated with 25 mg/kg b.w. of cyclophosphamide.

[2.] A total of 600 cells from each animal were analyzed to record chromosome aberrations, CA (%) = (No. of aberrant metaphases/Total No. of metaphase cells counted) X 100.

[3.] A total of 6000 cells from each animal were analyzed to record mitotic index, MI (%) = (No. of dividing cells/Total No. of bone marrow cells) X 100.

[4.] A total of 1200 polychromatic erythrocytes (PCE) from each animal were analyzed to record micronucleated polychromatic erythrocytes (MNPCE) frequencies and PCE /normochromatic erythrocytes (NCE) ratio using a light microscope under 1000 x magnification

%PCE = (PCE / (PCE + NCE))×100; %NCE = (NCE / (NCE + PCE)) ×100; % MNPCE= No. of PCE with micronuclei/Total No. of PCE (1000) X 100.

Table 3: Correlation coefficient between biomarkers.

	SOD	LPO	GST	CAT	GSH	CA	MI	MNPCE
SOD	1							
LPO	0.856	1						
GST	0.827	0.823	1					
CAT	0.679	0.836	0.391	1				
GSH	-0.765	-0.819	-0.993**	-.372	1			
CA	0.907	0.971*	0.735	0.905	-0.707	1		
MI	-0.906	-0.888	-0.610	-0.920	0.559	-0.972*	1	
MNPCE	0.828	0.715	0.398	0.872	-0.324	0.862	-0.957*	1

*Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

Conclusions

The current study focused on the toxicity of ACE to persuade the oxidative stress and genotoxicity to understand the mechanism of its toxicity by using various biomarkers, and also to evaluate the extent of the protective effect of AMB as an antioxidant agent. In the present study, oxidative damage and genotoxicity is a hallmark of ACE toxicity, as denoted by the injury markers including increasing the levels of SOD, CAT, GST, and LPO as well as reducing the level of GSH indicating genotoxicity. Also, our study exhibit that the ACE increased the frequency of chromosomal aberrations and MNPCE in rat bone marrow as well as decreased the number of dividing cells indicating genotoxicity. These effects may be prompted by the oxidative stress resulting from the free radical release (ROS) and reduction of the antioxidants. However, the use of AMB was ascertained to reduce the harmful effects of ACE in the investigated parameters. Because ambroxol easily enters the subcellular compartments, so we believe that the ambroxol supply is a putative protector against ACE effects and could prevent humans from intoxication by this pesticide. More studies are needed to identify the appropriate non-toxic dose and the mode of action of ambroxol.

Declarations

Data availability The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Ethical approval: All animal housing, handling, breeding and bioassays were conducted with the guidelines of the Institutional Animal Care and Use Committee (IACUC), Alexandria University, Egypt.

Consent to participate: 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.

Consent to publish: Informed consent was obtained from all individual participants included in the study.

Plant reproducibility This article does not contain any studies with Plant Reproducibility performed by any of the authors.

Clinical trials registration: This study does not contain any studies with clinical trials performed by any of the authors.

Author contributions Eltoweissy: Conceived, designed and revised this study; **Ezz El-Din** carried out the experiments and statistical analysis; **Osman** wrote the draft of the manuscript. All authors read and approved the final manuscript.

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