

Original Research

Vincamine Counteracts Gentamicin-Induced Parotid Gland Toxicity: Insights Into Antioxidant Defense, TRPC1/CHOP Modulation and Ferroptosis-Related Pathways

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Abstract

Background: Gentamicin (GET) is a widely used antibiotic, but its administration can cause parotid gland toxicity. Vincamine (VIN), a purified alkaloid with antioxidant and cytoprotective properties, may exert protective effects against gentamicin-induced toxicity. This study investigated the involvement of ferroptosis in GET-induced parotid gland toxicity and evaluated the protective effects of VIN and its potential underlying mechanisms. **Methods:** Adult male rats were divided into four groups: control, GET (100 mg/kg, i.p.), VIN (40 mg/kg, p.o.), and GET/VIN. Treatments were administered for 7 days. Parotid gland injury was evaluated by measuring serum lactate dehydrogenase (LDH) levels. Oxidative stress and cellular damage were assessed by measuring total antioxidant capacity (TAC), glutathione homeostasis (glutathione (GSH)/oxidized glutathione (GSSG)), and lipid peroxidation based on malondialdehyde (MDA) levels. Ferroptosis-related markers (glutathione peroxidase 4 (GPX4), solute carrier family 7 member 11 (SLC7A11), transferrin receptor 1 (TFR1), ferroportin 1 (FPN1), ferritin heavy chain 1 (FTH1) and Nrf2/HO-1 expression), were analyzed. Endoplasmic reticulum stress-related markers, including C/EBP homologous protein (CHOP), PERK, GRP78, and transient receptor potential canonical 1 (TRPC1), were also evaluated. Histopathological examinations were also performed. **Results:** GET significantly increased LDH levels and oxidative stress and induced parotid tissue damage. VIN treatment markedly reduced LDH levels, and attenuated oxidative stress. These protective effects were associated with modulation of Nrf2/HO-1 signaling and ferroptosis-related markers, including GPX4 and SLC7A11. VIN treatment was also associated with reduced markers of endoplasmic reticulum stress (CHOP, GRP78 and PERK) as well as restored TRPC1. **Conclusions:** VIN exerted protective effects against GET-induced parotid gland toxicity, accompanied by reduced oxidative stress and modulation of ferroptosis- and endoplasmic reticulum stress-related pathways. These findings suggest that VIN may have potential for further investigation as a protective agent against gentamicin-induced parotid gland injury.

Keywords: vincamine; gentamicin; parotid toxicity; ferroptosis

1. Introduction

Aminoglycosides (AGs) constitute a crucial class of antibiotics specifically designed to target a wide range of bacterial pathogens, with particular efficacy against aerobic Gram-negative bacilli [1]. Among the AGs, Gentamicin (GET) is particularly noteworthy, as it is widely used in clinical settings to treat severe, broad-spectrum bacterial infections [2]. Regrettably, its robust therapeutic efficacy is frequently limited by multi-organ toxicity [3]. Conspicuously, the parotid glands represent a major, yet underappreciated, target of GET-induced adverse effects [4].

The toxicity of GET is intimately associated with the accumulation of reactive oxygen species (ROS) [5], which leads to raised malondialdehyde (MDA) levels and a diminution in antioxidant defenses, including glutathione (GSH) [6]. This imbalance enhances lipid peroxidation and

promotes the surge of deleterious species particularly hydroxyl radicals and hydrogen peroxide [7]. The resulting oxidative stress damages mitochondria, induces DNA fragmentation, triggers inflammatory pathways, ultimately culminating in cell death via multiple, potentially overlapping mechanisms, including apoptosis, necroptosis, and ferroptosis [8,9].

Ferroptosis is a morphologically and biochemically distinct, iron-dependent form of regulated cell death driven by uncontrolled lipid peroxidation that compromises membrane integrity [10]. This process is closely linked to GET-disrupted redox homeostasis, wherein lipid-derived ROS accumulation outpaces impaired antioxidant counter-mechanisms [11,12]. Recent studies have highlighted the role of ferroptosis in GET-induced acute kidney injury, providing mechanistically compelling support for ferroptosis as an operative pathway in GET toxicity [13,14]. Ferropto-



sis is fundamentally governed by glutathione peroxidase 4 (GPX4). Deactivation of GPX4, coupled with GSH depletion, drives the lethal accumulation of lipid peroxides that triggers ferroptosis, whereas GPX4 overexpression completely thwarts it [15,16]. Ferroptosis is also modulated by several other key regulators, such as transferrin receptor 1 (TFR1), ferroportin 1 (FPN1), solute carrier family 7 member 11 (SLC7A11), GPX4, and ferritin heavy chain 1 (FTH1) [17,18].

TFR1 is a vital protein that controls intracellular iron transport, playing a fundamental role in regulating iron homeostasis and metabolism [19]. FPN functions as a transporter that mediates the export of ferrous ions (Fe^{2+}) from cells, thereby contributing to systemic iron balance [20]. Intracellularly, GPX4 and SLC7A11 form an essential antioxidant defense system wherein SLC7A11 facilitates cystine import for GSH biosynthesis, and GPX4 later utilizes GSH to neutralize lipid peroxides. Together, they prevent ferroptotic cell death by maintaining redox homeostasis [21].

In previous studies, protective agents including lactoferrin and epigallocatechin gallate consistently alleviate gentamicin-induced injury by directly suppressing these ferroptotic pathways, firmly establishing ferroptosis as a central mechanism of GET toxicity. The reproducibility of these findings across independent studies underscores that ferroptosis is not an incidental phenomenon but a robust, dose-dependent outcome of GET exposure [13,14].

Given this compelling evidence, it is highly plausible that ferroptosis also underlies GET-induced parotid gland toxicity. This is further reinforced by prior reports implicating ferroptosis in radiation-induced parotid gland dysfunction, highlighting ferroptosis as a convergent mechanism of salivary gland injury [22]. Together, these data provide a strong mechanistic rationale for investigating an anti-ferroptotic intervention in the parotid gland, positioning ferroptosis not as a speculative pathway but as a critical driver of GET-induced tissue damage.

As a central orchestrator of the cellular antioxidant defense mechanism, Nrf2 plays an indispensable role in preserving redox balance and physiological homeostasis [23]. Oxidative stress-induced ROS accumulation triggers Nrf2 nuclear import, driving the transcription of downstream antioxidant enzymes such as HO-1, GPX4, and SLC7A11 [24,25]. HO-1 and its byproducts protect cells by scavenging harmful radicals [26]. Nrf2 activation has been shown to confer protection against ferroptosis in acute lung injury secondary to intestinal ischemia-reperfusion, via induction of HO-1 and SLC7A11 [27], and similarly limits ferroptosis in hepatic ischemia-reperfusion injury, underscoring the Nrf2/HO-1 axis as a candidate therapeutic target for ferroptosis-associated tissue injury more broadly.

The human transient receptor potential canonical 1 (TRPC1) protein forms functional, nonselective cation channels characterized by high calcium (Ca) permeability. Ubiquitously expressed across diverse human tissues and

cellular lineages, TRPC1 serves as a critical arbiter of intracellular calcium homeostasis. TRPC1 orchestrates this vital messenger system either by directly facilitating Ca^{2+} influx pathways or by modulating membrane potential depolarization [28]. Consequently, these channels have emerged as highly promising therapeutic targets for a spectrum of debilitating pathologies, including oncogenesis, epilepsy, chronic pain, rheumatoid arthritis, and adverse cardiac remodeling [29]. Within the exocrine system, TRPC1 acts as a primary molecular driver of fluid secretion within the acinar and ductal cells of the salivary glands. Disruptions to TRPC1 integrity trigger a pathological cascade: depletion of endoplasmic reticulum (ER) calcium stores induces severe ER stress, culminating in the upregulation of C/EBP homologous protein (CHOP) and ultimately driving salivary gland cell death [30].

Vincamine (VIN), a monoterpenoid indole alkaloid derived from the leaves of the *Vinca minor* plant, has been recognized for its medicinal properties, including free radical scavenging, cytoprotection, and organ preservation in the liver, kidneys, and neurons [31,32]. VIN exhibits antioxidant, antitumor, and neuroprotective properties, enhances cerebral blood flow, and modulates microcapillary circulation [33] while bolstering monoaminergic neurotransmission via redox modulation. In preclinical models of Parkinson's disease, VIN exhibits robust neuroprotective effects by simultaneously suppressing NF- κ B-induced inflammatory cascades and triggering the Nrf2/HO-1 signaling axis to counteract oxidative stress [34]. Overall, VIN mitigates oxidative stress, inflammation, and apoptosis across various central and peripheral disease models [35,36].

This study presents a novel investigation into ferroptosis as a key mechanism underlying GET-induced parotid gland toxicity. It also examines the protective effects of VIN, demonstrates that VIN suppresses ferroptosis and stimulates the Nrf2/HO-1 antioxidant cascade and the TRPC1/CHOP-mediated ER stress/calcium homeostasis pathway, ultimately mitigating oxidative stress and subsequent tissue injury.

2. Materials and Methods

2.1 Drugs and Chemicals

GET was obtained from Memphis (Garamycin® ampule, Memphis, Egypt). Vincamine was generously provided by Egyphar (Cairo, Egypt). For all other chemical reagents, the highest analytical grade commercially available was used in this study.

2.2 Experimental Animals

This study was performed with the utmost ethical rigor. The protocol acquired full approval from the Ethics Committee of the Deraya Center for Scientific Research (DCSR), Deraya University, Minia, Egypt (Ethical approval No. DCSR-010-024-25). This research is reported

following ARRIVE guidelines [37]. The total number of rats used in the study was calculated using G*Power (power 80%, $\alpha = 0.05$). A total of 24 male Wistar rats (220–250 g), were procured from the animal facility at Deraya University. Animals were healthy adult male Wistar rats (8–10 weeks). No animals met pre-specified exclusion criteria (e.g., signs of illness during acclimatization, >10% weight loss) during the study. The animals were individually housed in cages with a controlled 12-hour light/dark cycle (humidity of 55–65 %, and a temperature of 22–26 °C) and ad libitum access to food and water.

2.3 Experimental Design

Following a week of acclimatization, the 24 rats were distributed across four distinct sets, each consisting of six individuals, and treated for a duration of seven days. All experimental procedures, sample collection, biochemical analyses, histopathological evaluations, and data analyses were performed by investigators blinded to the group allocation and not involved in outcome assessment to minimize potential bias:

(1) Group I (Normal Control): Received the vehicle solution (0.5% carboxymethyl cellulose, CMC) administered orally once daily.

(2) Group II (VIN): Given Vincamine at a dosage of 40 mg/kg in 0.5% CMC, delivered orally via gavage [35,38].

(3) Group III (GET): Treated with GET at a dosage of 100 mg/kg/day via intraperitoneal injection [39,40].

(4) Group IV (GET/VIN): Treated with Vincamine (40 mg/kg) through oral gavage in conjunction with GET (100 mg/kg/day) via intraperitoneal injection.

Upon completion of the treatment period, rats were anesthetized by intraperitoneal injection of thiopental sodium (50 mg/kg; at a concentration of 25 mg/mL). The depth of anesthesia was verified by absence of pedal withdrawal reflex, absence of corneal reflex and no response to a noxious stimulus. Subsequently rats were euthanized by decapitation. Parotid gland tissue was harvested and dissected into three sections: the first section was fixed in 10% neutral buffered formalin for histopathological evaluation, the second was utilized for assessing oxidative stress biomarkers, and the third was preserved at -80°C for future RNA and protein analyses.

2.4 Biochemical Marker of Parotid Gland Injury

To evaluate systemic cellular damage, serum lactate dehydrogenase (LDH) activity was quantified using a commercial diagnostic kit (Human Diagnostics, Magdeburg, Germany; Cat. No. 12214). Spectrophotometric measurements were performed using a HumaLyzer Primus spectrophotometer (Human Diagnostics) following the manufacturer's specified guidelines.

2.5 Ferrous Iron Assay Kit

The levels of iron were evaluated by an Iron Assay Kit (Assay Genie, Dublin, Ireland & London, UK; Cat. No. MAES0206) in agreement with the manufacturer's protocol.

2.6 Levels of Oxidative Stress-Related Biomarkers.

Total antioxidant capacity (TAC) in serum was determined following the manufacturer's protocol provided by Biodiagnostic (Giza, Egypt).

To measure MDA concentration in parotid tissues, we utilized the rat MDA kit from Bio-Diagnostic (Giza, Egypt; Cat. No. MD2529). In this procedure, MDA reacts with thiobarbituric acid, yielding pink-colored thiobarbituric acid reactive species. The color intensity was quantified using a colorimetric method at 534 nm. MDA concentrations were calculated and reported as nmol/g of tissue, with the intensity of the pink hue directly related to MDA levels [41].

For the quantification of reduced GSH in parotid tissue homogenate, the rat GSH kit from (Bio-Diagnostic in Giza, Egypt; Cat. No. GR2511) was used. This assay depends on the colorimetric reaction based on glutathione's interaction with 5,5'-dithiobis(2-nitrobenzoic acid). The concentration of the produced colored product was assessed using a spectrophotometric method [42]. Furthermore, levels of oxidized glutathione (GSSG) in parotid tissue were evaluated as per the manufacturer's guidelines from (Elabscience®, Wuhan, China; Cat. No. E-BC-K097-M).

2.7 Enzyme-Linked Immunosorbent Assay

ELISA kits were employed to quantify the expression levels of GPX4 and TFR1 (BT-LAB, Shanghai, China; Cat. Nos. E1787Ra and E1656Ra, respectively), FPN (Arsh Biotech, New Delhi, India; Cat. No. LTaR1721EA), SLC7A11 (AFG Bioscience, Northbrook, IL, USA; Cat. No. EK246718), FTH1 (ABclonal, Wuhan, China; Cat. No. RK0666), PERK (Assay Genie, Dublin, Ireland, Cat. No. RTFI01385), GRP78 (ELK biotechnology, Wuhan, China, Cat. No. ELK6834) and nuclear Nrf2 (BT-LAB, Shanghai, China: Cat. No. E1083Ra) in parotid tissue, according to the manufacturers' instructions.

2.8 Assessment of TRPC1 and CHOP Levels

To evaluate the protein expression of TRPC1 and CHOP in parotid tissues, ELISA was conducted in strict accordance with the manufacturers' instructions. TRPC1 levels were determined using a Rat TRPC1 ELISA kit (MyBioSource, CA, USA; Catalog No. MBS9338839), while CHOP levels were analyzed using a Sunlong (Hangzhou, China, SL1663Ra) ELISA kit. The resulting absorbance (optical density) for both assays was measured at a wavelength of 450 nm.

Table 1. Primer sequences of intended genes.

Gene	Type of primer	Primer sequences	Accession number	Product length
<i>Nfe2l2</i>	Forward Primer	5' GCA CAT CCA GAC AGA CAC CA 3'	NM_001399173.1	276
	Reverse Primer	5' TCC TGC CAA ACT TGC TCC AT 3'		
<i>Hmox1</i>	Forward Primer	5' TAC GGC CCT GGA AGA GGA GA 3'	NM_012580.2	165
	Reverse Primer	5' TGC TGT GTG GCT GGT GTG TA 3'		
<i>Gapdh</i>	Forward Primer	5' CTCTCTGCTCCTCCCTGTTC 3'	NM_017008.4	351
	Reverse Primer	5' CGACATACTCAGCACCAGCA 3'		

2.9 Quantitative Real-Time PCR (qRT-PCR) for Gene Expressions

To precisely quantify the transcriptional profiles of *Nfe2l2* and *Hmox1* mRNA expression, qRT-PCR was performed. Briefly, total RNA was extracted from parotid tissue samples (~100 mg) homogenized in TRIzol reagent using a Branson digital homogenizer, and subsequently reverse-transcribed utilizing the RevertAid H Minus First Strand cDNA Synthesis Kit. Real-time PCR amplification was performed with Maxima SYBR Green Master Mix (Thermo Scientific, MA, USA, K0221) on an Applied Biosystems Step One Real-Time PCR System. Target gene expression levels were quantified using the comparative CT method, with GAPDH serving as the internal reference gene for normalization [43]. Primers are provided in Table 1.

2.10 Western Blotting Analysis

Parotid gland tissues were homogenized in hypotonic buffer supplemented with 1% protease and phosphatase inhibitor cocktails (Santa Cruz Biotechnology Inc., Texas, USA; Cat. Nos. sc-29130 and sc-45044, respectively). Cytosolic and nuclear proteins were extracted and total protein concentration was quantified spectrophotometrically using the bicinchoninic acid (BCA) assay (Santa Cruz Biotechnology Inc., Dallas, TX, USA; Cat. No. sc-278767A). Protein samples were denatured by boiling in 4× Laemmli buffer, and equal amounts of protein were separated on SDS-polyacrylamide gels (8%, 10%, or 14%) depending on the molecular weight of the target proteins. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using the Mini Trans-Blot® Cell system (Bio-Rad, Hercules, CA, USA). Membranes were then blocked with 5% bovine serum albumin (BSA) in 1× Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated overnight at 4 °C with primary antibodies diluted in 1× TBST containing 3% BSA. The primary antibodies used included mouse monoclonal anti-Nrf2 (Santa Cruz Biotechnology, Cat. No. sc-518036; 1:500) and β-actin (Cat. No. sc-47778; 1:1000). After four washes with 1× TBST, membranes were incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse secondary antibody (SeraCare, Milford, MA, USA; Cat. No. 5220-0310; 1:10,000). Detection was performed using the 1-Step™ NBT/BCIP substrate solution (Thermo Fisher Sci-

entific, Waltham, MA, USA). Band intensities were quantified using ImageJ software and protein expression was normalized to β-actin.

2.11 Histopathological Study

The right parotid salivary glands were dissected from all rats. They were fixed in neutral 10% formalin, dehydrated, embedded in paraffin, cut into 5-μm sections, and stained with H&E to verify histological structure [44,45].

Morphometric Study

Slides were captured using a high-resolution color digital camera placed on a BX51 microscope (Olympus, Japan), connected to a computer programmed with LC micro application software in the Histology and Cell Biology Department, Faculty of Medicine, Minia University, Egypt. ImageJ (<http://rsbweb.nih.gov/ij/>; NIH, Bethesda) was used to assess semiquantitative histopathological scoring in H&E-stained sections. Irregular acini, vacuolated cells, leukocytic infiltration, and vascular congestion were examined. Score scale: 0 = no lesions; 1 = less than or equal 25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100%. Six different non-overlapping randomly selected fields from each slide at a magnification of 400 were examined to evaluate quantitatively [46].

2.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 9, GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SD and analyzed using one-way ANOVA. Intergroup comparison was performed using Tukey's post hoc test. Histopathological scores were expressed as median with range for each group. Comparisons were performed using the Kruskal-Wallis test, followed by Dunn's multiple comparisons post hoc test with Bonferroni correction. Spearman's rank correlation analysis was performed to evaluate the association among the parameters investigated. Correlation coefficients and their corresponding significance levels were calculated using a two-tailed test.

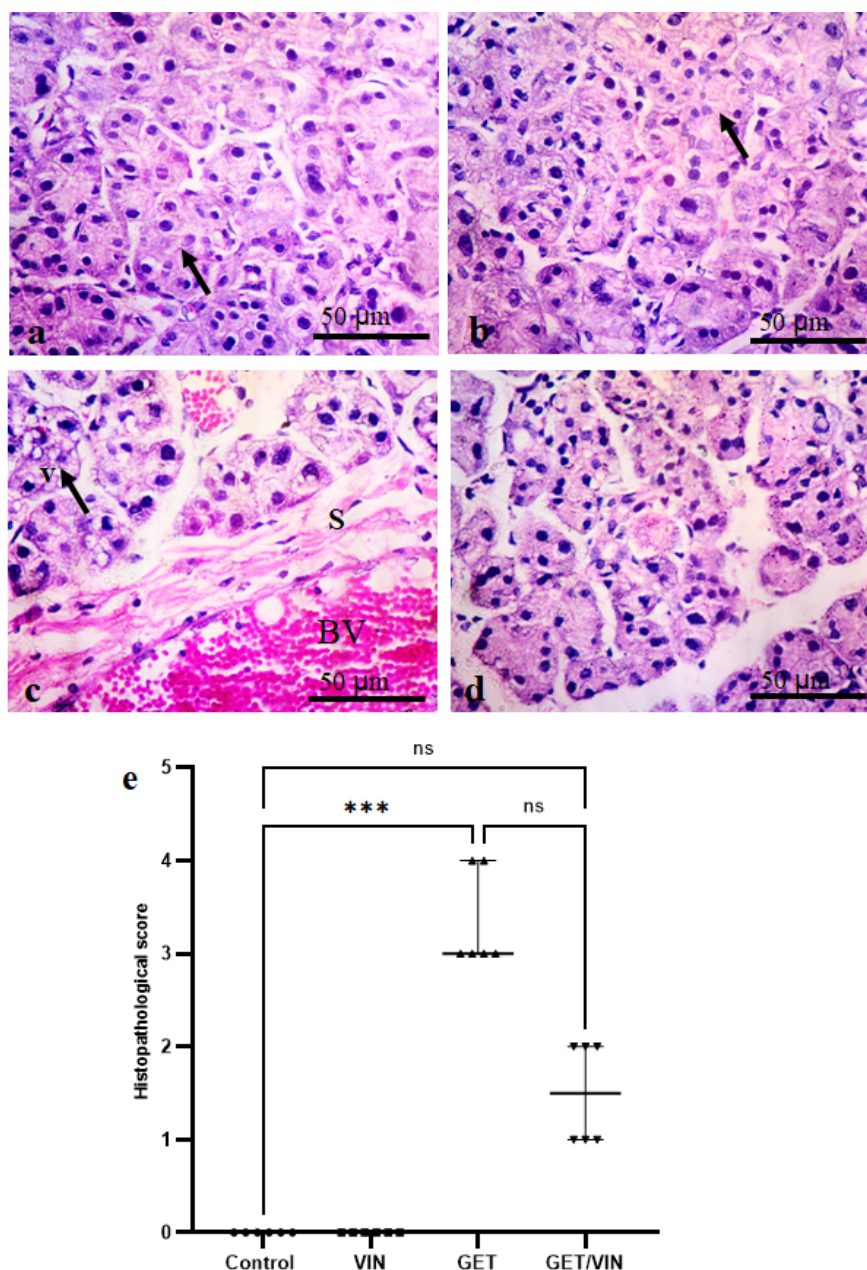


Fig. 1. Photomicrographs of rat parotid gland sections. (a,b) Control and VIN groups showing normal histological architecture with groups of serous acini (arrows). (c) GET group showing disorganized acini (black arrow) with cytoplasmic vacuolation (V), widened interlobular spaces (S), and dilated, congested blood vessels (BV). (d) GET/VIN group showing parotid acini closely resembling the control structure (H&E, $\times 400$). (e) Semi-quantitative scoring analysis. Data are expressed as median with range ($n = 6$, biological replicates). *** $p < 0.001$, ns: not significant. Statistical comparisons were performed using the Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni correction. Scale bar = 50 μm . VIN, vincamine; GET, gentamicin.

3. Results

3.1 Histopathological Results

Histological examination of the rat parotid glands revealed that both the Control and VIN groups exhibited similar architectural features, characterized by clusters of serous acini with basophilic cytoplasm. In contrast, the GET group demonstrated disorganized acini, with prominent widening of interlobular septa observed in the sections. Addition-

ally, cytoplasmic vacuolations and dilated, congested blood vessels were notable findings in this group. Remarkably, the parotid glands in the GET/VIN group showed structural characteristics that closely resembled those of the control group. The median histopathological scores and their statistical analysis were reported as median with range (Fig. 1).

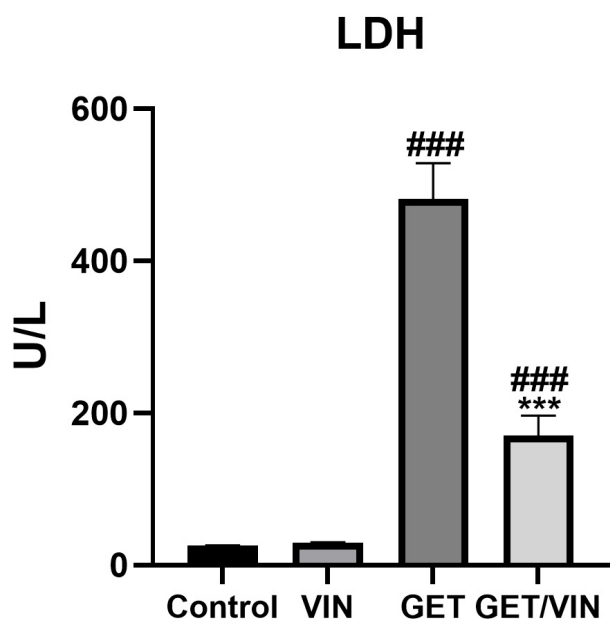


Fig. 2. Serum LDH levels. All values are expressed as the mean $\pm$ SD ($n = 6$). Statistical evaluations were done by one-way ANOVA with Tukey's post-hoc comparisons. ### $p < 0.001$ vs. control; *** $p < 0.001$ vs. GET. LDH, lactate dehydrogenase.

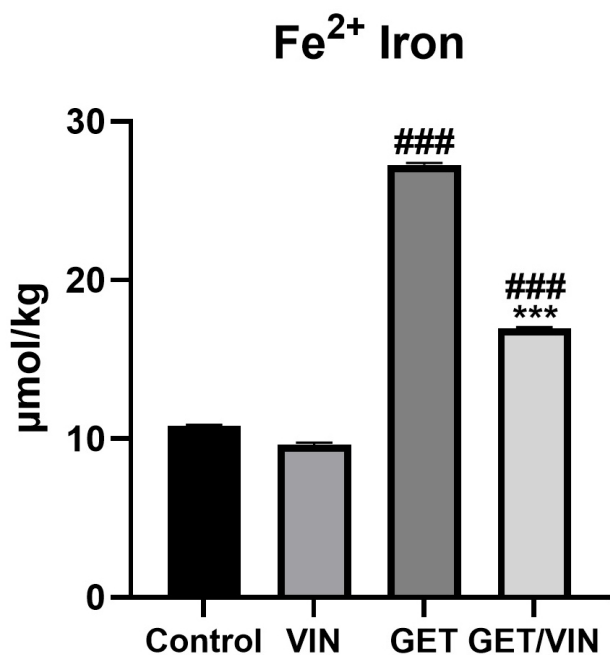


Fig. 3. Levels of ferrous iron (Fe²⁺) in the different experimental groups. All values are reported as mean $\pm$ SD ($n = 6$). Inter-group differences were analyzed utilizing one-way ANOVA with Tukey's post-hoc multiple comparison test. ### $p < 0.001$ vs. control group; *** $p < 0.001$ vs. GET group.

3.2 The Impact of Vincamine on Parotid Injury Markers

Serum LDH activities were quantified to assess parotid gland injury (Fig. 2). GET administration induced a profound cytotoxic insult, characterized by a stark elevation in serum LDH levels to 481.8 U/L ($p < 0.001$) relative to the control rats. On the contrary, therapeutic co-treatment with VIN significantly attenuated this tissue damage, eliciting a marked reduction in LDH levels to 170.3 U/L ($p < 0.001$) compared with the GET-alone cohort.

3.3 Vincamine Regulates Ferrous Iron Level Within the Parotid Tissue

GET treatment showed a significant accumulation of ferrous iron within the parotid tissue (27.25 $\mu\text{mol/kg}$; $p < 0.001$) compared to controls. Conversely, in the GET/VIN group, the levels of ferrous iron and its associated toxic effects were markedly reduced (16.96 $\mu\text{mol/kg}$; $p < 0.001$) compared to the GET group. Notably, VIN alone revealed no significant impact on iron levels, as depicted in Fig. 3.

3.4 Vincamine Attenuates Oxidative Stress Biomarkers in GET-Triggered Parotid Injury

We investigated how VIN combats oxidative stress generated by GET in rats by measuring serum TAC alongside tissue levels of GSH, GSSG, and MDA. A striking plunge in TAC was noted in the GET-treated group (0.6 mmol/L; Fig. 4a, $p < 0.001$), indicating severe oxidative stress. However, VIN dramatically reversed this decline, boosting TAC to a remarkable 0.73 mmol/L (Fig. 4a, $p < 0.01$) compared to GET alone, highlighting its potent protective effect.

Delving deeper into the parotid tissue, we observed a dramatic disruption of glutathione homeostasis in GET-treated rats. A precipitous drop in protective GSH levels (1.01 mg/g; Fig. 4b, $p < 0.001$) coincided with a surge in oxidized GSSG (4.6 $\mu\text{mol/g}$; Fig. 4c, $p < 0.001$), resulting in a severely imbalanced GSH/GSSG ratio (0.707; Fig. 4d, $p < 0.001$). Remarkably, VIN effectively restored this delicate balance. GSH levels rebounded to 2.2 mg/g (Fig. 4b, $p < 0.001$), GSSG plummeted to 2.10 $\mu\text{mol/g}$ (Fig. 4c, $p < 0.001$), and the GSH/GSSG ratio soared to 3.420 (Fig. 4d, $p < 0.001$), highlighting VIN's ability to re-establish crucial antioxidant defenses.

To further assess the extent of GET-induced oxidative damage, we quantified MDA, a key indicator of lipid peroxidation, in parotid tissue. GET exposure triggered a significant surge in MDA levels (3.91 nmol/g; Fig. 4e, $p < 0.001$), indicating substantial membrane damage. Remarkably, VIN effectively mitigated this damage, dramatically reducing MDA levels to 1.98 nmol/g (Fig. 4e, $p < 0.001$), underscoring its potent protective effect against lipid peroxidation.

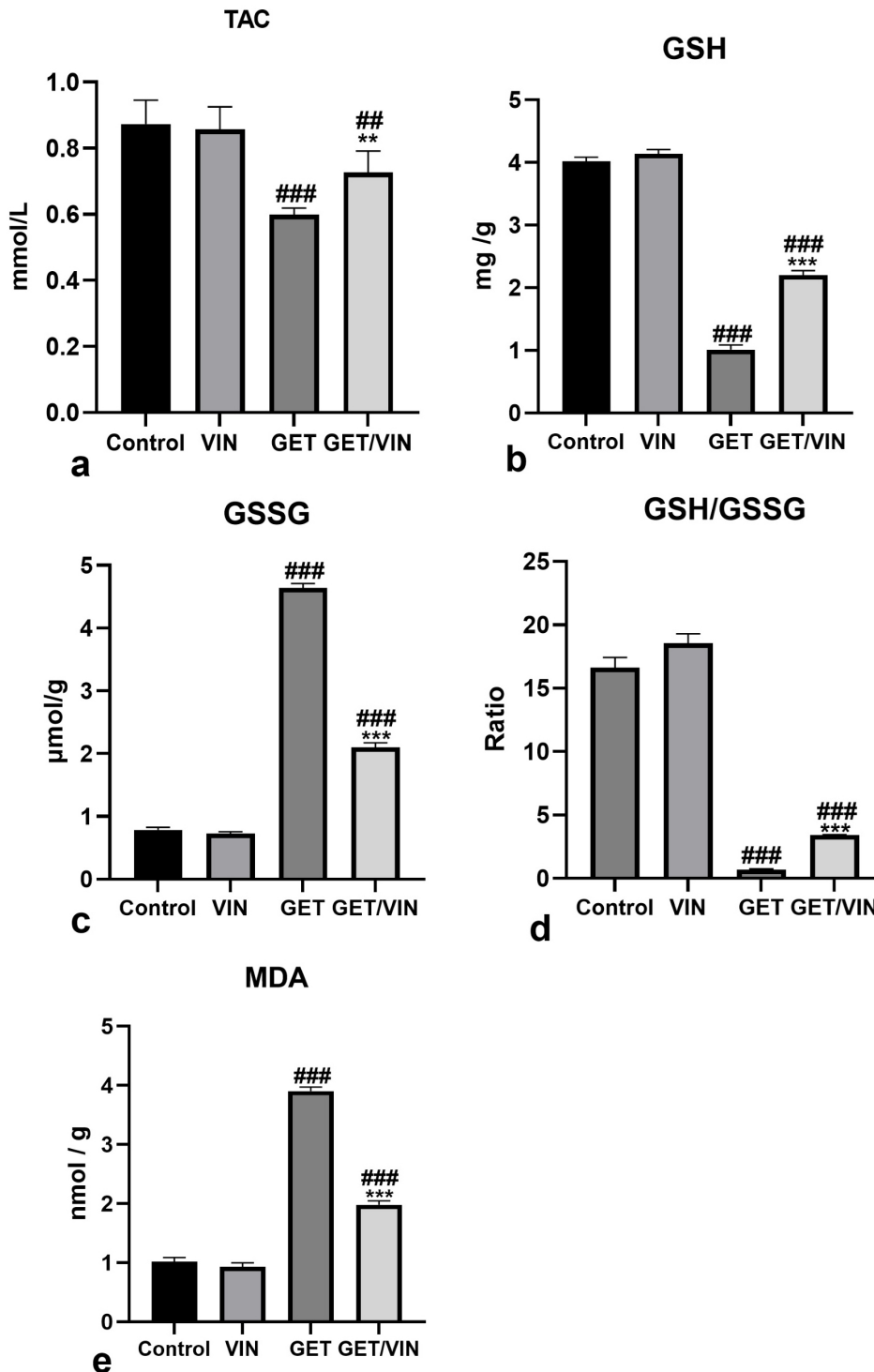


Fig. 4. Levels of (a) TAC, (b) GSH, (c) GSSG, (d) GSH/GSSG, and (e) MDA. All values represent mean $\pm$ SD (n = 6). One-way ANOVA with Tukey's post hoc multiple comparison test was performed. ### $p < 0.001$, ## $p < 0.01$ vs. control group; *** $p < 0.001$, ** $p < 0.01$ vs. GET group. TAC, total antioxidant capacity; GSH, glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde.

3.5 Vincamine Has a Potent Regulatory Effect on Ferroptosis in GET-Induced Parotid Injury

Ferroptosis, a distinctive model of highly orchestrated cell death, is primarily driven by lipid peroxidation due to an imbalance between oxidative stress and antioxidant de-

fenses [47]. Our study, having established ferroptosis's role in the toxic effects of GET, aimed to evaluate various ferroptosis markers to assess the protective effects of VIN.

We found a meaningful drop in GPX4 levels in the GET group (10.74 mU/L; Fig. 5a; $p < 0.001$) compared to

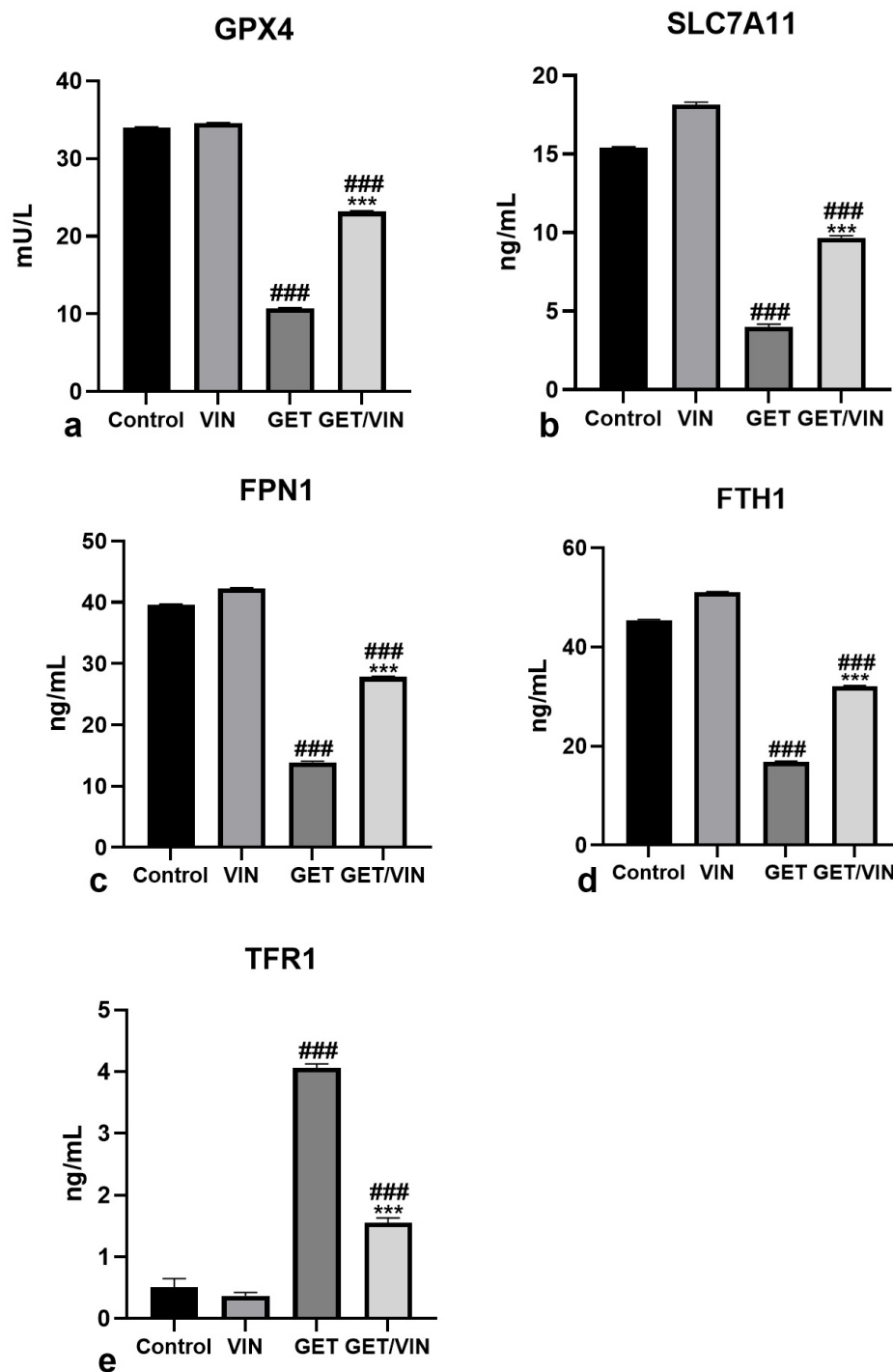


Fig. 5. Levels of GPX4 (a), SLC7A11 (b), FPN1 (c), FTH1 (d), and TFR1 (e). Bars represent mean $\pm$ SD (n = 6). Statistical significance was analyzed via ANOVA, with Tukey's post hoc test. ### p < 0.001 vs. control group; *** p < 0.001 vs. GET group. GPX4, glutathione peroxidase 4; SLC7A11, solute carrier family 7 member 11; FPN1, ferroportin 1; FTH1, ferritin heavy chain 1; TFR1, transferrin receptor 1.

normal rats. Interestingly, the GET/VIN group displayed a substantial rise in GPX4 levels (23.20 mU/L; Fig. 5a; p < 0.001) in contrast with the GET-treated animals. Similarly, SLC7A11 levels were noticeably diminished in the GET group (4.01 ng/mL; Fig. 5b; p < 0.001) relative to the

control animals, while the GET/VIN-treated rats displayed a substantial elevation (9.68 ng/mL; Fig. 5b; p < 0.001) relative to GET-alone-treated rats.

Furthermore, FPN1 concentrations were significantly depleted in the GET group (13.92 ng/mL; Fig. 5c; p

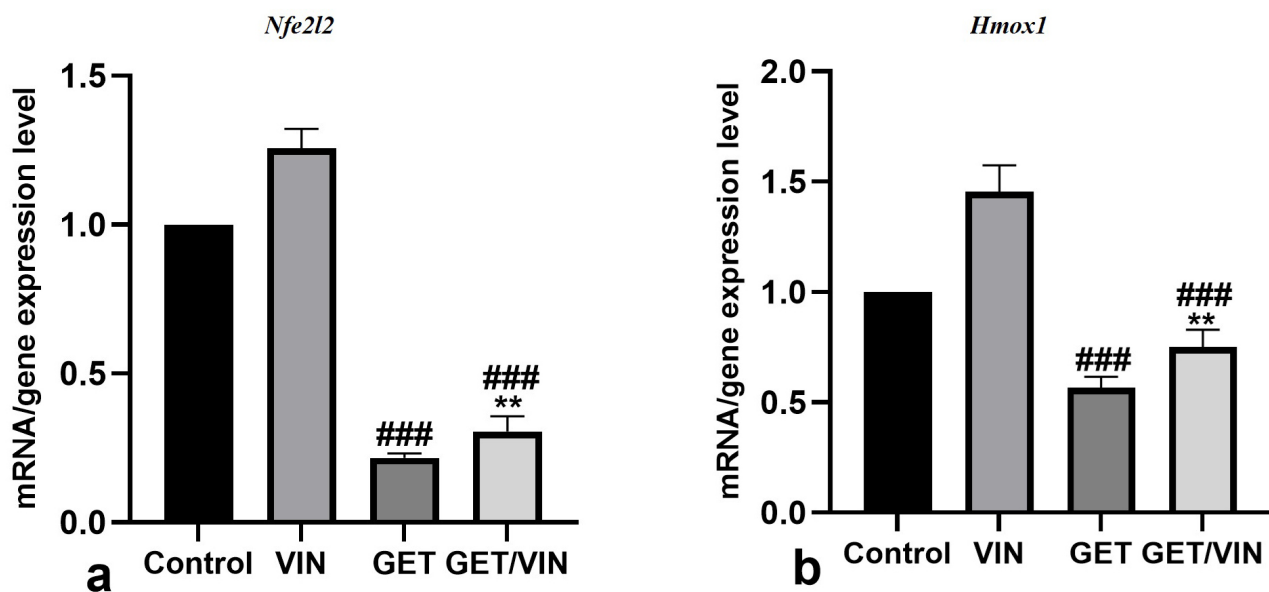


Fig. 6. Levels of *Nfe2l2* (a) and *Hmox1* (b). Bars represent mean $\pm$ SD ($n = 6$). Statistical significance was determined using one-way ANOVA with Tukey's post hoc test. ### $p < 0.001$ vs. control group; ** $p < 0.01$ vs. GET group.

< 0.001) compared with the controls. On the contrary, the GET/VIN-treated rats exhibited a robust restoration of FPN1 levels (27.81 ng/mL; Fig. 5c; $p < 0.001$) in comparison with GET-treated rats. Furthermore, FTH1 levels were diminished in the GET group (16.87 ng/mL; Fig. 5d; $p < 0.001$), while the GET/VIN group demonstrated a notable increase (32.11 ng/mL; Fig. 5d; $p < 0.001$) relative to the GET-alone-treated animals.

Conversely, TFR1 level was drastically upregulated in the GET group (4.06 ng/mL; Fig. 5e; $p < 0.001$) relative to controls. This pathological increase was effectively attenuated in the GET/VIN cohort, which displayed a highly significant decline in TFR1 expression (1.55 ng/mL; Fig. 5e; $p < 0.001$) compared to the GET group.

3.6 Vincamine Has a Potent Regulatory Effect on Nrf2/HO-1 Pathway That Antagonizes Ferroptosis in GET-Induced Parotid Injury

The Nrf2 signaling pathway functions as a direct downstream mechanism of ROS, regulating antioxidant response element (ARE)-dependent gene transcription to neutralize oxidative mediators and preserve cellular redox homeostasis. Consequently, we examined whether VIN's inhibitory effect on ferroptosis is mediated via the Nrf2/HO-1 signaling axis. In comparison with the control group, GET treatment gave rise to a profound suppression of both *Nfe2l2* (0.22-fold; Fig. 6a; $p < 0.001$) and *Hmox1* expression (0.57-fold; Fig. 6b; $p < 0.001$). Conversely, intervention with VIN successfully upregulated this pathway; the GET/VIN cohort demonstrated a statistically significant restoration in the mRNA expression levels of *Nfe2l2* (0.31-fold; Fig. 6a; $p < 0.01$) and *Hmox1* (0.75-fold; Fig. 6b; $p < 0.01$) relative to the GET-alone group.

3.7 Vincamine Modulates CHOP and TRPC1

In this study, CHOP and TRPC1 levels were assessed by ELISA. The GET group exhibited a significant increase in CHOP expression (65 pg/mL $p < 0.001$) along with a significant decrease in TRPC1 level (0.36 ng/mL) compared to the control group. In contrast, treatment with VIN significantly suppressed CHOP expression and elevated TRPC1 in the GET/VIN group (49 pg/mL & 0.62 ng/mL; Fig. 7a,b; $p < 0.001$).

3.8 Vincamine Modulates PERK and GRP78

To gain more insights into the role of endoplasmic reticulum stress, the levels of PERK and GRP78 in parotid tissues were assessed by ELISA. The GET group exhibited a significant elevation in PERK and GRP78 expression (154.5 pg/mL and 21.5 ng/mL, $p < 0.001$) compared to the control group. In contrast, treatment with VIN significantly suppressed PERK and GRP78 expression in the GET/VIN group (119.5 pg/mL & 15.35 ng/mL; Fig. 8a,b; $p < 0.001$).

3.9 Vincamine Induces Nuclear Translocation of Nrf2 in Parotid Gland

Nuclear Nrf2 translocation from the cytoplasmic compartment is a critical step for activating antioxidant gene transcription. In this study, nuclear Nrf2 levels were assessed through ELISA and western blotting. Relative to the control group, the GET group demonstrated a profound deficit in nuclear Nrf2 expression as evidenced by a western blotting assay (0.50; Fig. 9a,b; $p < 0.001$). Remarkably, intervention with VIN effectively reversed this suppression, yielding a highly significant elevation of nuclear Nrf2 levels in the GET/VIN cohort (1.26; Fig. 9a,b; $p < 0.001$). Detec-

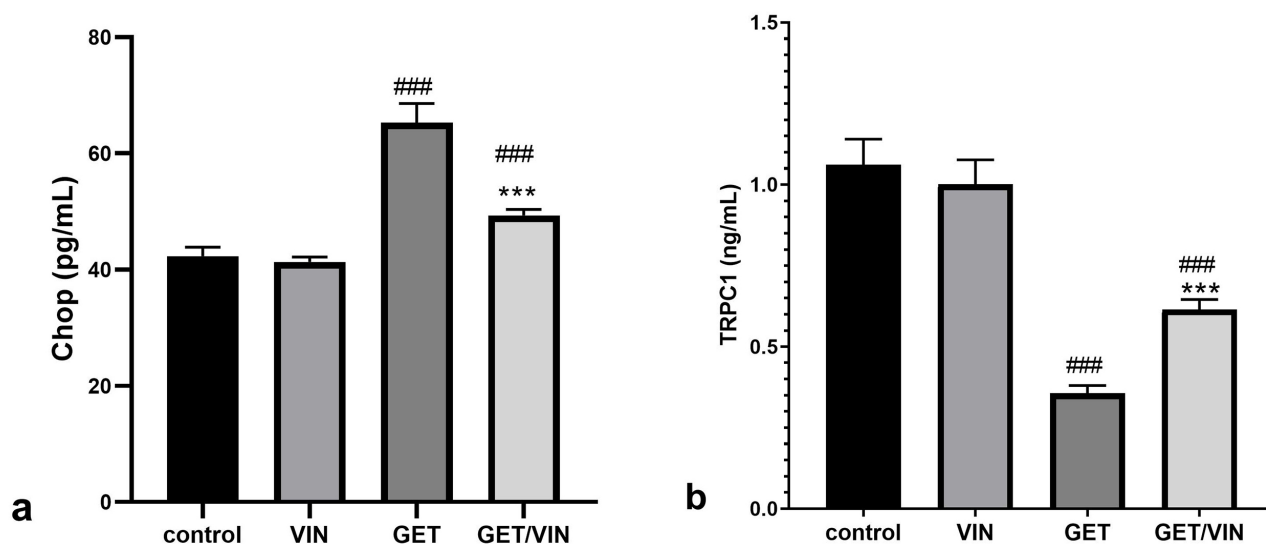


Fig. 7. Levels of CHOP (a) and TRPC1 (b). Bars represent mean $\pm$ SD ($n = 6$). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. ### $p < 0.001$ vs. control group. *** $p < 0.001$ vs. GET group. CHOP, C/EBP homologous protein; TRPC1, transient receptor potential canonical 1.

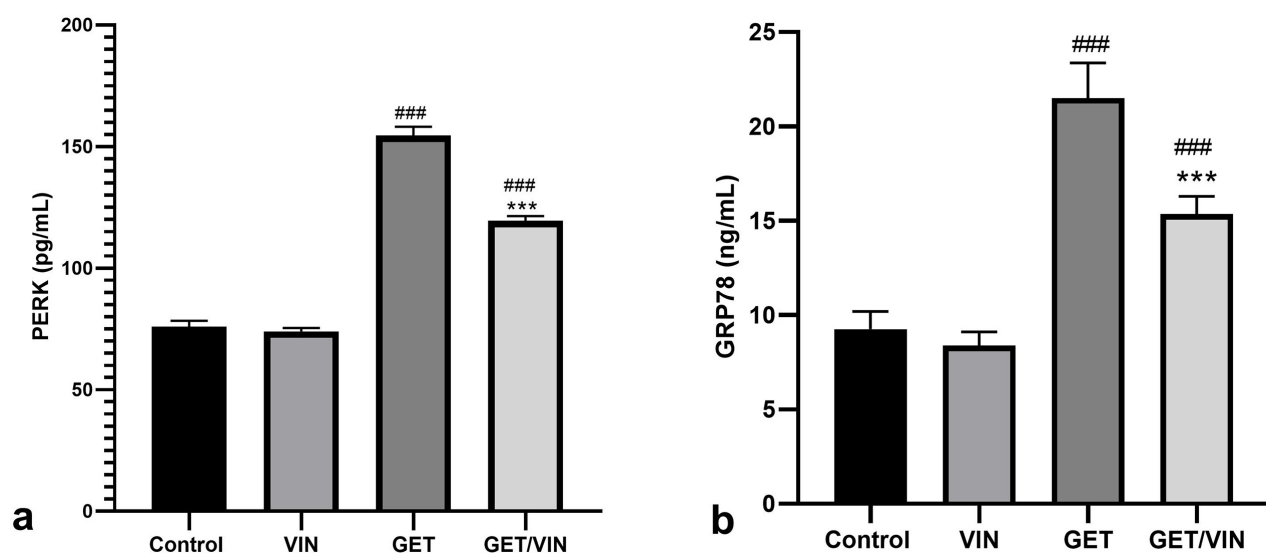


Fig. 8. Levels of PERK (a) and GRP78 (b). Bars represent mean $\pm$ SD ($n = 6$). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. ### $p < 0.001$ vs. control group, *** $p < 0.001$ vs. GET group. PERK, protein kinase RNA-like endoplasmic reticulum kinase; GRP78, Glucose-Regulated Protein 78.

tion of nuclear Nrf2 using ELISA showed similar results as in the GET-treated group, Nrf2 was significantly decreased to 12.5 ng/mL ($p < 0.001$) as compared to the control group (Fig. 9c). The administration of VIN significantly elevated Nrf2 protein levels to 18.2 ng/mL as compared to the GET-treated group.

3.10 Correlation Analysis

Spearman's rank correlation revealed significant positive correlations between histopathological injury scores and tissue MDA ($r = 0.90$), Fe^{2+} ($r = 0.9035$), TFR1 ($r =$

0.91), PERK ($r = 0.92$), GRP78 ($r = 0.9067$) and CHOP ($r = 0.91$), and a significant negative correlation with GSH ($r = -0.91$), TAC ($r = -0.8692$), GPX4 ($r = -0.91$), SLC7A11 ($r = -0.9005$), FTH1 ($r = -0.9089$), FPN1 ($r = -0.9117$) and TRPC1 ($r = -0.93$), indicating that the severity of parotid tissue injury is closely associated with the magnitude of iron accumulation, lipid peroxidation, ER stress activation, and ferroptosis related marker suppression across all experimental groups (Fig. 10).

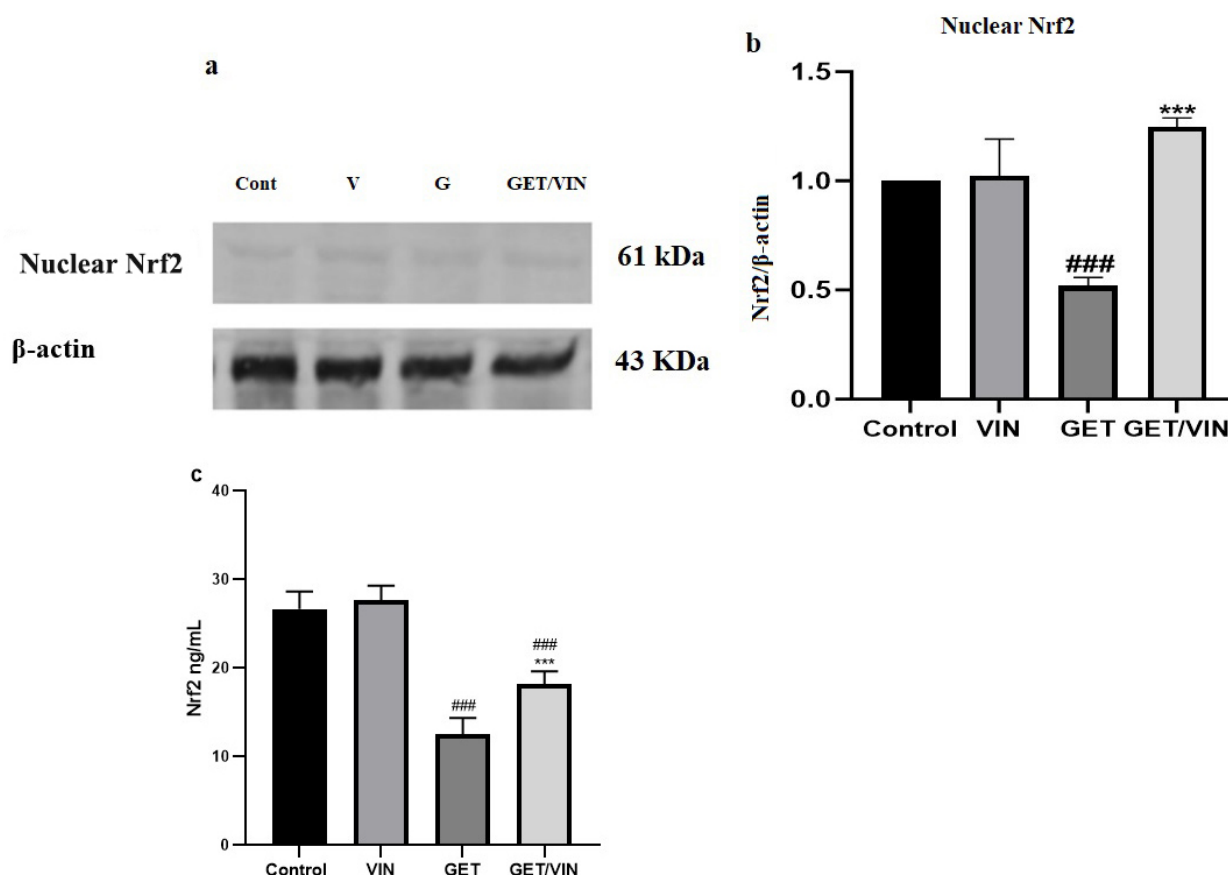


Fig. 9. Effect of VIN on Nrf2 protein expression (a) representative immunoblots for nuclear Nrf2 and β-actin (b): expression of Nrf2 was densitometrically expressed as fold change relative to the control group. (c): assessment of Nrf2 using ELISA. Bars represent mean ± SD (n = 6). Statistical significance was assessed through one-way ANOVA, with Tukey's test applied for post hoc comparisons. ###*p* < 0.001 vs. control group and ****p* < 0.001 vs. GET group.

4. Discussion

GET, an aminoglycoside isolated from *Micromonospora purpurea*, is extensively utilized in managing diverse bacterial etiologies, especially those attributed to Gram-negative pathogens, positioning it as a crucial therapeutic option for life-threatening conditions [48]. However, its clinical utility is frequently constrained by significant adverse effects, including nephrotoxicity [2], ototoxicity [49], and parotid toxicity [4] effects broadly attributed to oxidative stress, inflammation, and apoptosis [9,50].

Our histopathological analysis revealed that the parotid gland in the GET group exhibited disorganized acini, widened interlobular septa, cytoplasmic vacuoles, and dilated congested blood vessels, consistent with previous findings [4]. Conversely, the parotid glands in the GET/VIN group closely resembled the control structure. Consistent with this, serum LDH, a recognized marker of membrane integrity loss and tissue injury [51] was significantly elevated in the GET group and significantly reduced by VIN co-treatment, in line with prior models of glandular injury [52].

In salivary gland physiology, the TRPC1 channel serves as a master regulator of calcium dynamics. Sukumaran et al. [30] demonstrated that TRPC1 contributes to Ca^{2+} homeostasis in salivary gland cells and that TRPC1 deficiency reduces ER Ca^{2+} levels and promotes ER stress, accompanied by increased CHOP expression. Our data reveal a striking downregulation of parotid TRPC1 alongside elevated CHOP expression in the GET group. TRPC1 facilitates sustained Ca^{2+} influx essential for activating TMEM16A (Ca^{2+} -activated Cl^- channels, or CaCC), which drives Cl^- efflux critical for salivary secretion. ER stress and Ca^{2+} dysregulation often coincide with salivary pathologies; in this context, CHOP activation has been associated with suppression of TRPC1, apoptosis, and cell death [30]. In contrast, VIN significantly enhanced parotid TRPC1 levels while concurrently reducing parotid CHOP levels. The VIN-associated changes in TRPC1 and CHOP expression may reflect attenuation of ER stress signaling.

To extend this evidence beyond a single downstream effector, we additionally measured GRP78 and PERK, the ER stress sensor and its principal downstream transducer [53]. GET treatment significantly increased both GRP78

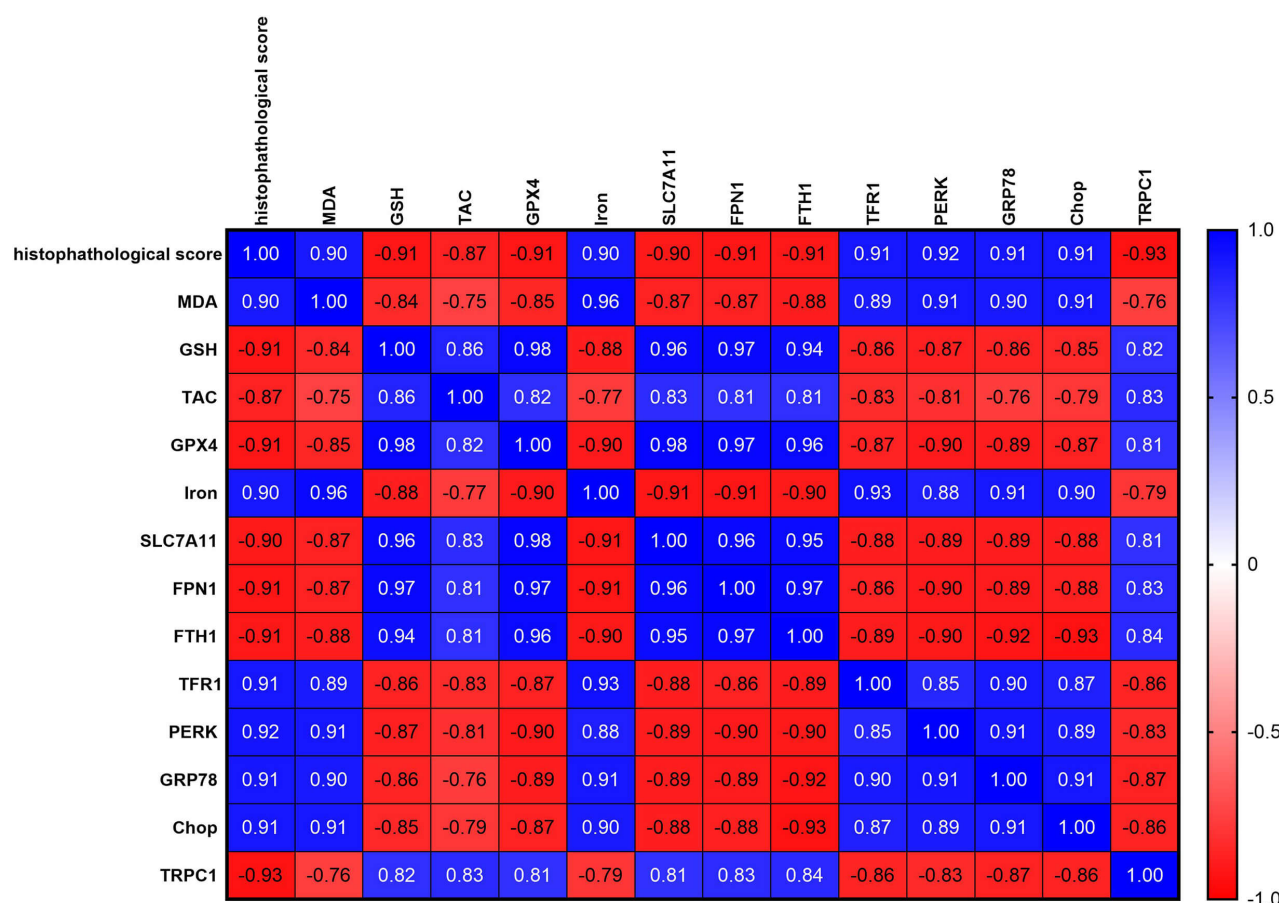


Fig. 10. Heatmap illustrating Spearman's rank correlation coefficients among histopathological score, oxidative stress, ferroptosis and endoplasmic stress markers. Positive correlations are represented by blue color and negative correlations are represented by red color. Correlation strength is expressed as Spearman's correlation coefficient (all $p < 0.0001$, $n = 24$).

and PERK expression relative to control, indicating genuine activation of the unfolded protein response rather than an isolated change in CHOP alone. VIN co-treatment significantly attenuated GRP78 and PERK upregulation, in parallel with its effects on CHOP, suggesting a coordinated modulation of this signaling axis. Together, these expression-level findings across GRP78, PERK, CHOP, and TRPC1 support the possible involvement of ER stress in GET-induced parotid injury and its attenuation by VIN.

Ferroptosis has emerged as a distinct modality of regulated cell death, driven by iron deposition and lipid peroxidation, both of which are instigated by ROS [54]. The accumulation of lipid-derived ROS, coupled with the disruption of membrane integrity, has been linked to ferroptosis induction, primarily due to a diminished endogenous antioxidant capacity. Iron plays a basic and necessary part in the execution of ferroptosis; factors such as increased iron uptake, impaired function of iron exporters, or degradation of proteins that contain iron can culminate in iron overload. This excess iron drives the transformation of hydrogen peroxide into hydroxyl radicals, as well as lipid peroxide into lipid alkoxyl radicals through the Fenton re-

action, a process implicated in lipid peroxidation. Key biomarkers including iron, GPX4, TFR1, SLC7A11, GSH, and MDA are frequently used to provide a composite assessment of ferroptosis-related changes [55,56,57]. Recent investigations have highlighted the involvement of ferroptosis-related mechanisms in GET-induced nephrotoxicity [14] and ototoxicity [54]. However, the specific role of ferroptosis in GET-induced parotid toxicity is yet to be elucidated. Our investigation is the first to describe a ferroptosis-related molecular signature in this context, with our findings suggesting that the protective effect of VIN may be associated with, and correlates with, alterations in this pathway.

Ferroptosis is triggered by GET-induced ROS, resulting in damage to salivary glands [4]. ROS indiscriminately target cellular molecules, particularly polyunsaturated fatty acids within cell membranes, via a cascade of reactions that lead to membrane lipid peroxidation [58]. This process is exacerbated by cross-linking with MDA and GSSG, which not only enhances lipid peroxidation but also inactivates crucial antioxidant enzymes [4,59,60]. In our study, VIN treatment was associated with reduced markers of oxidative

stress following GET exposure, reflected by higher GPX4 and GSH levels and lower MDA and GSSG levels. These findings are in line with previous research suggesting a role for vincamine in modulating oxidative stress [61].

Iron plays a pivotal role in ferroptosis by promoting ROS generation through enzymatic and non-enzymatic reactions [62]. The levels of iron are maintained in a dynamic equilibrium within and outside the cell, and any disruption to this balance can significantly influence a cell's susceptibility to ferroptosis [63]. Here, we demonstrated that the GET group triggered a robust intracellular iron influx, significantly surpassing control levels. Conversely, the VIN group successfully rescued this phenotype, suggesting a possible role for VIN in modulating iron accumulation.

Circulating Fe^{3+} binds to transferrin (TF) to form the TF- Fe^{3+} complex, which is recognized by transferrin receptor 1 (TFR1/CD71), the major mediator of cellular iron uptake [64]. Following internalization, Fe^{3+} is reduced to Fe^{2+} , increasing the intracellular labile iron pool [65]. Our study found a significant increase in TFR1 levels in the GET group, consistent with heightened iron uptake and a possible association with ferroptosis-related changes. However, VIN effectively disrupted this process, markedly reducing TFR1 levels in the VIN/GET group. These findings highlight VIN's ability to modulate iron transport, thus mitigating GET-induced parotid toxicity may be associated with and correlate with a ferroptosis-like molecular signature.

The ferritin complex, comprised of 24 polypeptide subunits from FTH1 and ferritin light chain (FTL), is crucial for the storage and release of intracellular iron [66]. Through its ferroxidase activity, FTH1 converts intracellular Fe^{2+} to Fe^{3+} , thereby reducing oxidative damage caused by Fe^{2+} -induced ROS. Additionally, intracellular ferrous iron can be oxidized and exported by the membrane protein FPN1, a specialized iron efflux pump that facilitates iron release from cells [67]. Impaired iron export and storage can lead to ferroptosis, as demonstrated by the reduced expression of ferritin and FTH1 in ferroptosis-sensitive cells [68]. Intriguingly, our research unveiled a significant decrease in FTH1 and FPN1 levels, key players in iron homeostasis, following GET treatment. Conversely, VIN effectively counteracted this decline, significantly boosting both FTH1 and FPN1 levels in the VIN/GET group. This finding provides compelling new evidence that VIN provides robust cellular protection against iron-induced oxidative stress, suggesting potential clinical implications for mitigating GET-related toxicity.

SLC7A11, the cellular gatekeeper for cystine, orchestrates its import in exchange for glutamate, thereby supporting GSH synthesis and protecting cells against oxidative stress and ferroptosis [69,70]. We noted a remarkable depletion of SLC7A11 in the GET-treated group, consistent with compromised expression of this defense-related marker. However, VIN effectively restored SLC7A11 lev-

els in the VIN/GET group, significantly surpassing those in the GET group. Given that elevated SLC7A11 is known to inhibit ferroptosis, these findings highlight the possible role for VIN in modulating these ferroptosis-related alterations [71,72].

Nrf2 is a master regulator of the cellular antioxidant defense system, coordinating protection against ROS-induced oxidative damage by transcriptionally activating cytoprotective genes, including *HO-1*, in response to oxidative stress [73,74]. Disrupting the Nrf2/Keap1 interaction triggers Nrf2 migration to the nucleus, culminating in triggering ARE [75]. Numerous studies have demonstrated that activating Nrf2 can mitigate the damage associated with ferroptosis [25,76]. Our study revealed a meaningful downregulation of Nrf2/HO-1 expression in the GET-treated group, reflecting compromised cellular protection. These results suggest the involvement of the Nrf2/HO-1 pathway in the protective pattern associated with VIN.

Emerging evidence indicates that ferroptosis and ER stress processes are bidirectionally linked: ferroptosis initiation can induce or amplify ER stress via iron overload and lipid peroxidation, while ER stress reciprocally regulates ferroptosis. Notably, ER stress can further promote iron accumulation via calcium-dependent signaling pathways, offering a plausible mechanistic bridge between TRPC1/calcium and TFR1/iron findings [77]. In our study, concordant GRP78/PERK/CHOP activation alongside TFR1 elevation, Fe^{2+} accumulation, and GPX4/Nrf2/HO-1 suppression in the GET group, all normalized by VIN and correlating with injury severity, suggests a single, interconnected stress network rather than two independent pathways, with the PERK-Nrf2-HO-1 axis as a plausible convergence point.

5. Limitations

This study has certain limitations that should be acknowledged. First, no ferroptosis inhibitor was included as a rescue/mechanistic control arm. Second, this study was conducted exclusively in an experimental animal model, and no clinical investigation in human subjects was performed. Third, only a single dose of VIN was tested; dose-response relationships were therefore not established. Fourth, direct functional indices of parotid gland activity, such as salivary flow rate or amylase activity, were not assessed in this study.

6. Conclusions

In conclusion, we have demonstrated for the first time that vincamine attenuates parotid gland degeneration and iron accumulation, associated with reduced ROS production and ferroptosis-related alterations induced by lipid peroxides following GET toxicity. This, in turn, is associated with improved cell survival and histological and biochemical improvements. Mechanistically, our results indicate that the protective effects of VIN are associated with ac-

tivation of the Nrf2/HO-1 pathway and may be linked, in part, to ferroptosis-related and ER-stress-related alterations. Our findings highlight VIN's previously unrecognized role in addressing GET-induced parotid toxicity.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization, methodology, software, validation, DHA and AA; formal analysis, DHA, SMNA, and AA; investigation, DHA, AA, SMNA, AAKE and NEMS; resources, DHA, AAKE and AA; data curation, DHA and AA; writing—original draft preparation, AA, AAKE, SMNA, and NEMS; writing—review and editing, DHA; visualization, DHA; supervision, DHA; project administration, AAKE; funding acquisition, AAKE. All authors have read and agreed to the published version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures were conducted in accordance with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments) and in adherence to the NIH Guide for the Care and Use of Laboratory Animals. The study protocol received full approval from the Ethics Committee of the Deraya Center for Scientific Research (DCSR), Deraya University, Minia, Egypt (Ethical approval No. DCSR-010-024-25).

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used ChatGPT-3.5 in order to check spelling and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL55517>.

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