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Therapeutic effect of vitamin D_3 against fipronil-induced oxidative damage, hematological disorders, EPO, RAS abnormalities, and renal impairments in male rats

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Abstract

Fipronil is a highly effective insecticide that belongs to the phenylpyrazole chemical group. It has been widely used to control crop pests and veterinary pests with toxic effects on non-target organisms. Vit. D_3 has been involved in numerous biological activities within the body. This study explored the potential ameliorative effects of Vitamin. D_3 against fipronil's detrimental effects on hematological, EPO, RAS, and kidney function in male rats. Animals were divided into 4 groups and orally treated daily for 28 days with Vitamin. D_3 (500 IU/kg), Fipronil (10 mg/ kg), or a combination of both as mentioned doses previously. The findings demonstrated that administering Fipronil resulted in a noticeable reduction in various hematological parameters, erythropoietin, albumin, total protein, and globulin levels, along with diminished renal antioxidant activity. Also, rats treated with Fipronil exhibited increased levels of kidney malondialdehyde, nitric oxide, and kidney function. Notably, Fipronil induced severe histopathological alterations in the kidney tissues. Immunohistochemical analysis revealed a robust positive expression of Caspase-3 in Fipronil-treated rats. Conversely, supplementation of Vitamin. D_3 to Fipronil-treated rats alleviated all the aforementioned effects induced by Fipronil. This study suggests that Vit. D3 has therapeutic effectiveness against Fipronil-induced oxidative damage, hematological disorders, EPO irregularities, RAS disruptions, and renal impairments due to its antioxidant and anti-apoptotic properties.

Keywords: phenylpyrazole insecticide, Vitamin D_3, EPO, apoptosis.

Introduction

Pesticides play a vital role in enhancing public health, food production, and aesthetics; however, they also contribute significantly to environmental and food pollution **(1)**. In the realm of insecticides, which are categorized into groups like herbicides, insecticides, fungicides, fumigants, and rodenticides based on their target pests, various chemical compounds are used. These include organochlorines, organophosphorus, carbamates, pyrethroids, neonicotinoids, and phenylpyrazoles. Phenylpyrazoles, a newer class of insecticides, were developed to replace older, more hazardous pesticides and combat resistance in parasitic insects **(2,3)**.

Fipronil (FIP) is a broad-spectrum phenylpyrazole insecticide used extensively in agriculture, veterinary

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practices, and sanitation efforts (4). Despite its widespread applications, FIP, classified as a Class C probable carcinogen, has raised concerns about its safety (5). Recent research has indicated that the use of FIP and its metabolites can lead to toxicity in vital organs. This toxicity is primarily driven by the inhibition of the mitochondrial respiratory chain, disrupting bioenergetic balance and giving rise to adverse events such as calcium imbalance and excessive production of free radicals (6). FIP has been linked to metabolic, mutagenic, and genotoxic effects in both humans and mice, earning it a place on the list of endocrine disruptors (7). The insecticide damages lipids, proteins, and DNA, resulting in neurotoxic effects to insects by blocking the ionotropic gamma-aminobutyric acid receptor (GABAR) within the central nervous system. This blocking hinders the usual transmission of nerve signals, leading to excessive stimulation at low levels and causing convulsions that lead to the death of insects at high levels. Also, it caused hepatotoxic, cytotoxic, and reproductive effects in both vertebrates and invertebrates (8). Notably, FIP results in oxidative injury to the renal and cerebral tissues (9).

Pesticides are potent endocrine disruptors; their exposure is involved in an imbalance of erythropoiesis and the renin-angiotensin system (RAS). These pathways have been deregulated by oxidative stress, which affects renal cortical interstitial and juxtaglomerular cells responsible for the secretion of erythropoietin and renin hormones, respectively, resulting in dyserthropoiesis and RAS over-stimulation promotes several diseases (10).

Cells can combat oxidative damage through mechanisms such as removing damaged molecules and using non-enzymatic and enzymatic antioxidant systems to neutralize free radicals (11). Antioxidants have proven effective in treating conditions arising from oxidative stress (12).

Vitamin D (Vit. D) is a steroid vitamin that is acquired either through exposure of the skin to UV light or from a diet that is high in cholecalciferol (Vit. D3) or ergocalciferol (Vit. D2) (13,14). Vit.D_3 is the preferred form, functioning as a hormone and hormone precursor, regulating tasks like calcium and phosphorus metabolism (15). Kidneys are the main producers of the active form of vitamin D, called 1, 25-dihydroxyvitamin D3 (or calcitriol for short). This activated vitamin D needs a special receptor in the body's cells (VDR) to play its role. Vitamin D has many important functions, including acting as an antioxidant and anti-apoptotic (16,17).

The current study aims to investigate the ameliorative effect of vitamin D3 against fipronil-induced oxidative damage, hematological disorders, EPO, RAS abnormalities, and renal impairments in male rats through the determination of renal oxidant and antioxidant markers, renal function parameters, histopathological and immunohistochemical analysis for caspase-3 expression.

Materials and Methods

Chemicals

The technical grade Fipronil (FIP) at a purity of 97%, with the chemical name 5-amino-1-(2,6-dichloro-4-(trifluoromethyl)phenyl)-4(1, R, S)trifluoromethylsulfinyl)-1-H-pyrazole-3-carbonitrile and CAS No. 120068-37-3, was sourced from the ISAGRO company located in the USA. Vitamin D3 (Vit.D3) in the form of oral drops with a concentration of 2800 IU/ml, where each drop contained 100 IU of Vitamin D3, was procured from Medical Union Pharmaceuticals, Alexandria, Egypt, under the product name "Vidrop." All other reagents were purchased from Biodiagnostic Co. in Cairo, Egypt. Sodium (Na^+) and anti-caspase-3 antibody were obtained from Abcam Co. based in Cambridge, UK. Erythropoietin (EPO) was sourced from Sigma-Aldrich Co. situated in Missouri, USA. Renin and angiotensin-II (ANG-II) were acquired from MyBioSource Co. located in San Diego, USA.

Animal groups

A total of twenty-eight adult male albino Wistar rats, weighing around 160 grams with a deviation of ± 10 grams, were utilized for the study. These rats were

sourced from the experimental animal facility at Alexandria University, Alexandria, Egypt. The animals were accommodated in cages, with seven rats per cage, and they were provided with a standard diet and tap water ad libitum. The housing conditions maintained a constant temperature, humidity, and a 12-hour light/dark cycle throughout the entire experimental period.

All procedures and handling of the animals were carried out following the guidelines set forth by the Alexandria University Institutional Animal Care and Use Committee (ALEXU-IACUC) under the approval number AU 04 18 12 02 MSc 3. Also, this research confirmed that all methods were carried out following relevant guidelines and regulations. It is also confirmed that all methods are reported following ARRIVE guidelines (https://arriveguidelines.org).

After two weeks of acclimatization, the rats were randomly assigned to four groups. Each group consisted of seven rats, and the assignment was as follows:

-Group I (Control group): Rats were orally administered distilled water via gavage each day for 28 days as a vehicle.

-Group II (FIP-treated group): Rats were orally administered fipronil alone via gavage at a dosage of 10 mg/kg b.w. (1/10 of LD50, approximately 100 mg/kg). This was done by dissolving fipronil in distilled water and administering it daily for 28 days.

-Group III (Vit.D3 + FIP-treated group): Rats supplemented orally with Vit. D_3 at a dose of 500 IU/kg b.w. /day for 28 days, in addition to oral administration of FIP at a dose of 10 mg/kg b.w. /day.

- Group IV (Vitamin D3 group): Rats received oral supplements of Vit. D_3 at a dose of 500 IU/kg b.w. /day for 28 days.

The supplementation of Vit. D_3 preceded FIP administration by 30 min.

The doses of Vit. D3 and FIP were chosen according to previous reports **(18,19)**.

Blood and tissue sampling

24 hours after the last FIP dose, blood samples were collected from rats under ketamine (100 mg/kg) and xylazine (20 mg/kg) anesthesia, and the rats were then sacrificed by cervical dislocation. Blood samples were acquired from all rat groups, with some collected in regular test tubes and others in tubes containing the anticoagulant Ethylene Diamine Tetra Acetic Acid (EDTA) for hematological analysis. All tubes were promptly placed on ice. After collection, the blood samples were subjected to centrifugation at 3000 revolutions per minute (r.p.m) for 15 minutes. The resulting sera were separated and stored at a temperature of -20 °C until they were utilized for subsequent biochemical assessments.

The kidneys from all experimental animals were swiftly extracted, rid of any attached blood residues, and then rinsed using chilled saline solution (0.9%). Following this, the kidneys were dried, and a quarter of a gram from each kidney was homogenized in 5 milliliters of a cold buffer solution (composed of 50 mM potassium phosphate, pH 7.5, and 1 mM EDTA). The resulting homogenates were subjected to centrifugation at 4000 r.p.m for 15 minutes at a temperature of 4 °C, using a cooling centrifuge (Hettich model EBA 12R, Germany). The supernatants obtained were then preserved at a temperature of -80 °C for subsequent biochemical assessments. Sections of the kidneys from each group were sliced and immediately immersed in 10% formalin for subsequent histological and immunohistochemical analyses.

Biochemical Analysis

Immediately following sample collection, blood specimens underwent analysis for various parameters. Red blood cell (RBC), hemoglobin (Hb), hematocrit (Ht), white blood cell (WBC), and platelet counts were determined using an Alfa swelab cell counter from Sweden.

The quantification of serum erythropoietin hormone (EPO, Cat. No: RAB0654) was conducted utilizing a sandwich enzyme-linked immunosorbent assay

(ELISA). Measurement of serum renin levels (Cat. No: MBS041519) was executed using the approach detailed by Eltablawy et al**. (20).** For serum angiotensin II (ANG-II, Cat. No: MBS703599), a sandwich enzyme immunoassay technique, as outlined by Eltablawy et al. **(20)**, was employed.

Detection of serum albumin (Cat. No: AB 10 10) followed the method devised by Doumas et al. **(21)** Serum total protein (T. Protein, Cat. No: TP 20 20) was evaluated following Sapan et al. **(22)** Calculation of serum globulin was based on the equation Total protein = Albumin - Globulin (g/dL), as presented by Busher **(23)**.

For serum urea (Cat. No: UR 21 10) and serum creatinine (Cat. No: CR 12 50), commercially available kits were employed. Determination of serum uric acid (Cat. No: UA 21 20) adhered to the protocol established by Kageyama. **(24)** Serum levels of sodium ion (Na^+, Cat. No: ab211096), potassium ion (K^+, Cat. No: PT 18 20), and calcium ion ($Ca2^+$, Cat. No: CA 12 10) were assessed using methods described by McCance et al. **(25)**, Radhika et al. **(26)** and Gindler & King **(27)**, respectively.

The extent of lipid peroxidation was gauged through the end-product malondialdehyde (MDA, Cat. No: MD 25 29) using the methodology outlined by Ohkawa et al. **(28).** Nitric oxide levels (NO, Cat. No: NO 25 33) were detected following the procedure put forth by Montgomery and Dymock. **(29)** Reduced glutathione (GSH, Cat. No: GR 2511) was quantified spectrophotometrically at 405 nm, employing the approach detailed by Crowley et al. **(30)**.

Enzymatic activities of superoxide dismutase (SOD; EC: 1.15.1.1), catalase (CAT; EC: 1.11.1.6), and glutathione peroxidase (GPx; EC: 1.11.1.9) were assessed within the kidney using methods established by Nishikimi et al. **(31)**, Sindhu et al. **(32)**, and Khan et al. **(33)**, respectively.

Histological assessment

Kidney tissue samples were immersed in 10% formalin solution for 12 hours for fixation. Subsequently, the tissues were dehydrated by gradually increasing concentrations of ethyl alcohol until they were completely immersed in alcohol (1 hour). They were then moved to xylol through a series of three changes, each lasting 5 minutes. The kidneys were placed in a mixture of melted wax and xylol in a 1:1 ratio for approximately 10 minutes and then transferred to paraffin at 56°C. This paraffin transfer process involved three changes, each lasting around 2 hours. After these steps, the tissues were sliced into sections and subjected to staining using hematoxylin and eosin (H&E). Under a microscope, the sections were carefully examined to assess any histopathological alterations following the method described by Carleton et al **(34)**.

Immunohistochemical evaluation:

Thin (5 µm) sections of kidney tissue embedded in paraffin were utilized for immunohistochemical analysis of caspase-3 using the avidin-biotin-complex (ABC) technique as outlined by Hsu et al. **(35).** A rabbit polyclonal antibody targeting caspase-3 (ab4051) was employed for this purpose. The sections were deparaffinized and subjected to a series of steps involving rinsing, blocking, antibody incubation, and visualization through various solutions. Finally, the sections were counterstained and cover-slipped.

Employ image analysis software to measure staining intensity and distribution. Then, analyze quantified data statistically to conclude protein expression or localization.

Statistical analysis

The results were presented as the mean ± standard error (S.E.). To assess the significance of treatment effects, a one-way analysis of variance (ANOVA) was conducted using SPSS version 22 (SPSS, IBM, USA). Subsequent pairwise comparisons between groups were performed using Duncan's Multiple Range Test. Statistical significance was determined based on a significance level of $P<0.05$, following the criteria established by Duncan. **(36).**

Results

Measures of hematological parameters and serum erythropoietin (EPO) in rats exposed to vit. D3, FIP, or a combination of both

In Figures 1A - 1F, Rats exposed to fipronil alone had significantly lower levels of hemoglobin (Hb), red blood cells (RBCs), hematocrit (Ht), serum erythropoietin (EPO), white blood cells (WBCs), and platelets (PLTs) compared to control rats.

Notably, the concurrent administration of Vitamin D3 with fipronil yielded noteworthy increases in all hematological parameters and EPO level, demonstrating a restorative effect compared to the animals exposed to fipronil alone.

Measures of renal oxidant and antioxidant status in rats exposed to vitamin D3, fipronil, or a combination of both

As illustrated in Figure 2 A - 2F, the oral application of FIP exhibited significant increases in renal levels of MDA and NO. Simultaneously, there were noteworthy decreases observed in renal GSH levels, as well as reduced activities of SOD, CAT, and GPX when compared to the control. Conversely, when Vitamin D3 was co-administered with FIP, substantial reductions were observed in MDA and NO levels, while remarkable enhancements were noted in GSH levels and the activities of antioxidant enzymes, in contrast to the group treated with FIP alone.

Measures of kidney function, serum albumin, total protein (T. Protein), and globulin concentrations in rats exposed to vit. D3, fipronil, or a combination of both

As depicted in the FIP-treated group Figure 3A - 3F, the levels of urea, creatinine, and uric acid exhibited considerable elevations coupled with significant reductions in serum albumin, T.Protein, and globulin concentrations when compared to control rats. Conversely, in rats treated with a combination of vitamin D3 and FIP, there was a notable reduction in serum concentrations of urea, creatinine, and uric acid, together with substantial increases in serum albumin, T. Protein, and globulin concentrations compared to the FIP-treated group.

Measures of renin, angiotensin II (Ang. II) and electrolyte levels, in rats exposed to vit.D3, FIP or a combination of both

The FIP-treated group exhibited marked elevations in renin, Ang. II and Na^+, K^+, Ca^{2+}concentrations within the serum, in contrast to the control. Conversely, administration of Vit.D3 + FIP resulted in notable reductions in serum renin, Ang. II and electrolyte levels, when contrasted with the FIP-treated group (Figure 4A - 4E).

Histopathological assessment

Upon microscopic evaluation of kidney sections derived from the control and Vitamin D3-treated groups (Figure 5A, B), a normal histological arrangement was observed. In the control group, the kidney sections exhibited an ordinary pattern, with intact glomeruli encircled by Bowman's capsules, along with proximal and distal convoluted tubules that displayed no indications of inflammatory alterations. Conversely, the group subjected to FIP treatment (Figure $5C_1$, $5C_2$) displayed evident degeneration in the renal tubules, accompanied by disruption of the basement membranes situated between these tubules. The majority of renal tubules showcased dilatation with cystic luminal enlargement, and the cells lining these tubules appeared flattened. Furthermore, the glomeruli in the FIP-treated group exhibited signs of degeneration and atrophy. In contrast, the introduction of Vitamin D3 in conjunction with FIP treatment (Figure 5D) yielded a modest enhancement in kidney histology. However, certain proximal and distal tubules still exhibited dilation, albeit to a lesser extent. Additionally, a few glomeruli displayed a capsular space.

Immunohistochemical evaluation

Figures 6 and 7 display immunostaining analyses for caspase-3 within kidney sections obtained from various experimental groups, as detailed below:

Microscopic images of kidney sections from both the control group and the Vitamin D3-treated group (Figure 6A, 6D, and 7) displayed faint caspase-3 immunoreactivity. In stark contrast, immunohistochemical assessment of kidney sections from animals subjected to FIP treatment (Figure 6B and 7) showcased intense staining indicative of caspase-3 presence. Conversely, when Vitamin D3 was administered concurrently with FIP to rats, there was evident reduction in caspase-3 immunoreactivity (Figures 6C and 7), signifying a marked decrease in its presence.

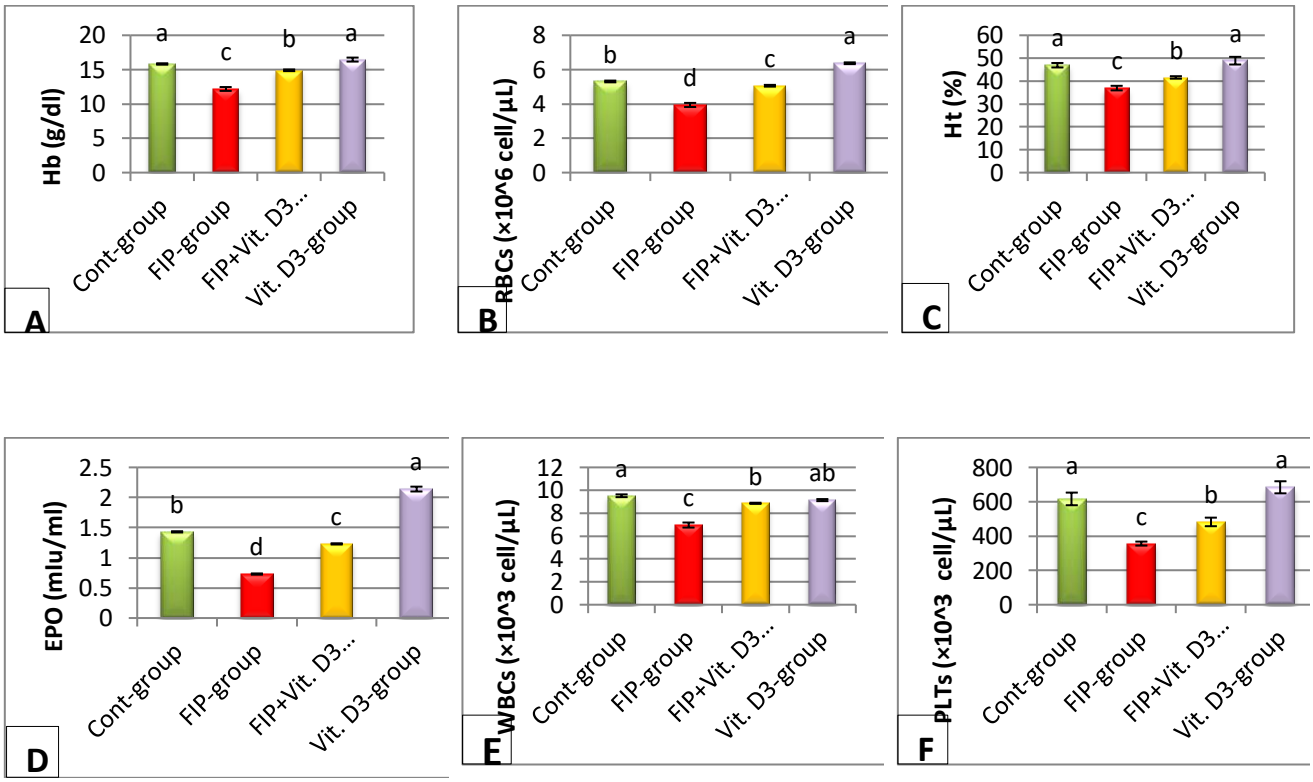

Figure (1): Effect of fipronil (FIP), vitamin D_3 (Vit. D_3) and their combination on hematological parameters: hemoglobin (Hb), red blood cells count (RBCs), hematocrit (Ht), erythropoietin (EPO), Leukocytes (WBCs), and platelets (PLTs) counts of male rats.

All the data were analyzed using one-way ANOVA followed by Duncan post Hoc test. Values are expressed as means ± S.E.; n=7 for each group. Mean values within the columns not sharing a common superscript letter (a, b, c, d) were significantly different, $p < 0.05$. For Hb: variation between groups=17.773, variation within groups=0.233 So, F=76.28. For RBCs: variation between groups=4.959, variation within groups=0.028 So, F=175.72. For Ht: variation between groups=146.356, variation within groups=6.343. So, F=23.075. For EPO: variation between groups=1.712 , variation within groups=0.001 So, F=2454 . For WBCs: variation between groups=6.489, variation within groups=0.097 So, F=67.25. For PLTs: variation between groups=106037.7, variation within groups=4189.225, so, F=25.312.

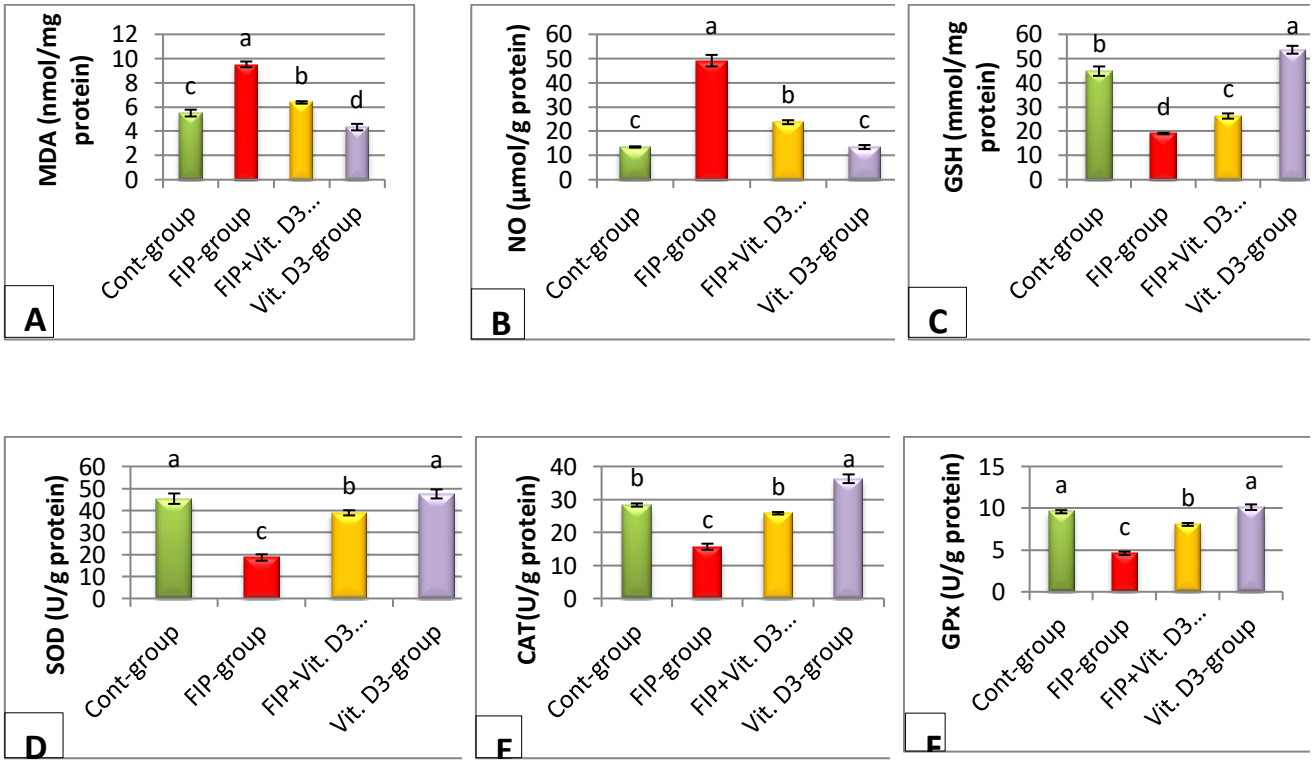

Figure (2): Effect of fipronil (FIP), vitamin D_3 (Vit. D_3) and their combination on lipid peroxidation end product malodialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH), together with superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) levels in kidney tissues of male rats.

All the data were analyzed using one-way ANOVA followed by Duncan post Hoc test. Values are expressed as means ± S.E.; n=7 for each group. Mean values within the columns not sharing a common superscript letter (a, b, c, d) were significantly different, $p < 0.05$. For MDA: variation between groups=24.639, variation within groups=0.258 So, F=95.50. For NO: variation between groups=1415.471, variation within groups=8.872, so, F=159.544. For GSH: variation between groups=1282.274, variation within groups=9.578, so, F=159.544. For SOD: variation between groups=867.678, variation within groups=16.597 So, F=52.278. For CAT: variation between groups=360.822, variation within groups=3.705 So, F=97.40. For GPx: variation between groups=30.870, variation within groups=0.279 So, F=110.546.

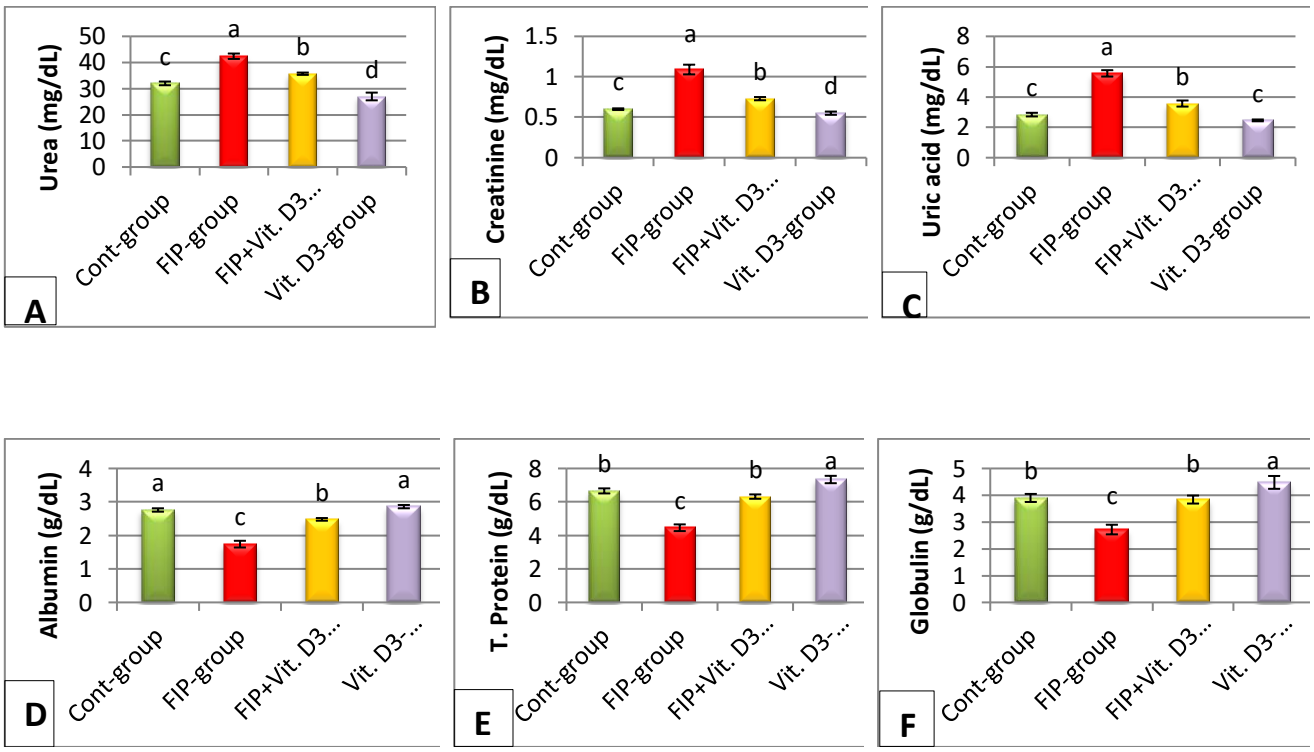

Figure (3): Effect of fipronil (FIP), vitamin D_3 (Vit. D_3), and their combination on the serum urea, creatinine, uric acid, albumin, total protein, and globulin concentrations in the serum of male rats.

All the data were analyzed using one-way ANOVA followed by Duncan post Hoc test. Values are expressed as means ± S.E.; n=7 for each group. Mean values within the columns not sharing a common superscript letter (a, b, c, d) were significantly different, $p < 0.05$. For urea: variation between groups=210.246, variation within groups=4.938 So, F=42.58. For creatinine: variation between groups=0.298, variation within groups=0.005 So, F=60.71. For uric acid: variation between groups=9.533, variation within groups=0.129 So, F=73.79. For albumin: variation between groups=1.281, variation within groups=0.020 So, F=63.276. For total protein: variation between groups=7.589, variation within groups=0.164 So, F=46.27. For globulin: variation between groups=2.706, variation within groups= 0.167. So, F=16.23.

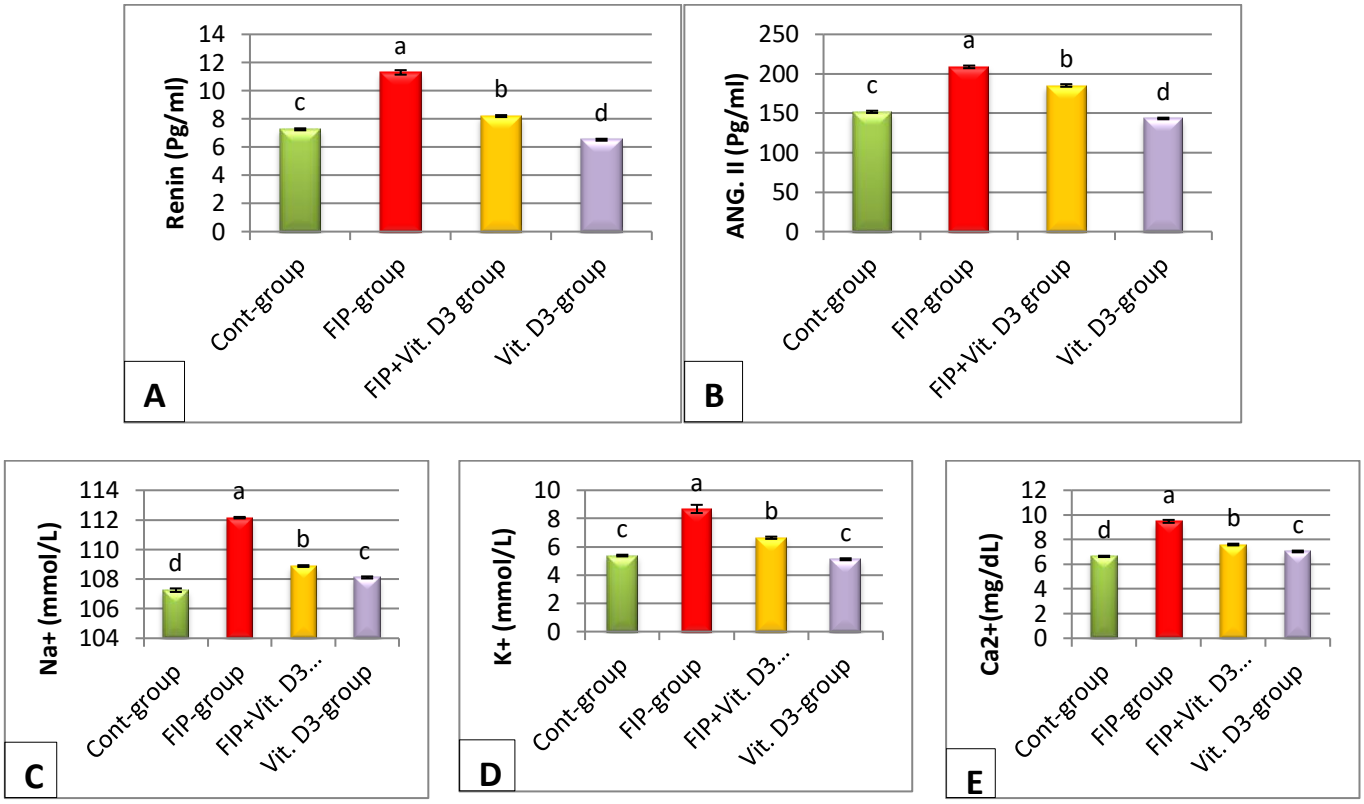

Figure (4): Effect of fipronil (FIP), vitamin D_3 (Vit. D_3), and their combination on renin, angiotensin II (ANG. II), sodium (Na^+), potassium (K^+), and calcium (Ca^{+2}) ions, concentrations in serum of male rats.

All the data were analyzed using one-way ANOVA followed by Duncan post Hoc test. Values are expressed as means ± S.E.; n=7 for each group. Mean values within a column not sharing a common superscript letter (a, b, c, d) were significantly different, $p < 0.05$. For renin: variation between groups=19.505, variation within groups=0.068 So, F=285.79. For ANG. II: variation between groups=4141.686, variation within groups=85.536. So, F=48.42. For Na^+: variation between groups=22.936, variation within groups=0.034 So, F=684.60. For K^+: variation between groups=13.196, variation within groups=0.132 So, F=99.765. For Ca+2: variation between groups=7.895, variation within groups=0.032 So, F=246.729.

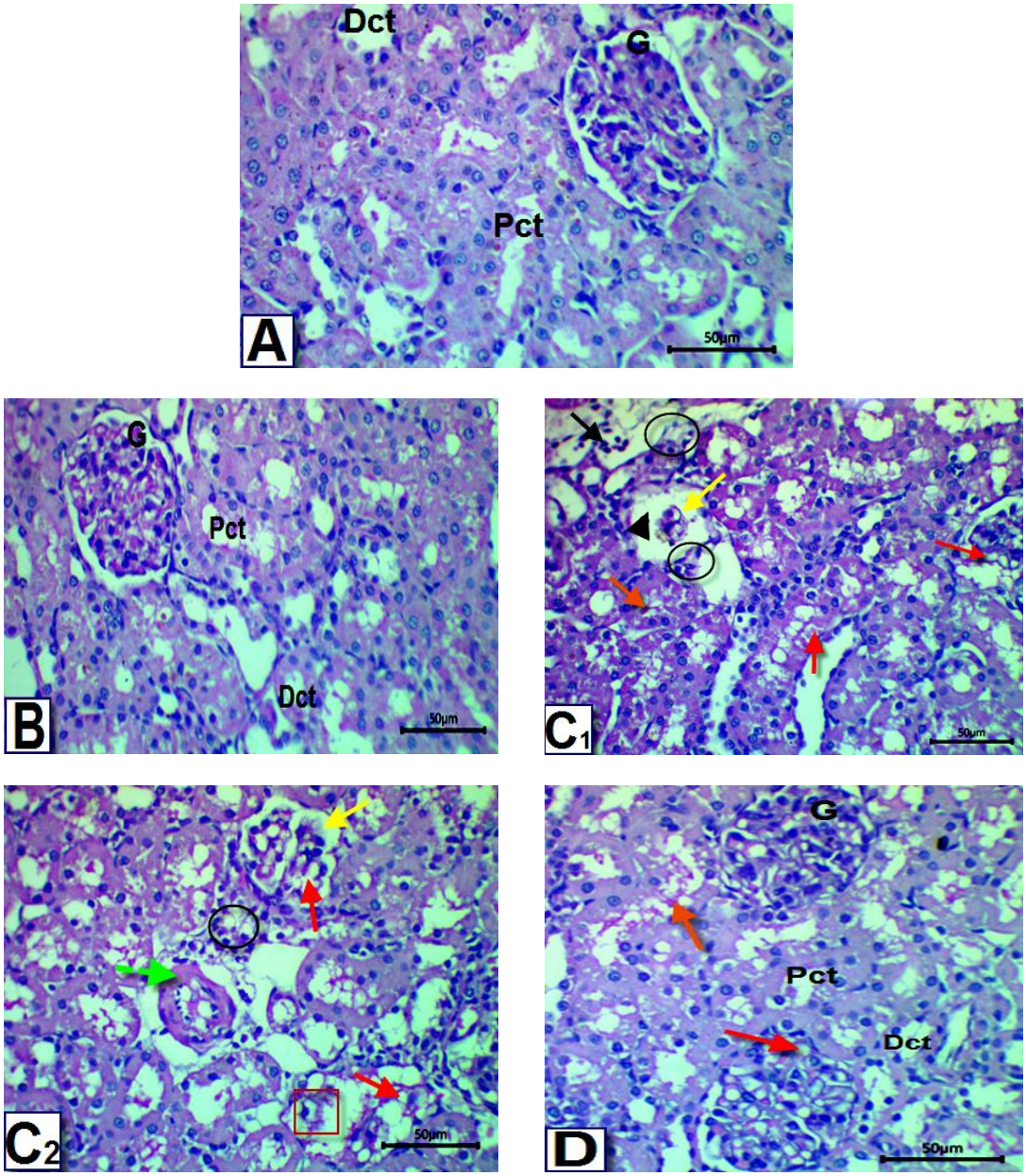

Figure (5): Photomicrographs of kidney sections (A&B), indicating: control & Vit. D3-treated groups, showing normal cortical architecture with normal glomerulus (G), proximal convoluted tubules (Pct), and distal convoluted tubules (Dct). FIP-treated group **(C_1&C_2)**, showing: shrunken glomeruli (yellow arrow) with capsular space (black head arrow), degenerative changes in the glomerulus and tubular cells (black circle), pyknotic nuclei (black arrow), and hemorrhage (orange arrow), vacuolization (red arrows), thick blood vessel (green arrow) and tubular lumen with cellular debris & congestion (red square). Vit. D_3+FIP-treated group (D), showing marked improvements in kidney tissue with the presence of few vacuoles and hemorrhage (H & E X400).

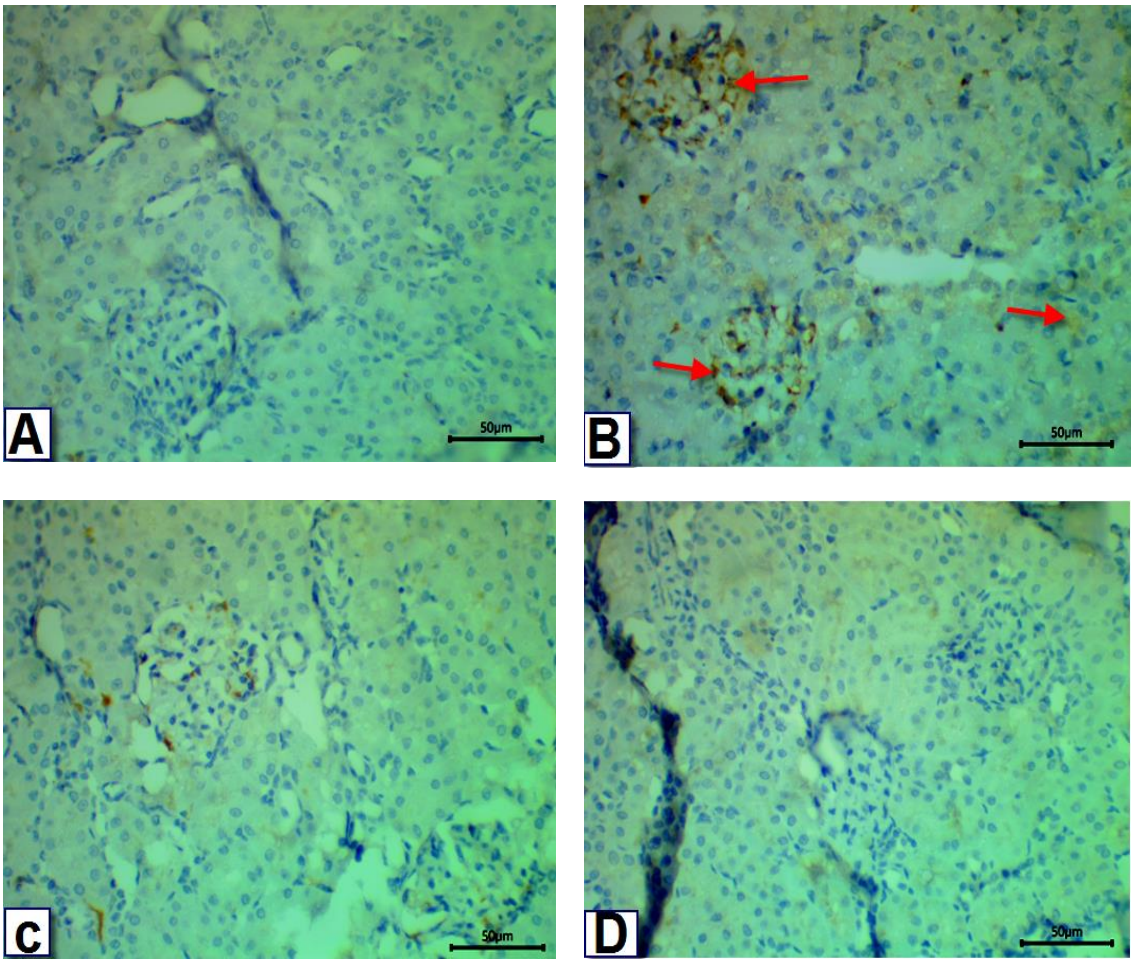

Figure (6): Photomicrographs of sections in the kidney of control and Vit. D3- treated groups (A&D) showing weak immunostaining for caspase-3. Positive immunostaining for caspase-3 (red arrows) in the kidney of the FIP-treated group (B), in the kidney of Vit. D_3+FIP-treated group (C) showing weak immunostaining for caspase-3 in most kidney tissues (Caspase-3, 400).

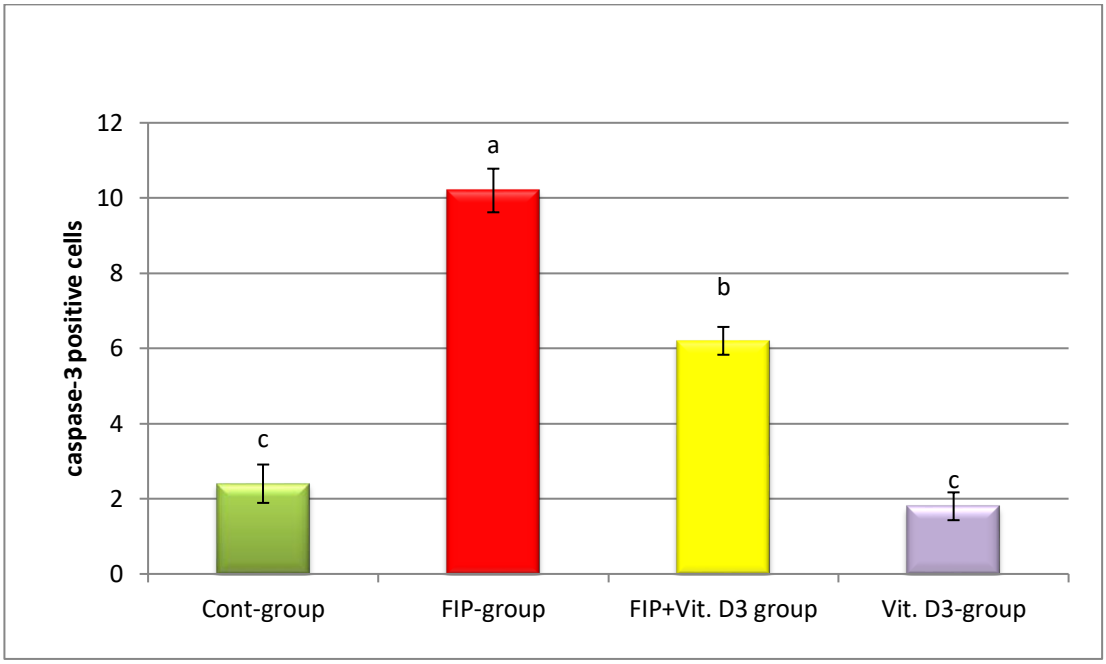

Figure (7): Effect of vitamin D3 (Vit. D3), fipronil (FIP), and their combination on mean number caspase-3 -positive cells in the kidney of male rats.

All the data were analyzed using one-way ANOVA followed by Duncan post Hoc test. Values are expressed as means ± S.E.; n=7 for each group. Mean values within a column not sharing a common superscript letter (a, b, c, d) were significantly different, p <0.05. For caspase-3-positive cells: variation between groups=77.933, variation within groups=1.150 So, F=67.77.

Discussion

The lipophilic nature of Fipronil (FIP) allows for the maintenance of distinct cellular damage, with its sulfone form being more toxic, persistent, and prone to bioaccumulation compared to regular FIP **(37)**. Studies have indicated that administering vitamin D3 before ischemia can ameliorate renal dysfunction and restore normal glomerular filtration rate **(38)**.

In the present research, rats exposed to FIP exhibited remarkable reductions in various hematological parameters, including Hb, RBCs, Ht, WBCs, and PLTs, along with serum EPO levels. This decrease in Hb, Ht, and RBCs could be attributed to the oxidative damage promoted by FIP exposure, leading to the destruction of erythrocytes in the rats' hematopoietic organs **(39)**. This aligns with the findings of Al-Harbi et al. **(40)**, who documented oxidative stress in rat erythrocytes caused by FIP ingestion. Gong et al. **(41)** also noted hemolytic anemia in rats treated with FIP. Likewise, FIP-induced alterations in calcium homeostasis through the Ca^{2+} intrinsic pathway could lead to increased Ca^{2+} influx into RBCs, resulting in cellular hyperosmolarity that triggers eryptosis, characterized by membrane blebbing and cell shrinkage. **(42)** Besides, the reduction in EPO observed by Wahed et al. **(43)** was associated with impaired erythropoiesis and the emergence of microcytic hypochromic anemia, marked by RBC lysis and subsequent decreases in RBC count, Hb, and Ht levels.

Conversely, the concurrent administration of Vitamin D3 with FIP led to elevated hematological parameters and EPO levels compared to the group treated only with FIP. These findings align with the research conducted by Bella et al. **(44),** which validated that diabetic mice treated with Vitamin D3 exhibited enhancements in RBC counts, Ht percentage, and Hb levels. Vitamin D receptors (VDRs) expressed by specific subsets of bone marrow cells, such as stromal and accessory cells, are believed to enhance the erythropoiesis process **(45)**. Additionally, Aucella et al. **(46)** reported increased Hb and Ht levels in chronic kidney disease patients undergoing hemodialysis after Vitamin D3 treatment. Vitamin D3 also positively influences serum and kidney EPO levels during renal injury repair **(47)**. The expression of VDRs in hematological organs, as well as the synergistic effect of Vitamin D3, promotes higher EPO receptor expression **(45).** These findings align with the observations of Bella et al. **(44)**, who found that Vitamin D3 restored WBC and mononuclear cell counts. Additionally, Vitamin D3 supplementation exhibits a broad antioxidant effect and stimulates VDRs present in immune cells (B and T cells, macrophages, and monocytes) **(48)**. Moreover, the current results are in line with the suggestions of Leytin and Freedman **(49)**, who proposed that Vitamin D3 could restore platelet count by modulating VDR activity, thereby influencing megakaryocytopoiesis and PLT activation through its role in calcium flux modulation.

The present study established that the administration of FIP led to increased concentrations of MDA and NO in the kidney tissues, while concurrently leading to a reduction in GSH levels. These effects are likely due to the lipophilic nature of FIP, which, independently of GABA receptors, leads to the rapid distribution of FIP and its cytotoxic metabolites to mitochondrial membranes. This, in turn, causes uncoupling of oxidative phosphorylation, inhibiting he generation of cellular energy and accelerating the rate of respiration **(18)**. Despite this disruption, cellular ATP levels might remain sufficient to facilitate FIP-induced cell death through apoptosis **(37)**. FIP has been reported to stimulate ROS production through the protein kinase B (AKT)/glycogen synthase kinase 3 beta (GSK3β) signaling pathway, which reduces anti-apoptotic proteins and activates apoptotic mediators such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and cAMP-response element-binding protein (CREB) **(8)**. This increase in cellular oxidative products overwhelms the antioxidant defense system, causing damage to cellular macromolecules such as lipids, DNA, and proteins, ultimately leading to apoptotic or necrotic mechanisms **(8,33)**.

In contrast, co-administration of Vit. D3 alongside FIP resulted in a reduction in MDA and NO levels in kidney tissues, while simultaneously elevating GSH levels. These outcomes can be attributed to the antioxidant properties of Vitamin D3, which act to mitigate oxidative damage by regulating the NADPH oxidase-4 (NOX-4)/nuclear factor erythroid 2-related factor-2 (NrF2)/thioredoxin (Trx) signaling pathway and dampening stress-induced kinase signaling cascades **(50)**. This antioxidative action suppresses MDA levels and the activity of inducible nitric oxide synthase (iNOS)-generated nitric oxide (NO) **(51)**. Consequently, 1,25(OH)2 D3 and its analogues are promising contenders for ROS detoxification due to their capacity to decrease MDA levels and boost GSH activity in damaged tissues **(52)**.

The results of the current study indicated that FIP-treated rats exhibited reduced activities of kidney antioxidant enzymes, including SOD, CAT, and GPx. This is likely due to FIP-induced oxidative damage, leading to a decline in the capacity of these defense enzymes **(53)**. Notably, CAT, SOD, and GPx play crucial roles in protecting cellular macromolecules and are considered the primary line of defense against oxidative and cellular injury **(40)**. Additionally, AlBasher et al. **(6)** suggested that the oxidative damage induced by FIP could be attributed to its

stimulating effect on the generation of ROS and/or RNS.

On the other hand, the supplementation of Vit. D3, alongside FIP, helped regulate the alterations in kidney antioxidant enzyme activities. These outcomes are likely due to the physiological and pharmacological actions of Vitamin. D3, which leads to the upregulation of the redox-sensitive transcription factor NrF2 and the suppression of NF-κB activation through the NrF2/heme oxygenase-1 (HO-1)/transcription factor BTB and CNC homology-1 (BACH) pathway **(19)**. Furthermore, Vit.D3's antioxidative effect significantly reduced ROS generation, thereby enhancing the cellular defense systems **(54)**.

In the present study, rats treated with FIP demonstrated raised levels of serum urea, creatinine, and uric acid. This could be attributed to the oxidative stress induced by FIP, leading to an increase in kidney injury markers in the serum. This, in turn, might impair the kidneys' ability to eliminate catabolic by-products and break down purine and pyrimidine bases (associated with DNA degradation), as suggested by Sun et al. **(55)**. Additionally, FIP appeared to disrupt the glomerular filtration rate (GFR), thus hindering renal function and resulting in higher levels of serum creatinine, a reliable filtration marker as supported by Badgujar et al. **(9)** These observations align with Mossa et al. **(53)** study, which linked elevated urea and creatinine levels to tubular cell necrosis in FIP-treated rats. In addition, AlBasher et al. **(6)** proposed that the elevation in urea levels might be due to increased enzyme synthesis, leading to the conversion of ammonia to urea, or enhanced protein breakdown in the FIP-treated group.

When Vit.D3 was administered alongside FIP, there was a reduction in serum urea, creatinine, and uric acid levels compared to the FIP-treated group. Vitamin D3, also known as cholecalciferol, has diverse roles including anti-inflammatory, anti-fibrotic, and anti-apoptotic properties, as stated by Arfian et al. **(56)** Additionally, Vitamin D3 appeared to provide renal protection by alleviating kidney podocyte injury-related glomerular dysfunction through the nephrin/phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, thus preventing podocyte apoptosis, as indicated by Trohatou et al. **(57)** Consequently, calcitriol therapy seems to effectively address glomerular filtration defects, renal parenchymal damage, and function, ultimately decreasing the risk of acute kidney injury (AKI), as highlighted by Sari et al. **(58)** Besides, Reis et al. **(59)** suggested that Vitamin D3 treatment can inhibit plasma creatine kinase and blood urea nitrogen levels.

Also, treatment with FIP led to a decrease in serum protein levels, including albumin, globulin, and total protein. These findings align with Abdel-Daim et al.'s **(18)** study, which indicated that FIP-induced oxidative damage to the liver resulted in a notable reduction in albumin and total protein levels in rat serum. The rise in hepatic enzyme levels is known to contribute to protein degradation, as suggested by AlBasher et al. **(6).** However, when Vit.D3 was supplemented alongside FIP, there was an improvement observed in albumin, total protein, and globulin concentrations. These outcomes are consistent with El-Boshy et al. **(60)**, where Vitamin D3 supplementation was shown to enhance total protein and albumin levels in rat blood. This effect was attributed to Vitamin D3's regulatory impact on hepatic enzymes, oxidative stress, inflammation, and apoptotic markers.

The current investigation demonstrated that rats exposed to FIP experienced an increase in their serum renin and ANG-II levels as compared to the control group. This abnormal elevation leads to the impending disruption of various physiological processes involving circulation, metabolism, and respiration, primarily by triggering an upsurge in the production of ROS **(61)**. Various external triggers induce hyperactivity in the renin-angiotensin system (RAS) by causing an excessive release of renal renin, which follows the RAS pathway involving angiotensin-

converting enzyme II and the angiotensin type 1 receptor. This cascade ultimately raises ANG-II levels, contributing to several inflammatory and fibrotic conditions **(62)**.

However, the findings of this study contradict those presented by Chaguri et al. **(63)**, who indicated that FIP could lead to elevated hypertension by promoting the release of vasopressin-induced endothelin-1 expression. This vasoconstrictive effect and activation of the RAS exhibit a direct relationship, and both their decreased activities are utilized in the management of chronic kidney diseases **(64)**.

Interestingly, the combined administration of Vitamin D3 with FIP resulted in a decrease in both serum renin and ANG-II hormone levels in male rats. Vitamin D3 operates through a protective mechanism that involves regulation by the RAS and various receptors **(65-68)**. Moreover, Eltablawy et al. **(20)** proposed that Vitamin D3 can moderate the increase in cytokine production, injuries to glomerular and tubular cells, accumulation of extracellular matrix, and the generation of ROS. This modulation is achieved by notably reducing serum renin and ANG-II levels.

The current results indicated that administration of FIP led to an increase in the levels of sodium, potassium, and calcium ions in the serum. This effect could be attributed to the hyperactivity of the renin-angiotensin system (RAS) triggered by FIP treatment, resulting in an upregulation of renal ion cotransporters and Na^+/K^+ ATPase subunits associated with hypertension, as suggested by Rozansky **(65)**. Similarly, La Merrill et al. **(66)** demonstrated elevated concentrations of sodium, potassium, and calcium ions in prenatal rats exposed to dichlorodiphenyltrichloroethane pesticide. This exposure induced the mRNA expression of $Na^+/K^+/Cl^-$ cotransporters, a Na^+/Ca^{2+} exchanger, and various Na^+/H exchangers in hypertensive kidneys. Besides, the production of ATP, essential for the energy-dependent Na^+/K^+ ATPase pump on the cell membrane, was hindered due to reduced oxygen pressure. Consequently, this disruption in energy pumps led to the efflux of potassium from the cells, influx of sodium along with water (hydropic degeneration), cell rupture, and subsequent hypernatremia and hyperkalemia, as described by Brar et al. **(67)** FIP's actions further inhibited ATPase activity, as suggested by Gupta et al. **(68)** and Ardeshir et al. **(69)** Additionally, FIP or its metabolites notably increased mitochondrial calcium through the calcium pathway, causing the release of accumulated calcium from the mitochondria into the cytosol and ultimately inducing apoptosis, according to Tavares et al. **(70)** FIP's action also altered the plasma membrane permeability, leading to the leakage of calcium from cells into the bloodstream, as indicated by Sun **(71)**.

However, when rats were treated with a combination of Vitamin D3 and FIP, there was a suppression observed in serum sodium, potassium, and calcium levels. The amelioration of serum electrolyte levels during Vit.D3 treatment can be attributed to its hormonal influence in mitigating RAS activation, countering the alteration in glucose metabolism that causes ATP depletion, and addressing disruptions in calcium hemostasis, as proposed by Abu el Maaty et al. **(72)**, Ferder et al. **(62)**, and Graidis et al. **(51)** In line with this, Kapil et al. **(73)** confirmed that cholecalciferol treatment significantly reduced fractional excretion of sodium (serum and urine sodium levels) and serum potassium levels, since Vitamin D3 exhibited a protective influence against ischemia-reperfusion-induced acute kidney injury (AKI) in rats.

The latest histological examination found that FIP treatment resulted in kidney abnormalities, including the enlargement and degeneration of twisted tubules, as well as congestion in the capillaries within the glomeruli, accompanied by a decrease in glomerular size. These alterations in the kidney tissue structure are likely due to the increased oxidative damage to the kidneys and higher levels of programmed cell death markers induced by FIP, as previously reported by

Khalaf et al. (5) However, when Vitamin D3 was administered alongside FIP, the kidney exhibited a more or less normal appearance. These findings align with the research conducted by Elbassuoni et al. (19), which demonstrated that Vitamin D3 effectively alleviated these histological changes.

In particular, the current findings revealed that FIP-intoxicated rats had substantially elevated caspase-3 expression levels in contrast to the control group. This heightened caspase-3 activity is likely a result of FIP-induced activation of caspase-3 due to the production of reactive oxygen species (ROS) and the triggering of the MAPK/caspase-3 signaling pathway, as reported by Wang et al. (74). Conversely, the combined administration of Vitamin D_3 and FIP remarkably decreased the expression of caspase-3. Vitamin D3 has been shown to inhibit glomerular cell death induced by puromycin aminonucleoside by activating the PI3K/Akt signaling pathway. This activation subsequently leads to decreased caspase-3 levels and a shift in the Bax/Bcl-2 ratio, as demonstrated by Xiao et al. (75).

Conclusion

Vitamin D_3 (cholecalciferol) shows promising therapeutic potential as a regulator in mitigating the harmful effects of FIP exposure on erythropoietin (EPO), the renin-angiotensin system (RAS), and renal health. FIP exposure can disrupt EPO levels, leading to hematological abnormalities and oxidative damage, while also impacting the RAS and contributing to renal dysfunction. Vitamin D_3 supplementation holds promise in modulating EPO levels, regulating the RAS, and preserving renal function, potentially counteracting the adverse effects of FIP through its antioxidant and anti-apoptotic properties. Further research is needed to fully understand the mechanisms involved and optimize supplementation strategies. Nevertheless, these findings highlight the importance of considering nutritional interventions to address environmental toxin exposures and maintain hematological and renal health.

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Author Contributions

Heba Mohamed Abdou contributed to the conceptualization and study design, writing—original draft preparation, writing—review and editing, visualization and supervision; Mohamed Mounir Hammad contributed to the experimentation and statistical analysis, writing—original draft preparation, and all authors; Heba Mohamed Abdou, Mohamed Mounir Hammad, Hussein Khamis Hussein, Maha Moustafa Attia and Nema Mohammed Abd El-Hameed have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

Data Availability Statement

The datasets generated during the current study are available from the corresponding authors upon reasonable request.

Ethical Statements

All procedures and handling of the animals were carried out in accordance with the guidelines set forth by the Alexandria University Institutional Animal Care and Use Committee (ALEXU-IACUC) under the approval number AU 04 18 12 02 MSc 3.

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